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Purification of microsomal signal peptidase as a complex

(canine pancreatic microsomes/posttranslational precursor processing/gradient sievorptive chromatography/multisubunit membrane protein/glycoprotein subunits)

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ABSTRACT **We report here the purification to near homogeneity of signal peptidase from canine pancreatic microsomes. Purification was monitored using an improved posttranslational assay. A 42-fold enrichment over starting membranes was achieved by selective solubilization in nonionic detergent/high-salt buffer followed by gradient sievorptive anion and cation exchange chromatography, hydroxylapatite chromatography, gel filtration, and sucrose gradient velocity sedimentation. When examined by NaDodSO$_4$/PAGE, the purified enzyme consisted of a complex of six polypeptides with apparent molecular masses of 25, 23, 22, 21, 18, and 12 kDa. The 22- and 23-kDa subunits were shown to be glycoproteins based on their sensitivity to endoglycosidase H and their ability to bind concanavalin A. We suggest that only one subunit of this complex carries out signal peptide cleavage. The structural association of the other subunits in stoichiometric amounts may reflect their requirement in chain translocation across the microsomal membrane.**

Most secretory, lysosomal and some membrane proteins contain a signal sequence that targets polysomes synthesizing them to the endoplasmic reticulum (1, 2) and is endoproteolytically (3, 4) cleaved by signal peptidase during translocation of the nascent chains (5). Signal peptidase is an integral membrane protein (6) whose activity can be monitored after detergent solubilization using a posttranslational cleavage assay (3, 6–8) in which phospholipid is required for activity of the enzyme (8). Several papers have described schemes to enrich microsomal signal peptidase activity (4, 6, 9, 10) but the enzyme has never been identified or purified.

In this paper, we report the purification to near homogeneity of canine microsomal signal peptidase. We present evidence that the enzyme activity is associated with a complex of six polypeptides and that two of these are glycosylated. The significance of these findings for protein translocation across the endoplasmic reticulum membrane is discussed.

MATERIALS AND METHODS

Materials. Hydroxylapatite (Biogel-HTP) was from Bio-Rad. Sephacryl S-300 and Sephadex G-25 fine were from Pharmacia. QAE-Sephadex A-25, SP-Sephadex C-25, peroxidase type IV-labeled Con A and type V-E L-α-phosphatidylcholine (from egg yolk; PtdCho) were from Sigma. NaDodSO$_4$ to be used in signal peptidase assays was from BDH. NaDodSO$_4$ (≈95%) to be used in NaDodSO$_4$/PAGE was from Sigma. Nikkol (octaethyleneglycol mono-*n*-dodecyl ether) was from Nikko Chemicals (Tokyo, Japan). [^{35}S]Methionine (1000 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was from Pierce. Trasylol was obtained from Mobay Chemical (New York). 3,3′-Dimethoxybenzidine dihydrochloride was from Eastman Kodak. *Streptomyces griseus* endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96) was a gift from P. Robbins.

General Procedures. Stock solutions of PtdCho at 10 mg/ml were prepared by drying 5 ml of PtdCho (supplied in 9:1 chloroform/methanol) under a stream of nitrogen in a 37°C water bath followed by resuspension, with mild sonication, in 50 ml of double-distilled water. PtdCho stock solutions were stored at −20°C. All pH adjustments were carried out at 25°C. The pH of triethanolamine and Tris buffers was adjusted with acetic acid. The pH of the Hepes and glycylglycine buffers was adjusted with KOH. The 5 M KOAc stock solution was adjusted to pH 7.5 with acetic acid. The 0.2 M EDTA stock was adjusted to pH 7.5 with NaOH. Sodium phosphate stock solutions were prepared by mixing 0.5 M monobasic and dibasic sodium phosphate solutions to reach the desired pH. All stock solutions were filtered through 0.45 μm Millipore filters. All steps, unless otherwise noted, were carried out at 4°C or on ice. Samples to be stained for monitoring the polypeptide profile were prepared for NaDodSO$_4$/PAGE as described (11), except that the trichloroacetic acid pellets were washed with 95% acetone/5% HCl and the samples were not alkylated; 12% polyacrylamide slab gels were used. Protein assays were performed using the method of Schaffner and Weissman (12).

Preparation of Stripped Microsomal Membranes. Canine rough microsomal membranes (RM) were prepared as described (13) and resuspended at 50 A_{280} units/ml [1 equivalent (equiv)/μl] (13) in RM buffer (0.25 M sucrose/50 mM triethanolamine, pH 7.5/1 mM dithiothreitol), frozen in liquid nitrogen, and stored at −80°C.

EDTA/salt-stripped RM (EK-RM) were prepared as described (11, 13). Two EDTA extractions were performed prior to the high-salt extraction, which was also performed twice. After the second high-salt extraction, the membranes were washed with RM buffer, resuspended to a concentration of 2 equiv/μl (2× EK-RM), frozen in liquid nitrogen, and stored at −80°C.

Step Solubilization. A 25-ml sample of 2× EK-RM was adjusted to a final concentration of 1 equiv/μl in 50 mM KOAc/50 mM triethanolamine, pH 8/0.125 M sucrose/2.5 mM Mg(OAc)$_2$/1% Nikkol/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/"1× PI" (chymostatin, leupeptin, pepstatin, and antipain at 0.1 μg/ml each, and 0.1% aprotinin). Nikkol was added after all other components. After a 15-min incubation on ice, 8.3 ml of this suspension was layered over a step gradient consisting of a 20.5-ml "spacer" layer (50 mM KOAc/50 mM triethanol-

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Abbreviations: RM, rough microsomes; EK-RM, EDTA/KOAc-stripped RM; equiv, equivalent; FT, flowthrough; PtdCho, L-α-phosphatidylcholine; SRP, signal recognition particle.

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amine, pH 8/0.375 M sucrose/1 mM dithiothreitol/1% Nikkol/PtdCho (1 mg/ml)/1 mM phenylmethylsulfonyl fluoride/1× PI) and a 7.5 ml "bottom" layer (0.5 M KOAc/50 mM triethanolamine, pH 8/0.625 M sucrose/1 mM dithiothreitol/PtdCho (1 mg/ml)/1 mM phenylmethylsulfonyl fluoride/1× PI]. The gradients were centrifuged for 5 hr at 27,000 rpm (96,300 × g_{av}) in a Beckman SW 27 rotor. An ISCO gradient fractionator equipped with a UV monitor (A_{280}) was used to fractionate the gradients. The A_{280} peak at the interface between the spacer and bottom steps was collected, divided into aliquots, frozen in liquid nitrogen, and stored at −80°C until use. The total volume from the step solubilization ranged from 51–54 ml, or about 1 equiv/μl.

Anion Exchange Chromatography. The gradient sievorptive technique (14) was used. Step-solubilized extract (105 ml) derived from 50 ml of 2× EK-RM was adjusted to 0.5 M KOAc with 5 M KOAc and to pH 8.3 with 2 M Tris base and loaded by reverse flow (100 ml/hr) onto a 2-liter (5 × 100 cm) column of QAE-Sephadex A-25 previously equilibrated with 20 mM KOAc/125 mM sucrose/0.4% Nikkol/PtdCho (0.4 mg/ml)/20 mM Tris, pH 8.3. The load was followed by 150 ml of 0.5 M KOAc/0.625 M sucrose/0.4% Nikkol/PtdCho (0.4 mg/ml)/20 mM Tris, pH 8.3 and then by 2 liters of 0.5 M KOAc/0.5 M sucrose/0.4% Nikkol/20 mM Tris, pH 8.3. The flowthrough (FT) peak fractions, identified by absorbance at 280 nm prior to the salt gradient, were pooled (260 ml).

SP-Sephadex C-25 Concentration Column. The QAE-Sephadex FT was diluted with an equal volume of 0.4% Nikkol/PtdCho (0.4 mg/ml)/1 mM dithiothreitol/40 mM Hepes and incubated overnight in batch with 20 ml of SP-Sephadex C-25 previously equilibrated with 20 mM KOAc/0.2 M sucrose/0.4% Nikkol/PtdCho (0.4 mg/ml)/1 mM dithiothreitol/20 mM Hepes, pH 7. After incubation, the resin was packed into a 1.5 × 40 cm column, washed with 40 ml of equilibration buffer and eluted (18 ml/hr) with 1 M KOAc/0.2 M sucrose/0.4% Nikkol/PtdCho (0.4 mg/ml)/1 mM dithiothreitol/20 mM Hepes, pH 7. The peak fractions identified by absorbance at 280 nm were pooled (18 ml).

Cation Exchange Chromatography. The gradient sievorptive technique (14) was used. The eluate from the concentration column was adjusted to 0.5 M KOAc with 5 M KOAc and to pH 9.1 with 1 M glycylglycine, pH 13, and applied by reverse flow (20 ml/hr) to a 200-ml (2.5 × 40 cm) column of SP-Sephadex C-25 previously equilibrated with 100 mM KOAc/125 mM sucrose/0.4% Nikkol/PtdCho (0.4 mg/ml)/1 mM dithiothreitol/50 mM glycylglycine, pH 9.1. The load was followed by 20 ml of 0.5 M KOAc/0.35 M sucrose/0.4% Nikkol/PtdCho (0.4 mg/ml)/1 mM dithiothreitol/50 mM glycylglycine, pH 9.1 and, then, by 250 ml of 0.5 M KOAc/0.35 M sucrose/0.4% Nikkol/50 mM glycylglycine, pH 9.1. The FT peak fractions were pooled (40 ml).

Hydroxylapatite Column I. The SP-Sephadex FT was diluted with an equal volume of buffer to a final condition of 100 mM KOAc/15 mM sodium phosphate, pH 6.8/25 mM glycylglycine/0.4% Nikkol/PtdCho (0.4 mg/ml)/62.5 mM sucrose/1 mM dithiothreitol and applied at 1–2 ml/hr to a 0.5-ml hydroxylapatite column previously equilibrated to the load condition. The column was washed with 1 ml of equilibration buffer and eluted with 0.5 M KOAc/0.2 M sodium phosphate, pH 6.8/0.4% Nikkol/PtdCho (0.4 mg/ml)/1 mM dithiothreitol. The eluted peak pool was 1 ml.

Sucrose Gradients. The hydroxylapatite column I eluate (1 ml) was layered on a 33.2-ml gradient of 5–20% sucrose in 0.15 M KOAc/50 mM triethanolamine, pH 8/1 mM dithiothreitol/0.4% Nikkol/PtdCho (0.4 mg/ml). The gradient was centrifuged 10 hr at 49,500 rpm (197,000 × g_{av}) in a Sorvall TV 850 rotor. The gradient was fractionated on an ISCO density gradient fractionator and 1.5-ml fractions were collected. The seven fractions comprising the peak of signal peptidase activity were pooled.

Hydroxylapatite Column II. The sucrose gradient pool was diluted 5-fold with buffer to a final load condition of 100 mM KOAc/20 mM sodium phosphate, pH 6.8/10 mM triethanolamine, pH 8/≈100 mM sucrose/1 mM dithiothreitol/0.4% Nikkol/PtdCho (0.4 mg/ml) and applied at 1–2 ml/hr to a 0.5-ml hydroxylapatite column previously equilibrated to the load condition. After washing with 100 mM KOAc/20 mM sodium phosphate, pH 6.8/1 mM dithiothreitol/5 mM CHAPS/PtdCho (0.08 mg/ml), the column was eluted with 0.5 M KOAc/0.2 M sodium phosphate, pH 6.8/1 mM dithiothreitol/5 mM CHAPS/PtdCho (0.08 mg/ml), and a 1-ml eluate was collected.

Gel Filtration. The hydroxylapatite column II eluate was adjusted to 1 M KOAc/0.1 M sucrose and applied by reverse flow (3.5 ml/hr) to a 1.5 × 100 cm Sephacryl S-300 column equilibrated with 1 M KOAc/50 mM triethanolamine, pH 8.5/50 mM sucrose/1 mM dithiothreitol/5 mM CHAPS/PtdCho (0.08 mg/ml). The column was eluted with 1 M KOAc/50 mM triethanolamine, pH 8.5/150 mM sucrose/1 mM dithiothreitol/5 mM CHAPS/PtdCho (0.08 mg/ml). Fractions comprising the peak of signal peptidase activity were pooled (9 ml) and desalted on Sephadex G-25 into 100 mM KOAc/20 mM sodium phosphate, pH 6.8/1 mM dithiothreitol/5 mM CHAPS/PtdCho (0.08 mg/ml).

Hydroxylapatite Column III. The desalted sample was loaded at 1 ml/hr onto a 0.5-ml hydroxylapatite column equilibrated with 100 mM KOAc/20 mM sodium phosphate, pH 6.8/1 mM dithiothreitol/5 mM CHAPS/PtdCho (0.08 mg/ml) and eluted with 0.25 M KOAc/0.2 M sodium phosphate, pH 6.8/1 mM dithiothreitol/0.4% Nikkol/PtdCho (0.4 mg/ml). The purified signal peptidase (1 ml) was frozen in liquid nitrogen and stored at −80°C.

Signal Peptidase Assay. Wheat germ translation mixtures (15), programmed with bovine pituitary RNA (16) (4 A_{260} units/ml), contained 160 μl of wheat germ extract per 400 μl of mixture and final concentrations of 2 mM $Mg(OAc)_2$ and 110 mM KOAc. The translation mixtures were stored at −20°C.

Standard signal peptidase assay conditions were as follows: 150 mM KOAc/50 mM triethanolamine, pH 8/1 mM EDTA/125 mM sucrose/0.2% Nikkol/PtdCho (0.5 mg/ml)/0.05% $NaDodSO_4$/1× PI. Aliquots of the completed wheat germ translation mixture were brought to 0.2% $NaDodSO_4$ and incubated for 15 min at 56°C immediately prior to addition to the assay mixture. The $NaDodSO_4$-treated translation mixture (5 μl per assay) was added last. The final assay volume was 20 μl. Assay mixtures were incubated for 90 min at 25°C.

After $NaDodSO_4$/PAGE (11) of the assay mixtures on 12% polyacrylamide slab gels and subsequent autoradiography, processing by signal peptidase was quantitated by cutting out the relevant bands and determining their radioactivity in a scintillation counter as described (11). Percent processing was determined by the following equation: [(8/7) (prolactin cpm − background cpm)]/{(preprolactin cpm − background cpm) + [(8/7) (prolactin cpm − background cpm)]} × 100.

Con A Blots. Gels were electrophoretically transferred to nitrocellulose as described by Towbin *et al.* (17). After electroblotting, the nitrocellulose was blocked in "Con A blot buffer" (0.5 M NaCl/50 mM Tris·HCl, pH 7.5/2.5 mM $MgCl_2$/1 mM $CaCl_2$/0.5% Tween 20) and incubated for 3 hr with horseradish peroxidase-conjugated Con A diluted 1:2000 in blot buffer. The blots were washed three times with excess Con A blot buffer for 30 min each and twice with 10 mM Tris·HCl, pH 7.5, for 2 min. Color was developed by incubation with a freshly prepared solution of substrate [3,3'-dimethoxybenzidine dihydrochloride (0.25 mg/ml)/10 mM Tris·HCl, pH 7.5/0.005% H_2O_2], for 1–10 min. The reaction was stopped by rinsing the blot in water. All incubations were done at room temperature.

Endoglycosidase H Treatment. Signal peptidase (430 equiv hydroxylapatite I eluate) was incubated with 0.055 unit of endoglycosidase H for 22 hr as described (18). Control incubations of signal peptidase and endoglycosidase H alone were carried out under the same conditions.

RESULTS

The purification of canine pancreatic microsomal signal peptidase was accomplished by selective solubilization, gradient sievorptive anion and cation exchange chromatography, hydroxylapatite chromatography, sucrose gradient velocity sedimentation, and gel filtration. Fractions from each step were monitored by activity assays (Table 1) and by $NaDodSO_4$/PAGE (Fig. 1). Brief descriptions of the assay and each purification step follow.

Posttranslational Assay. A posttranslational cleavage assay similar to that described by Jackson (19) was used to follow the purification. The assay involves incubating completed [^{35}S]methionine-labeled preprolactin (synthesized in a wheat germ cell-free translation system programmed with bovine pituitary RNA) with various fractions in 0.2% Nikkol and PtdCho at 0.5 mg/ml. Pretreatment of the substrate with 0.2% $NaDodSO_4$ caused a several-fold enhancement of observed processing (data not shown), probably by increasing the exposure of the cleavage site (6). Percent processing was linear with respect to added peptidase up to about 35% processing, although it was possible to obtain as much as 70% processing if sufficient peptidase was added (data not shown). Assays done to assess the degree of purification were performed in the linear region.

Solubilization of Signal Peptidase. Canine pancreatic rough microsomal membranes were extensively washed with EDTA and high-salt buffers to remove extrinsic proteins and ribosomes. Generally, 60% of protein was removed by these washes without loss of activity (Table 1). Signal peptidase, like the signal recognition particle (SRP) receptor (20), was solubilized by Nikkol only in the presence of high salt concentrations. In contrast, many EK-RM proteins were solubilized by 1% Nikkol in the presence of low salt concentrations. We capitalized on the insolubility of signal peptidase in Nikkol and low salt to effect a selective solubilization. EK-RM were incubated with 1% Nikkol/low-salt buffer and layered on a sucrose step gradient. The upper step served as a spacer and was also 1% Nikkol/low salt. The lower step was 1% Nikkol/high salt. Both steps contained PtdCho at 1 mg/ml. After centrifugation and fractionation two major A_{280} peaks were seen. Proteins solubilized by Nikkol at low salt remained at the top of the gradient. Proteins insoluble at low salt concentrations quickly sedimented as large aggregates,

Table 1. Purification balance sheet

Sample	Total activity,* units	Protein, mg	Specific activity, units/mg
RM	400,000	655	611
EK-RM	400,000	270	1,482
Solubilized peak	246,750	92	2,682
QAE-Sephadex FT	156,000	37	4,216
SP-Sephadex concentration column	79,926	21.2	3,767
SP-Sephadex FT	46,539	3.56	13,072
Hydroxylapatite column I	36,188	1.58	22,904
Sucrose gradient	29,403	0.85	34,633
Hydroxylapatite column III	8,773	0.34	25,736

*One unit of activity is defined as the amount of enzyme that gives 20% processing of preprolactin to prolactin under the standard assay conditions. One microliter of RM contains 4 units of activity and roughly corresponds to 1 mg of pancreas tissue.

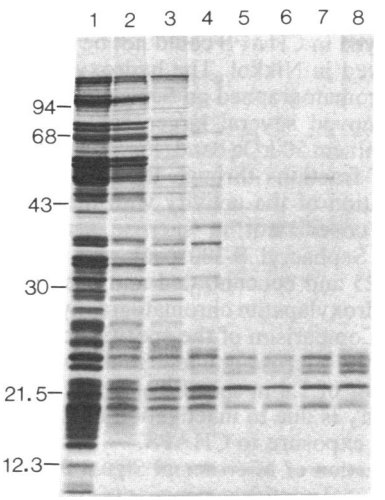

FIG. 1. Purification of signal peptidase activity as monitored by $NaDodSO_4$/PAGE and Coomassie blue staining. Lanes: 1, 15 equiv, RM; 2, 15 equiv, EK-RM; 3, 25 equiv, step-solubilized Nikkol/high-salt pool; 4, 40 equiv, QAE-Sephadex FT pool; 5, 250 equiv, SP-Sephadex FT pool; 6, 250 equiv, hydroxylapatite column I eluate; 7, 750 equiv, sucrose gradient peak pool; 8, 2000 equiv, hydroxylapatite column III eluate. Positions of molecular mass markers (kDa) are indicated. Equivalents of various fractions through the purification are defined with respect to RM (i.e., 1 equiv is that material that is derived from 1 μl of RM).

until becoming solubilized in the high-salt/Nikkol step. In a typical solubilization, approximately 40% of total protein was found in the load zone, 7% in the low-salt step, and 35–40% in the high-salt step, with the remainder pelleting (data not shown). Sixty to 70% of signal peptidase activity loaded was recovered in the high-salt step. The addition of PtdCho to the step gradient resulted in an approximately two-fold higher yield of activity (unpublished results). In light of this, and the requirement of PtdCho for signal peptidase activity (8), it was included in all our buffers. High-salt/EDTA stripping and step solubilization of RM resulted in 86% removal of the initial protein with a 62% recovery of activity, giving a 4.4-fold purification (Table 1).

Ion Exchange Chromatography. Gradient sievorptive chromatography (14) was used because conventional ion exchange chromatography resulted in low yields and poor resolution. The step-solubilized EK-RM sample was subjected to gradient sievorptive anion exchange chromatography at pH 8.3 on QAE-Sephadex A-25. The FT peak (20 mM salt) contained 63% of the activity and 40% of the protein loaded for a 1.6-fold purification (Table 1). After concentration, the sample was subjected to gradient sievorptive cation exchange chromatography at pH 9.1 on SP-Sephadex C-25. The FT peak (100 mM salt) contained 17% of the protein and 58% of the activity loaded, giving a 3.5-fold purification (Table 1).

Hydroxylapatite Column Chromatography I. A hydroxylapatite column was used to concentrate the SP-Sephadex FT. This step resulted in a 1.75-fold purification (Table 1), in addition to the 40-fold concentration achieved.

Sucrose Gradient Sedimentation. The hydroxylapatite peak pool was subjected to velocity sedimentation through 5–20% sucrose gradients. Signal peptidase activity cosedimented precisely and uniquely with six polypeptides ranging in molecular mass from 12 kDa to 25 kDa. This step gave a 1.5-fold purification (Table 1).

Gel Filtration. To prepare samples for gel filtration, the sucrose gradient peak was concentrated and exchanged into CHAPS buffer by hydroxylapatite chromatography (hydroxylapatite column II) because the large Nikkol micelle size (120 monomers per micelle) would be expected to obscure

small differences between proteins. The specific activity of fractions assayed in CHAPS could not be compared directly to those assayed in Nikkol. The hydroxylapatite column II eluate was chromatographed on Sephacryl S-300 in 1 M salt. This step removed several larger contaminants including most of a prominent 50-kDa band (Fig. 1, compare lanes 7 and 8). Assays of fractions through the elution profile clearly showed coelution of the activity with the six polypeptides observed to cosediment in sucrose gradients (data not shown). The Sephacryl S-300 peak was then desalted on Sephadex G-25 and concentrated and exchanged back into Nikkol by hydroxylapatite chromatography (hydroxylapatite column III). Comparison of the specific activity of this final fraction with the sucrose gradient peak pool shows a reduction by a factor of 1.4 in specific activity. This reduction in specific activity is due to inactivation of the enzyme caused by prolonged exposure to CHAPS.

Characterization of Microsomal Signal Peptidase. Purified microsomal signal peptidase consists of six polypeptides with apparent molecular masses of 12, 18, 21, 22, 23, and 25 kDa (Fig. 1, lane 8, and Fig. 2). Coomassie blue staining intensity of the 12-kDa band varied, even in duplicate lanes. This is probably due to variable precipitation and staining of this polypeptide. Analysis of signal peptidase by sucrose density gradient sedimentation showed exact cosedimentation of all six polypeptides with the processing activity (Fig. 2) at about 4 S. Monomers of the individual polypeptides would be expected to have substantially lower sedimentation coefficients.

Recently, we discovered that the 22- and the 23-kDa polypeptides are glycoproteins. Treatment of purified peptidase with endoglycosidase H reduced the molecular masses of these two polypeptides to 19 kDa (Fig. 3*B*, compare lanes 1 and 2). Further evidence that these bands are glycoproteins was obtained by probing protein blots of fractions throughout the purification with horseradish peroxidase-conjugated Con A (Fig. 3*A*). After the cation exchange step, the only glycoproteins remaining were the 22- and 23-kDa polypeptide components of the complex. Thus, it should be possible to replace the sucrose gradient and Sephacryl S-300 steps with a lectin column. Preliminary results of experiments to develop a simplified isolation protocol using Con A-Sepharose suggest that all six polypeptides can bind to such a column and be eluted with methyl α-D-mannoside (data not shown).

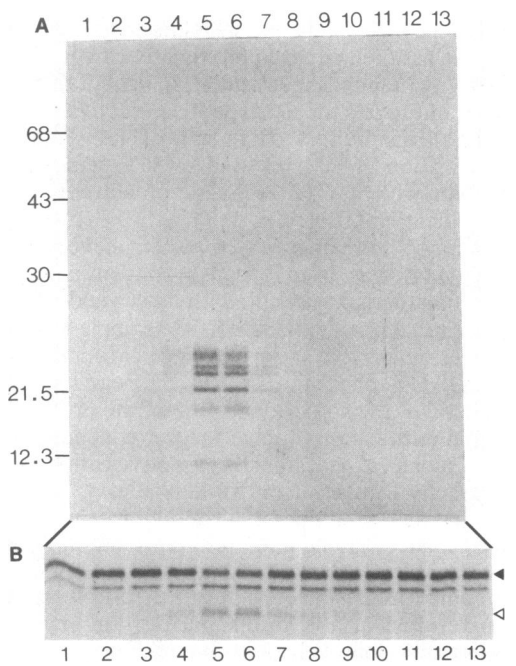

FIG. 2. Sucrose gradient sedimentation of purified signal peptidase. Two hundred microliters of hydroxylapatite column III eluate was layered on a 3-ml gradient of 5–20% sucrose in 0.15 M KOAc/50 mM triethanolamine, pH 8/1 mM dithiothreitol/0.4% Nikkol/PtdCho (0.4 mg/ml) and centrifuged for 17 hr at 49,900 rpm (234,000 × g_{av}) in a Beckman SW 50.1 rotor. Thirteen fractions were collected using an ISCO gradient fractionator. (*A*) The fractions were visualized by NaDodSO$_4$/PAGE and Coomassie blue staining. Positions of molecular size markers (kDa) are indicated. (*B*) An aliquot (4 μl) or each fraction was assayed for signal peptidase activity. ▲, Position of preprolactin; △, position of prolactin.

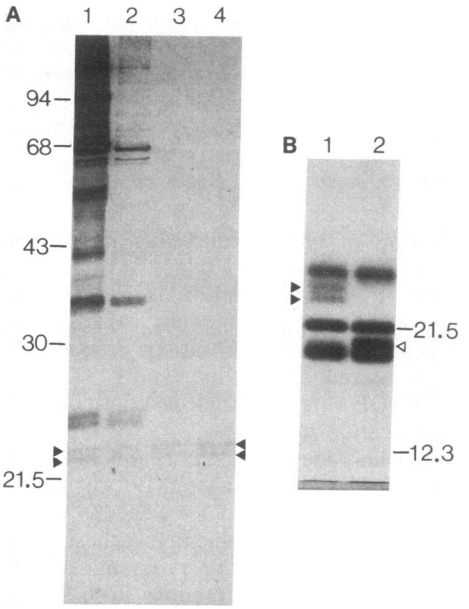

FIG. 3. Two polypeptide components of signal peptidase are glycosylated. (*A*) A protein blot of fractions from various stages of the purification was probed with horseradish peroxidase-conjugated Con A. Lanes: 1, 25 equiv, EK-RM; 2, 25 equiv, step-solubilized high-salt/Nikkol pool; 3, 25 equiv, QAE-Sephadex FT pool; 4, 150 equiv, SP-Sephadex FT pool. Positions of molecular mass markers (kDa) are indicated on the left. ▲, 22- and 23-kDa subunits of signal peptidase. (*B*) Endoglycosidase H treatment of signal peptidase. Partially purified signal peptidase was incubated in the absence (lane 1) and presence (lane 2) of endoglycosidase H. NaDodSO$_4$/PAGE/Coomassie blue staining of a control incubation of endoglycosidase H alone produced no visible bands in the region of the complex (data not shown). ▲, 22- and 23-kDa polypeptides of the complex; △, the 19-kDa band resulting from endoglycosidase H treatment. Positions of molecular mass markers (kDa) are indicated on the right.

DISCUSSION

We have described the purification to near homogeneity and the initial characterization of the canine microsomal signal peptidase. Enzyme activity was followed during purification using an improved posttranslational cleavage assay similar to that developed by Jackson (19). RM were stripped with high salt concentrations and EDTA, and the peptidase was selectively solubilized with Nikkol/high-salt buffer. After solubilization, anion and cation exchange chromatography, hydroxylapatite chromatography, sucrose gradient velocity sedimentation, and gel filtration steps were performed. When analyzed by NaDodSO$_4$/PAGE, the purified signal peptidase activity consists of six polypeptides ranging from 12 to 25 kDa. The 22- and 23-kDa polypeptides were shown to be glycoproteins by their endoglycosidase H susceptibility and their ability to bind Con A. In all purification steps, including sucrose gradient sedimentation and gel filtration in 1 M salt, the six polypeptides copurified with signal peptidase activity. In fact, we have yet to find a nondenaturing treatment that

will cause dissociation. Another line of evidence for the existence of a complex is the fact that all six polypeptides could be specifically bound to and eluted from a lectin affinity column even though only two polypeptides are glycosylated (data not shown).

PtdCho was added to all column buffers. Purification of several integral membrane proteins has been facilitated by the use of mixed detergent/phospholipid micelles (21–25). Interestingly, it has been shown (22, 25) that deletion of phospholipid from buffers caused two multisubunit membrane protein complexes to dissociate. We have not shown directly that this phenomenon occurs with the signal peptidase complex, but it is an intriguing possibility and might be a good way to separate the subunits without denaturation.

The subunit stoichiometry of the signal peptidase complex is uncertain at this point. Conversion of the 22- and the 23-kDa polypeptides by endoglycosidase H digestion to one band, which migrates slightly more slowly than the 18-kDa band, raises the possibility that they could be differentially glycosylated forms of one polypeptide, perhaps the 18-kDa band itself. Alternatively, they could be glycosylated forms of two different proteins that happen to have the same molecular weight. Peptide mapping and amino acid sequencing of these polypeptides would clarify this ambiguity and also rule out the possibility that one or more of these polypeptides are degradation products that nevertheless remain associated with the complex. We consider proteolytic degradation unlikely because a myriad of protease inhibitors was present during the membrane isolation and solubilization steps and because the ratio of all six polypeptides to each other remained constant throughout the purification. In addition, the population of complexes may be heterogeneous. For example, half the complexes may contain the 23-kDa polypeptide and the other half contain the 22-kDa polypeptide as their sole glycoprotein. Keeping the above caveats in mind, we propose that the purified signal peptidase consists of at least four but not more than six polypeptides.

Bacterial signal peptidase I (26) and II (27) consist of only one polypeptide each. Signal peptidase I (leader peptidase) can cleave eukaryotic preproteins (28). Bacterial preproteins can be cleaved by the eukaryotic peptidase (29). It is therefore likely that only one subunit of the isolated complex carries out signal peptide cleavage, with the other subunits performing related functions. The other polypeptides may be different processing enzymes, such as a putative signal peptide peptidase (30) and/or enzymes involved in glycosylation. Alternatively, this complex may represent all or part of a hypothetical pore (1, 31) through which the nascent chain crosses the membrane. Based on Coomassie blue staining intensities, we have estimated that each of the polypeptide chains of the signal peptidase complex is severalfold more abundant than either SRP (2) or the SRP receptor (20) and is roughly equivalent in amount to bound ribosomes (data not shown). If only catalytic amounts of the complex had been observed, as with the SRP receptor, then it could not function as a pore. According to the signal hypothesis (1, 31), a pore is present during the entire process of chain translocation. Polypeptide components of this putative pore would therefore be present in approximately stoichiometric amounts with bound ribosomes.

The complex we have isolated may only be the core of a larger complex containing other components, either transiently or permanently associated. Among the permanently associated components might be the ribophorins (32), which have been hypothesized to participate in translocation and are also present in stoichiometric amounts with bound ribosomes. A transiently associated component might be the SRP receptor (2), which has been shown to participate in translocation and is present in substoichiometric amounts compared to bound ribosomes. Both the ribophorins and the SRP receptor are separated from signal peptidase early in the purification (data not shown).

Assaying lipid vesicles reconstituted with the purified signal peptidase complex and the SRP receptor for their translocation competence would determine whether these components are necessary and sufficient to translocate nascent preproteins across the microsomal membrane.

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