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Yeast signal peptidase contains a glycoprotein and the *Sec11* gene product

(protein purification/yeast microsomal membranes/integral membrane proteins/posttranslational precursor processing)

JACQUES T. YADEAU*, CHARLES KLEIN†, AND GÜNTER BLOBEL

Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021

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ABSTRACT **Partially purified yeast microsomal signal peptidase appears to be a complex of four polypeptides of 13, 18, 20, and 25 kDa. The 18-kDa chain is the product of the *Sec11* gene, which is necessary for signal peptidase activity. The 25-kDa subunit is a glycoprotein that binds Con A. Two related methods for purification of the enzyme are presented; the first includes removal of peripheral membrane proteins from microsomes by alkali extraction, solubilization of the enzyme by nonionic detergent and high salt, and four different chromatographic procedures. An alternative method was developed based on lectin-affinity chromatography.**

The signal sequence that targets a protein to the endoplasmic reticulum is usually removed by a membrane protein, signal peptidase (SP), during or shortly after translocation of a preprotein into the endoplasmic reticulum (1). Canine rough microsomes contain approximately the same number of SP complexes and bound ribosomes (2), which suggests that SP may be part of the translocation site. SPs have been isolated from canine (2) and avian (3, 4) microsomal membranes. Two SPs (I and II) have been isolated from *Escherichia coli* plasma membranes (5, 6). We chose to study yeast SP (ySP) for several reasons, including the suitability of yeast for genetic analysis, which has lead to the isolation of secretory mutations (7). Additionally, we hoped that comparison of SP to other SPs would shed light on the mechanism of action. The disparate eukaryotic SP enzymes and SP-I of *E. coli* cleaved common substrates (8), which suggested that they share structural features. Recently ySP was characterized enzymatically (9) and shown to be distinct from the mammalian, avian, or bacterial SPs. Although these enzymes recognize common substrates, they exhibit different optima with respect to pH, temperature, and concentrations of salt, lipid, and detergent.

The purification data presented in this paper suggest that like the vertebrate enzymes, ySP may be a polypeptide complex. A subunit of the enzyme is immunologically related to the product of the *Sec11* gene, an essential gene required for ySP activity (10). The 21- and 18-kDa subunits of canine SP are homologous to *Sec11* (11, 12). Identification of a glycoprotein subunit in the yeast enzyme led to a second purification strategy based on lectin-affinity chromatography. Both canine SP (2, 13) and hen oviduct microsomal SP (3, 4) contain a glycoprotein subunit.

Although all eukaryotic SPs thus far characterized are multimeric, *E. coli* SP-I is a single 39-kDa polypeptide (5). Subunits of the eukaryotic complexes could be specialized to carry out several functions that might be performed by the larger bacterial protein. Alternatively, eukaryotic enzymes may possess activities not found in the bacterial enzyme. Presentation of the cleavage site and endoproteolytic cleavage of the peptide bond may be done by different subunits of eukaryotic complexes. Interaction of the signal sequence with more than one subunit could increase specificity or turnover rate, as has been proposed for the processing-enhancing protein of mitochondrial matrix SP (14). Subunits of SP could facilitate transport of proteins across the endoplasmic reticulum, hydrolyze signal peptides after removal from preproteins, or act as a signal sequence receptor.

MATERIALS AND METHODS

Materials. Most materials were as described (9). Zymolyase-100T from ICN was used for spheroplasting. Nitrocellulose for immunoblots was from Schleicher & Schuell. QAE-Sephadex, S Sepharose Fast Flow, Sephacryl S200 HR, a Mono S HR 5/5 column, a Mono Q HR 5/5 column, and a Superose 12 column were from Pharmacia. Bio-Gel HTP hydroxylapatite was from Bio-Rad. Fraction V bovine serum albumin and ^{14}C-labeled Con A were from Sigma. Keyhole limpet hemocyanin was from Calbiochem. ^{125}I-protein A was from NEN.

Assaying ySP. Assays were done as described (9), except that samples were incubated at 15°C for times based on the expected activity of the fraction. The KOAc concentration of each column fraction was estimated by measuring conductivity, and two assay mixtures were prepared that contained high or low concentrations of KOAc. Various amounts of the two assay mixtures were added to samples to reach a final concentration of 75 mM KOAc. Assays were analyzed by electrophoresis on 12.5% polyacrylamide gels without urea (15).

Isolation of Yeast Microsomal Membranes (yMMs). Crude membranes were isolated by differential centrifugation of lysed *Saccharomyces cerevisiae* cells as described (9, 15). All manipulations after lysis were done on ice or at 4°C. Crude membranes from a 30-liter culture were diluted with 20 mM Hepes, pH 7.5/1 mM dithiothreitol (buffer C) to 60 ml, homogenized in a Dounce homogenizer, and fractionated by sucrose density-gradient centrifugation. Ten milliliters of the membrane suspension was placed in each of six Beckman SW 28 tubes; a spacer layer (15 ml of buffer C/20% sucrose) and a cushion (buffer C/60% sucrose) were injected under the membrane suspension. After overnight centrifugation at 122,000 × g_{max}, yMMs were collected with a syringe from the 20%/60% interface, frozen in liquid nitrogen, and stored at −80°C. SP activity was enriched in membranes from the 20%/60% interface (data not shown).

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Abbreviations: PtdCho, L-α-phosphatidylcholine; SP, signal peptidase; yMM, yeast microsomal membrane; ySP, yeast signal peptidase.

*Present address: Department of Medicine, The New York Hospital, New York, NY 10021.
†Present address: Committee on Cellular and Developmental Biology, Harvard Medical School, Boston, MA 02115.

SP Purification by Method A. yMMs were extracted with alkali to remove peripheral membrane proteins. The membranes were then washed with detergent at low ionic strength, and activity was solubilized by extraction with detergent at high ionic strength. Anion- and cation-exchange chromatography was performed at pH 9, followed by hydroxylapatite and cation-exchange chromatography at pH 7.5. Further analytic scale separation was achieved by gel filtration in the presence of 0.3 M KOAc/0.1% Nikkol/0.00825% SDS.

Carbonate Extraction of Membranes. Two hundred and six thousand equivalents of sucrose-gradient-purified yMMs (1 equivalent per μl = 50 A_{280} units per ml), derived from three 30-liter cultures of yeast (which contained 572 g of packed yeast cells) was adjusted to 50 mM Na_2CO_3, pH 11.5/1 mM dithiothreitol in a volume of 200 ml. The suspension was mixed in a Dounce homogenizer, incubated for 15 min, and centrifuged for 2 hr at 286,000 × g_{max} in a Sorvall T865 rotor. The pellets were resuspended in 50 mM Na_2CO_3/5% glycerol/1 mM dithiothreitol, incubated for 15 min, and centrifuged through 50 mM Na_2CO_3/10% glycerol/1 mM dithiothreitol. This step was repeated, and the membranes were resuspended in buffer C/5% glycerol and pelleted again.

Differential Solubilization. Membrane proteins of carbonate-washed yMMs were differentially solubilized with Nikkol and phosphatidylcholine (PtdCho), first at low ionic strength and then at higher ionic strength. Membrane pellets were suspended with a Dounce homogenizer in 60 ml of buffer C 1% Nikkol/PtdCho at 1 mg/ml/10% (vol/vol) glycerol/1× protease inhibitors [protease inhibitor mixture is 0.1 mM EDTA/0.1 mM $Mg(OAc)_2$/Trasylol at 10 units/ml/0.1 μM antipain/0.1 μM leupeptin/0.1 μM pepstatin/1 mM phenylmethylsulfonyl fluoride]. After 15-min incubation, cushions of buffer C/0.1% Nikkol/PtdCho at 0.4 mg/ml/14% glycerol/1× protease inhibitors were underlaid, and the partially extracted membranes were collected by centrifugation as before. The pellets were suspended to 7.6 equivalents per μl with a Dounce homogenizer in 27 ml of 3% Nikkol/PtdCho at 1.5 mg/ml/0.3 M KOAc/50 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES), pH 9/0.1 M sucrose/1 mM dithiothreitol/1× protease inhibitors. After 15-min incubation insoluble material was removed by centrifugation at 286,000 × g_{max} for 1 hr. The supernatant, termed high salt/detergent extract, contained solubilized ySP activity.

Column Chromatography. Initial separations used anion and cation-exchange resins at pH 9. Two hundred milliliters of QAE-Sephadex in a 2.5 × 40 cm column was equilibrated to buffer D [50 mM 2-(cyclohexylamino)ethanesulfonic acid, pH 9/10% glycerol/0.1% Nikkol/PtdCho at 0.4 mg/ml/1 mM dithiothreitol]/50 mM KOAc. The column was loaded at 30 ml/hr with 27 ml of high salt/detergent extract, and developed with buffer D/0.3 M KOAc. Flow-through fractions, defined as fractions that contained protein (determined by absorbance at 280 nM) and eluted before the rise in conductivity, were pooled and applied at 3 ml/hr to a 2.5 × 30 cm column containing 30 ml of S Sepharose equilibrated with buffer D/50 mM KOAc. The column was washed with 45 ml of buffer D/50 mM KOAc followed by 120 ml of buffer D/150 mM KOAc. Bound proteins were eluted at 17 ml/hr with a 130-ml gradient of buffer D from 150 to 700 mM KOAc. The peak of activity extended from 335 mM to 410 mM KOAc.

Hydroxylapatite chromatography was more reproducible at pH 7.5 than at pH 9, and elution with a KOAc gradient gave better resolution than was obtained with a phosphate gradient (data not shown). Eight grams of hydroxylapatite was suspended in 150 ml of buffer E (40 mM Hepes, pH 7.5/10% glycerol/0.1% Nikkol/PtdCho at 0.4 mg/ml/1 mM dithiothreitol)/100 mM KOAc/5 mM sodium phosphate, pH 7.5. The supernatant was discarded after allowing the slurry to settle for 10 min. Batch equilibration was repeated, and the hydroxylapatite was poured into a 1.5 × 20 cm column that was equilibrated for 1 hr at 11 ml/hr and overnight at 8 ml/hr. The 26-ml S Sepharose peak pool was applied to the hydroxylapatite column at 6.2 ml/hr after being diluted to 100 mM KOAc/5 mM sodium phosphate, pH 7.5, by the slow addition of 67 ml of buffer E/6.94 mM sodium phosphate, pH 7.5. The column was washed with 24 ml of buffer E/100 mM KOAc/5 mM sodium phosphate, pH 7.5, and eluted at 5 ml/hr with a 90-ml gradient of buffer E/5 mM sodium phosphate, pH 7.5, from 100 to 600 mM KOAc. Peak ySP activity was found between 400 and 447 mM KOAc.

Further separation was achieved by cation-exchange chromatography at pH 7.5. A fast protein liquid chromatography Mono S column was equilibrated with buffer E/100 mM KOAc. The 21-ml hydroxylapatite peak pool was diluted with 69 ml of buffer E to 100 mM KOAc/1.2 mM sodium phosphate. After injection of the load, the column was washed with 3 ml of equilibration buffer and developed with a 25-ml gradient of buffer E from 100 to 450 mM KOAc.

A fast protein liquid chromatography Superose 12 gel filtration column was equilibrated with buffer E/0.3 M KOAc/0.00825% SDS. Two hundred microliters of fraction 15 from the Mono S column was injected into the column, which was developed with equilibration buffer. Then 0.75-ml fractions were collected. A 20-μl aliquot was reserved for assay of SP activity, and the remainder of each fraction was precipitated with trichloroacetic acid for electrophoretic analysis.

SP Purification by Method B. Peripheral membrane proteins were removed from yMMs by extraction with EDTA and KOAc. ySP activity was solubilized with Nikkol and 0.3 mM KOAc and applied to a Con A column. The Con A eluate was further purified by hydroxylapatite and cation-exchange chromatography.

Membrane Washing and Solubilization. Forty milliliters of yMMs (2 equivalents per μl) were diluted with 10 ml of buffer C and centrifuged for 90 min at 256,000 × g_{max} in a Beckman 50.2 Ti rotor. Membrane pellets were suspended in 40 ml of buffer C/25 mM EDTA/5% glycerol, and cushions of buffer C/25 mM EDTA/10% glycerol were underlaid. After 10-min incubation, membranes were collected by centrifugation as above. EDTA-stripped yMM pellets were suspended in 40 ml of buffer C/5 mM EDTA/0.5 M KOAc/5% glycerol, and cushions of buffer C/0.5 M KOAc/10% glycerol were underlaid. Membranes were pelleted as above after 10-min incubation. Washed membranes were solubilized by suspension to 8 equivalents per μl in 3% Nikkol/PtdCho at 1.5 mg/ml/0.3 M KOAc/2.5 mM $Mg(OAc)_2$/50 mM triethanolamine acetate, pH 8/0.1 M sucrose/1× protease inhibitors. After 15-min incubation, insoluble material was removed by 15-min centrifugation at 321,000 × g_{max}.

Chromatography. The solubilized material was adjusted to 1 mM $MnCl_2$/10 mM $CaCl_2$/0.5 M KOAc and applied at 5 ml/hr to a 5-ml column of Con A Sepharose equilibrated to 0.5 M KOAc/1 mM $MnCl_2$/10 mM $CaCl_2$/25 mM triethanolamine acetate, pH 8/10% glycerol/0.1% Nikkol/PtdCho at 0.4 mg/ml/1 mM dithiothreitol (buffer F). The flow-through fraction was collected and reapplied. The column was washed with buffer F at 45 ml/hr for 10 hr and eluted at 5 ml/hr with buffer F/0.5 M methyl α-D-mannopyranoside. Active fractions (60 ml) were diluted to 75 mM KOAc with buffer E and applied to a hydroxylapatite column prepared as for method A. The column was washed with 35 ml of buffer E/100 mM KOAc/5 mM sodium phosphate, pH 7.5, and eluted with a 90-ml gradient of buffer E/5 mM sodium phosphate, pH 7.5, from 100 to 700 mM KOAc. Active fractions were pooled (30 ml), diluted to 50 mM KOAc with buffer D, and applied at 10 ml/hr to an S Sepharose column prepared as described. The column was washed at 20 ml/hr with 60 ml of buffer D/50 mM KOAc and 130 ml of buffer

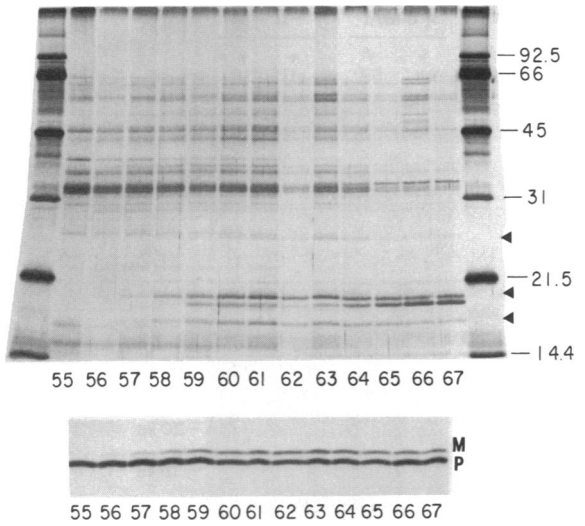

FIG. 1. Hydroxylapatite chromatography. yMMs were extracted at pH 11.5, selectively solubilized, and subjected to cation- and anion-exchange chromatography and then hydroxylapatite chromatography. (*Upper*) Silver-stained 15% polyacrylamide resolving gel that contained SDS and 4 M urea. Two hundred microliters of the hydroxylapatite column fractions was precipitated with trichloroacetic acid, washed with chilled acetone, and resuspended in sample buffer. Molecular mass standards were marked in kDa. (*Lower*) Autoradiogram of an assay gel. Ten microliters of each fraction was assayed at 15°C for 20 min. Cleaved pro-α-factor (M for mature) migrated above its precursor (P) in this 12.5% polyacrylamide gel. Fractions 60 (400 mM KOAc)–67 (447 mM KOAc) were combined as the hydroxylapatite peak pool. The 18-, 20-, and 25-kDa bands are indicated.

D/150 mM KOAc. Activity was eluted with a 120-ml gradient of buffer D from 150 to 1000 mM KOAc.

Antibody Production. A 13-residue peptide (Tyr-Leu-Asn-Lys-Ser-Lys-Glu-Ile-Val-Gly-Thr-Val-Lys-Cys) that contained the glycosylation site of *Sec11* (10), and an additional carboxyl-terminal cysteine was synthesized. Three rabbits (maintained by Pocono Rabbit Farm, Canadensis, PA) were repeatedly injected with peptide coupled via the cysteine residue to keyhole limpet hemocyanin (16) and were then boosted with peptide coupled with glutaraldehyde to bovine serum albumin (17).

Other Methods. Silver stains of polyacrylamide gels were performed with an ICN Rapid-Ag-Stain kit. Plasmid pDJ100 (a gift from David Julius, Columbia University, New York) was transcribed as described (15) and translated in a wheat germ lysate (18) by using prepro-α-factor mRNA at 60 ng/μl with [^{35}S]methionine as label. The translation mixture was frozen in aliquots in liquid nitrogen and stored at −80°C.

RESULTS

Method A Initial Steps. Density-gradient fractionation of crude yeast membranes resulted in 3.6-fold enrichment of ySP activity (data not shown). ySP activity remained associated with the membranes (9) after alkali extraction removed peripheral membrane proteins and proteins contained within the vesicles. After two carbonate extractions 1 equivalent of yMM contained 1.5 μg of protein (by the Pierce BCA protein assay); activity had been enriched 3-fold. yMMs were then extracted with Nikkol and lipid at a low ionic strength. ySP activity was solubilized with Nikkol and PtdCho in the presence of 0.3 mM KOAc. This selective solubilization further enriched activity.

Chromatography. Flow-through fractions from a QAE-Sephadex ion-exchange column were separated on an S Sepharose column. Active fractions were applied to a hydroxylapatite column. Three bands of 18, 20, and 25 kDa (arrowheads in Fig. 1 *Upper*) appeared in the silver-stained gel coincident with the rise in activity in fractions 57–59 (Fig. 1 *Lower*). The 25-kDa band runs as a doublet. Other major bands did not coelute with activity, except possibly a band at ≈19.5 kDa. Polypeptides of <14 kDa were not retained on this gel. Peak of activity from the hydroxylapatite column was fractionated at pH 7.5 on a Mono S ion-exchange column (Fig. 2). ySP activity began to elute at 210 mM KOAc (fraction 12). Four bands coeluted with activity: the three seen in the hydroxylapatite column fractions at 18, 20, and 25 kDa and a weakly staining 13-kDa band. None of the other polypeptides eluted with activity.

Immunoblot Analysis. Proteins of active fractions from the Mono S column were electrophoresed and either stained with Coomassie blue (Fig. 3 *Left*) or transferred to nitrocellulose and probed with anti-Sec11 peptide antiserum (Fig. 3 *Right*). The 18-, 20-, and 25-kDa bands bound similar amounts of Coomassie blue, although silver-staining of the 25-kDa band

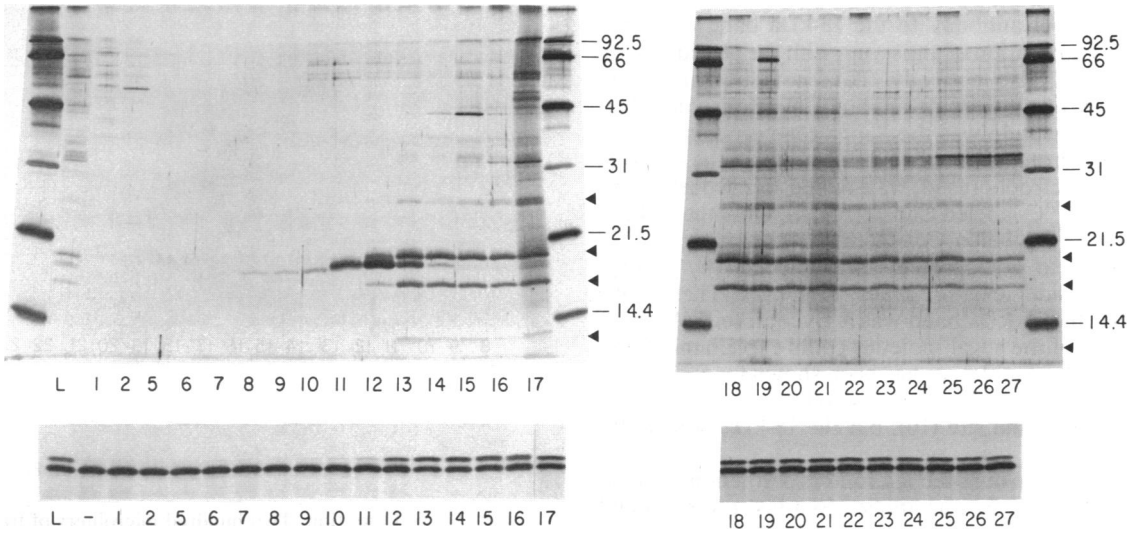

FIG. 2. Mono S chromatography. Load fraction (L) of the silver-stained gel (*Upper*) corresponded to 47 μl of the hydroxylapatite peak pool. Fraction 1 represented a late flow-through fraction. Two hundred microliters of each fraction was precipitated with trichloroacetic acid. Conductivity began to rise at fraction 6. Five microliters of the fractions was assayed for 10 min at 15°C (*Lower*). −, negative control assay incubated under identical conditions without yeast proteins. The 13-, 18-, 20-, and 25-kDa bands are indicated.

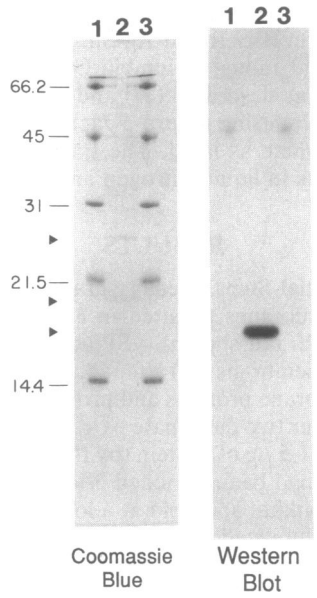

FIG. 3. Sec11 immunoblot. (*Left*) For Coomassie blue staining, 600 μl each from fractions 18 and 20–25 from the Mono S column was pooled, trichloroacetic acid-precipitated, acetone-washed, suspended in 40 μl of sample buffer, and electrophoresed in two lanes of a 14-cm 15% polyacrylamide gel containing SDS and 4 M urea. Lanes: 1 and 3, molecular mass markers; 2, ySP; these were stained with Coomassie blue. (*Right*) For immunoblot (Western blot), protein was incubated for 15 min in transfer buffer [192 mM glycine/25 mM Tris base/20% (vol/vol) methanol/0.01% SDS] and then transferred to 0.2 μM pore size nitrocellulose for 5 hr at 75 V at 4°C. The blot was blocked for 2 hr with phosphate-buffered saline/0.1% Tween 20/0.2% gelatin, incubated for 1 hr with antibody against Sec11 peptide (diluted 1:50 with phosphate-buffered saline/1% bovine serum albumin/0.1% Tween 20), washed with phosphate-buffered saline/0.1% Tween 20 (once in 200 ml and 4 times in 500 ml), and incubated with 1:1000 dilution of ^{125}I-protein A in phosphate-buffered saline/0.2% gelatin/0.1% Tween 20. The blot was washed as before, dried, and exposed to film at −80°C with an intensifying screen. Lanes: 1 and 3, molecular mass markers; 2, ySP.

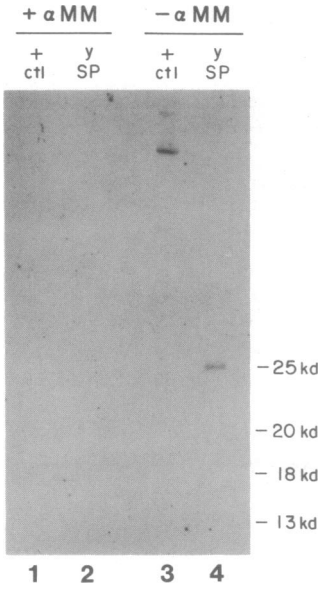

FIG. 4. Con A blot. Lanes: 2 and 4, 200 μl per lane of the Mono S peak pool (derived from fractions 18–23) that were precipitated, electrophoresed, and transferred to nitrocellulose as described for Fig. 3; 1 and 3, control canine microsomal glycoproteins (+ ctl), derived by Con A-affinity chromatography of Nikkol-solubilized canine rough endoplasmic reticulum (from F. Khakee and G. Greenburg, Rockefeller University). The nitrocellulose was incubated overnight in 0.5 M NaCl/50 mM Tris, pH 7.5/2.5 mM $MgCl_2$/1 mM $CaCl_2$/0.5% Tween 20 (wash buffer) and cut in two. The strip containing lanes 1 and 2 was incubated for 3 hr in wash buffer/1:1000 ^{14}C-labeled Con A/0.25 M methyl α-D-mannopyranoside. The strip containing lanes 3 and 4 was incubated in wash buffer/1:1000 ^{14}C-labeled Con A. The nitrocellulose strips were rinsed 5 times with 0.5 liter of wash buffer, impregnated for 10 min with a fluorophor (Enlightning, NEN), dried, and exposed to film. Location of the polypeptide chains was determined by colloidal gold staining of the second sheet of nitrocellulose (data not shown). kd, kDa.

was much less intense. The 13-kDa band was faintly visible on the original gel. The antibody reacted with the 18-kDa band. Preimmune serum contained little reactivity against solubilized carbonate-extracted yMM and did not react with any polypeptides in the Mono S column fractions (data not shown). Binding of antibody to the 18-kDa band could be blocked by preincubation of antiserum with the peptide (at 10 mg/ml) used to raise antibody (data not shown). From cross-reactivity of the peptide antibody, we concluded that the 18-kDa polypeptide was the *Sec11* gene product.

Con A Blots. Proteins of active fractions from the Mono S column were electrophoresed, transferred to nitrocellulose, and probed with ^{14}C-labeled Con A (Fig. 4). Only the 25-kDa subunit bound ^{14}C-labeled Con A (lane 4). Binding of ^{14}C-labeled Con A to glycoproteins was blocked by methyl α-mannopyranoside (Fig. 4, lanes 1 and 2). These data indicated that the 25-kDa band was a glycoprotein. Alternative processing of the sugar moieties could explain migration of the protein as a doublet, as has been shown for SPC 22/23 protein of canine SP (13). *Sec11* encodes a protein with a potential glycosylation site (10), but the 18-kDa protein did not bind Con A.

Gel Filtration. Two hundred microliters of fraction 15 of the Mono S column (Fig. 3) was applied to a fast protein liquid chromatography gel filtration column (Fig. 5). The column buffer contained 0.3 M KOAc to diminish aggregation and 0.0825% SDS to decrease size of the detergent/lipid/protein micelles. Fractions of peak activity (15 and 16) contained the 18-, 20-, and 25-kDa bands. The limited volume that could be injected into the column precluded loading enough material to visualize the weakly staining 13-kDa polypeptide. Additional bands above 45 kDa in each lane represented artifacts caused by long development of the silver stain.

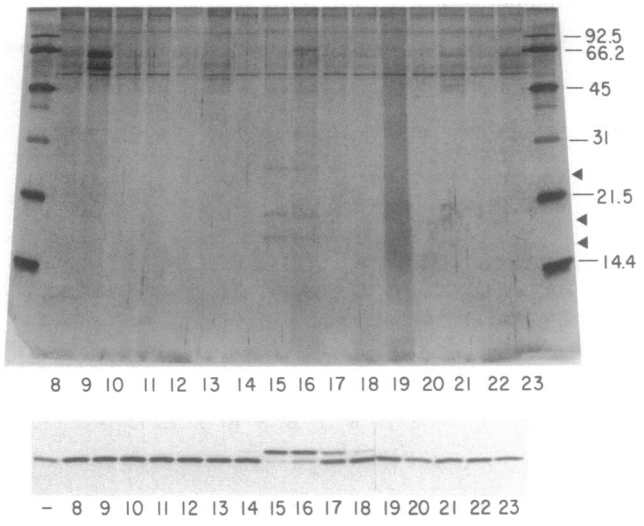

FIG. 5. Gel filtration. Two hundred microliters of fraction 15 of the Mono S column (see Fig. 2) was injected into a Superose 12 column. (*Upper*) Each lane contained the trichloroacetic acid precipitate of the entire 0.75-ml fraction after 20 μl of the fraction was removed for assay. The 18-, 20-, and 25-kDa bands are indicated. (*Lower*) Assay was incubated at 15°C for 6 hr.

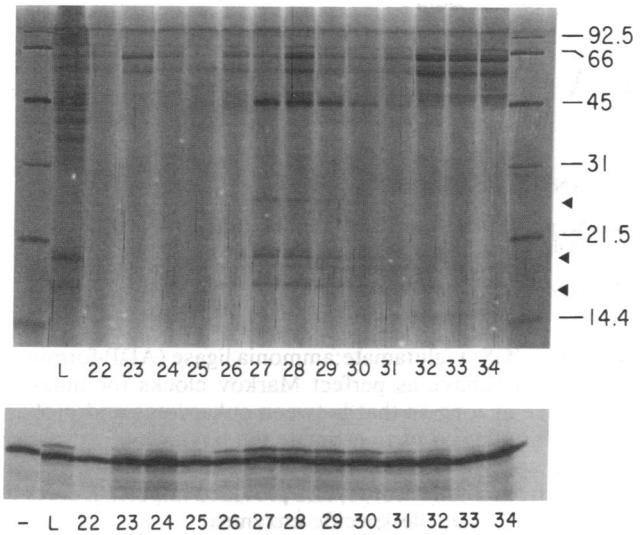

FIG. 6. Method B: S Sepharose chromatography. yMMs were extracted with EDTA and high-salt, solubilized, and subjected to lectin affinity, hydroxylapatite, and cation-exchange chromatography. (*Upper*) The load fraction (L) of silver-stained gel contained the trichloroacetic acid precipitate of 500 μl of the hydroxylapatite peak pool. Seven hundred and fifty microliters of the S Sepharose fractions was precipitated with trichloroacetic acid. The 18-, 20-, and 25-kDa bands are indicated. (*Lower*) Ten microliters of the fractions was assayed for 15 min at 15°C.

Method B. This alternative purification technique was based on the association of activity with a 25-kDa glycoprotein. We were unable to reliably separate solubilized ySP on lectin-affinity columns after incubation of yMMs with alkali (data not shown). The losses may have been caused by partial dissociation of the polypeptides at pH 11.5 (12). Therefore, yMMs were extracted with EDTA and high salt, solubilized, and separated on a Con A–Sepharose column. Further chromatography was done on hydroxylapatite and S Sepharose columns. Active fractions (27–30) from the S Sepharose column contained three bands of 18, 20, and 25 kDa (Fig. 6); a polypeptide of ≈45 kDa was also present. Further purification will be necessary to determine whether the 45-kDa band is a constituent of the ySP complex.

DISCUSSION

Active ySP fractions from two purification methods contained proteins of 18, 20, and 25 kDa. We hypothesize, in part by analogy with canine and avian SP complexes, that these proteins are associated *in vivo*. Method A included carbonate extraction and differential solubilization of yMMs followed by chromatography on five columns. We have four times seen the coelution from a Mono S column of activity and the 18-, 20-, and 25-kDa bands but only once scaled up sufficiently to see the weakly staining 13-kDa band; the association of the 13-kDa band with activity is tentative at present. The 25-kDa protein is glycosylated, as shown by its ability to bind Con A. This fact allowed development of an alternative purification based on Con A chromatography. Method B used high-salt and EDTA extraction of yMMs and chromatography on three columns (including a lectin-affinity column) to give higher yields but lower purity. The 18-, 20-, and 25-kDa proteins eluted together from a Con A column, although only the 25-kDa protein bound Con A on an immunoblot. This procedure provided additional evidence that the polypeptides may be a complex. Some impurities (e.g., at 45 kDa) found in method B could be loosely associated members of a larger complex. The carbonate extraction of yMMs used in method A may strip off proteins that remain associated through the milder washes of method B.

The association of the polypeptides was probably not from coaggregation, as they remained associated in 0.3 M KOAc/0.00825% SDS/0.1% Nikkol. Method B omitted alkali extraction of yMMs, showing the association was not caused by exposure to high pH. The proteins could have coeluted because of shared physical properties. However, the polypeptides were not separated from activity by four distinct separation principles: ion exchange (both anion and cation), hydroxylapatite, gel filtration, and lectin affinity. Cross-linking studies, further purification, or genetic data could provide more evidence of physical association.

Canine SP contains subunits of 12, 18, 21, 22/23, and 25 kDa (2), but electrophoresis of samples of canine SP and ySP in adjacent lanes revealed that only the yeast 13-kDa band and the canine 12-kDa band comigrated (data not shown). The 21- and 18-kDa subunits of canine SP are homologous to *Sec11* (11, 12), which encodes for the 18-kDa yeast protein. That Sec11 is required for ySP activity *in vivo* (10) does not necessarily indicate that it is physically part of the enzyme. For example, the *Sec11* gene could encode a protein responsible for a posttranslational modification necessary for ySP activity. Our data show that the *Sec11* gene product is physically present in active fractions. The finding that *Sec11* is conserved across such phylogenetically distinct organisms as yeast and dogs suggests that it plays a vital role in cellular physiology.

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