Two Subunits of the Canine Signal Peptidase Complex Are Homologous to Yeast SEC11 Protein*

Gregory S. Shelness and Günter Blobel
From the Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, New York, New York 10021

Canine microsomal signal peptidase activity was previously isolated as a complex of five subunits (25, 22/22, 21, 18, and 12 kDa). Two of the signal peptidase complex (SPC) subunits (23/23 and 21 kDa) have been cloned and sequenced. One of these, the 21-kDa subunit, was observed to be a mammalian homolog of SEC11 protein (Sec11p) (Greenburg, G., Shelness, G. S., and Blobel, G. (1989) J. Biol. Chem. 264, 15762-15765) a gene product essential for signal peptide processing and cell growth in yeast (Böhní, P. C., Desh-qiès, R. J., and Schekman, R. W. (1988) J. Cell Biol. 106, 1035-1042). cDNA clones for the 18-kDa SPC subunit have now been characterized and found to encode a second SEC11 homolog. Both the 18- and 21-kDa canine SPC subunits are integral membrane proteins by virtue of their resistance to alkaline extraction. Upon detergent solubilization, both proteins are found in a complex with the 22/23kDa SPC subunit, the only SPC subunit containing N-linked oligosaccharide. No steady-state pool of canine Sec11-like monomers is detected in microsomal membranes. Alkaline extraction of microsomes prior to solubilization or solubilization at alkaline pH causes partial dissociation of the SPC. The Sec11p-like subunits displaced from the complex under these conditions demonstrate no signal peptide processing activity by themselves. The existence of homologous subunits is common to a number of known protein complexes and provides further evidence that the association between SPC proteins observed in vitro may be physiologically relevant to the mechanism of signal peptide processing and perhaps protein translocation.

Most proteins destined for the secretory pathway contain amino-terminal signal peptides which specify their targeting to the ER membrane. Targeting is achieved by the interaction of ribosomes synthesizing secretory preproteins with the signal recognition particle (SRP). The ribosome-nascent chain-SRP complex is subsequently directed to the ER membrane via an interaction between SRP and the SRP receptor (docking protein), an integral ER membrane protein (for review, see Walter and Lingappa, 1989). Following the SRP receptor-mediated, GTP-dependent clamping of SRP (Comste and Gilmore, 1989), the signal peptide portion of the nascent chain is thought to interact with an ER membrane-associated signal sequence receptor (Wiedmann et al., 1987).

Although many of the components responsible for the targeting of proteins to the ER membrane have been identified, little is known of the factors or mechanisms responsible for the translocation of proteins across the ER membrane. We have focused attention on signal peptidase, an enzymatic activity which cleaves the amino-terminal signal peptide from secretory preproteins. This activity is an integral component of the ER membrane (Lively and Walsh, 1983) and is thought to act during or shortly after translocation (Blobel and Dobberstein, 1975). For this reason, the protein(s) involved in signal peptide processing may reside at protein translocation sites and associate with other components of the translocation apparatus.

Consistent with this possibility, canine microsomal signal peptidase activity was purified as a complex of five proteins with molecular masses of 26, 22/22, 21, 18, and 12 kDa (Evans et al., 1986a). By analogy to E. coli, where a single polypeptide chain leader peptidase (signal peptidase I) (Zwinzinski and Wickner, 1986; Wolfe et al., 1983) is sufficient to catalyze signal peptide processing of both prokaryotic and eukaryotic preproteins (Watts et al., 1983), it was suggested that only one or a subset of canine SPC subunits catalyzes signal peptide cleavage and that the remaining proteins play other roles related to protein translocation (Evans et al., 1986a).

To analyze the structure and function of the canine SPC, cDNA cloning of the individual subunits was undertaken. The deduced amino acid sequence of the glycosylated 22/23-kDa SPC subunit (SPC 22/23) (Shelness et al., 1988) was found to have homology to tryptic peptides derived from the hen oviduct signal peptidase glycoprotein, one of two possible proteins thought to catalyze signal peptide processing in the avian system (Baker and Lively, 1987). In addition, the amino acid sequence of SPC 23/23 indicated a single transmembrane segment with most of the protein mass located on the luminal side of the ER membrane (Shelness et al., 1988), consistent with the known ER luminal localization of the signal peptidase active site (Walter et al., 1979). Cloning and sequencing of the 21-kDa SPC subunit (SPC 21) (Greenburg et al., 1989) allowed its identification as a mammalian homolog of Sec11p (Böhní et al., 1988), a gene product required, but not necessarily sufficient, for signal peptide processing in yeast. Neither cloned protein demonstrated detectable amino acid sequence similarity to E. coli leader peptidase.
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In this report, the isolation and characterization of the cDNA for the 18-kDa signal peptide complex subunit (SPC 18) is described. The cDNA-derived amino acid sequence of SPC 18 was found to be strikingly similar to that of SPC 21. However, the canine SPC contains two yeast Sec11p homologs. Antisynthetic peptide antibodies specific for the 18-, 21-, and 22/23-kDa SPC subunits were used to examine the association of signal peptide complex proteins with the ER membrane and with each other. These results indicate that the 18- and 21-kDa Sec11p-like subunits of the canine SPC function exclusively as part of a hetero-oligomeric integral membrane-protein complex.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources: avian myeloblastosis virus reverse transcriptase, Life Sciences, Inc.; restriction enzymes, Boehringer Mannheim or New England BioLabs, Inc.; T4 DNA polymerase, E. coli DNA ligase, RNase H, and ConA-Sepharose 4B, Pharmacia LKB Biotechnology Inc.; λ in vitro packaging extract (Gigapack Giant), Strategen; modified T7 DNA polymerase (Sequenase), T7 RNA polymerase, and T4 DNA polymerase (Bethlehem, Inc., Gaithersburg, PA) which had been frozen in liquid nitrogen and stored at −80 °C. In some instances, use of frozen tissue gave rough microsomes which incompletely pelleted through a 1.3 M sucrose cushion (140,000 × g, 2.5 h) perhaps due to RNA degradation. In this case, the 1.3 M sucrose cushion was diluted 4-fold with homogenization buffer containing 0.25 M sucrose (Walter and Blobel, 1983) and centrifuged at room temperature, followed by a 10-min incubation at 15 °C (Baker et al., 1983). RNase treatment was performed exactly as described (Shelness et al., 1988). Solubilization of rough microsomes-Rough microsomes were washed with salt and EDTA as described previously (Walter and Blobel, 1983; Evans et al., 1986a). Solubilization of salt- and EDTA-washed rough microsomes—Salt- and EDTA-washed rough microsomes (5 ml, 2 eq/μl) at pH 11 (Fig. 8, condition 2) was performed as described previously (Shelness et al., 1988) and screened in an equal volume of ice-cold 0.1 M sodium carbonate, pH 11.5, using a Dounce homogenizer. The suspended membranes were further diluted with 0.1 M sodium carbonate, pH 11.5 (final volume of 160 ml), and pelleted as described above. The suspension was then frozen in liquid nitrogen and stored at −80 °C.

Polymerase Chain Reaction—First-strand cDNA synthesis was performed as described (Shelness and Williams, 1984), except that 4 μg of canine liver poly(A) RNA (Shelness et al., 1988) were used as template and 5 μg of unlabeled, partially degraded SPC 18 antisense oligodeoxynucleotide II (Fig. 1B) were used as a primer. Annealing was for 1 h at 45 °C; the extension reaction contained 10 μl of [α-32P]dCTP (3000 Ci/mmol; Du Pont-New England Nuclear). Following polymerase chain reaction, the reaction was ethanol-precipitated, and the dried pellet was dissolved in 50 μl of deionized water. Twenty-five μl of the reaction were subsequently subjected with 8 μg of oligo dT (5 μg of oligo dT II and subjected to PCR amplification using buffer conditions suggested by the enzyme supplier (AmpliTag, United States Biochemical Corp.) After 25 cycles of denaturation (94 °C, 1.5 min), annealing (42 °C, 2.5 min), and extension (72 °C, 3.0 min), (Perkin-Elmer-Cetus thermal DNA cyclers), the PCR amplification products were extracted with buffer-saturated phenol-chloroform (50:50), precipitated with ethanol, and digested with restriction endonucleases EcoRI and Sau3A following size fractionation on a 3% NuSieve G-agarose gel (FMC BioProducts) containing 0.5 μg/ml ethidium bromide, the amplification products were visualized under 450 nm UV light, and the region of the gel containing fragments of ~90 base pairs was excised and recovered by phenol-chloroform extraction of the melted gel slice. The PCR products were cloned into the EcoRI-Sau3A sites of pUC19 and sequenced.

Construction and Screening of cDNA Libraries—An unmodified canine liver cDNA library was constructed in Agt10 (Hygn et al., 1985) as described previously (Shelness et al., 1988) and screened with a 5′-32P-labeled oligonucleotide probe corresponding to the sequence (Fig. 1D) of the SPC 18 PCR amplification product (Fig. 1D). The hybridization and wash conditions were as described previously (Shelness et al., 1988). The hybridization buffer contained no dextran sulfate, and the concentrations of bovine serum albumin, Ficoll, and polyvinylpyrrolidone were 0.1% (5 × Denhardt's solution).

DNA Sequence Analysis—DNA sequencing of inserts from Agt10 clones were subcloned into pBluescript ISK(+) (Strategene). Plasmids were purified by CsCl density gradient centrifugation or with Qiagen plasmid purification cartridges and sequenced as described previously (Shelness et al., 1988).

Antisynthetic Peptide Antibodies—The following peptides specific for SPC subunit domains were synthesized on an Applied Biosystems 430A peptide synthesizer using β-butyrolactone chemistry and HF cleavage: RGDNPKLLKDDMTKCR (residues 112–126 of SPC 22/23), VRAGAVTGLPASGLC (residues 2–17 of the SPC 21 primary translation product), and SLDFLDDVRRMRRKR (residues 3–17 of SPC 18). The peptides were conjugated via the carboxyterminal cysteine residues (not present in the native protein sequence) to keyhole limpet hemocyanin (Calbiochem) using the heterobifunctional cross-linker-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Chemical Co.) (Green et al., 1982). Approximat-
Signal Peptide Assay—Detergent-solubilized signal peptide was assayed using the method of Jackson and Blobel (1977) as modified by Evans et al. (1986a). Bovine preprotease mRNA was synthesized in vitro from plasmid pGEM-BP1 using T7 RNA polymerase (Nicchita and Blobel, 1989). Approximately 12 μg of in vitro synthesized mRNA was translated in a 500-μl reaction containing 200 μl of reticulocyte lysate and 50 μl of [35S]methionine (1000 Ci/mmol; Du Pont-New England Nuclear). The preprotease was activated by the addition of 2 volumes of saturated ammonium sulfate; and after a 30 min incubation on ice, the precipitate was collected by centrifugation at 12,000 × g for 10 min at 4°C. The pellet was dissolved in 100 μl of 110 mM KOAc, 20 mM triethanolamine, pH 7.5, 2 mM Mg(OAc)2, 1 mM DTT and loaded on a Sephacryl G-25 (medium) column (0.5 × 10 cm) to remove residual ammonium sulfate. A column was pre-equilibrated and eluted in the same buffer as described above. The void volume fractions were pooled, aliquoted, and stored at −20°C. The reaction conditions used to preprotease to prolactin were identical to those of Evans et al. (1986a), except that the final DTT concentration was 20 mM (Baker and Lively, 1987).

RESULTS

Amino Acid Sequencing—Purified signal peptide complex (50 μg) (Evans et al., 1986a) was fractionated by preparative 12% SDS-PAGE. The 18-kDa protein was electrophoresed and subjected to automated Edman degradation. Two amino acids were detected at most cycles, indicating the presence of a second amino terminus. The 18-kDa protein was then purified by a procedure described previously (Shelness et al., 1988) in which rough microsomes were washed with salt and EDTA, detergent-solubilized, and subjected to ConA-Sepharose affinity chromatography, followed by hydroxylapatite chromatography in the presence of 0.1% SDS (Moss and Rosenblum, 1972). Sample preparation for the SDS-hydroxylapatite column involved denaturation at 70°C in the presence of 2% SDS, 0.1 M DTT. Following this treatment, the 18-kDa protein appeared to elute from the hydroxylapatite column as a single discrete peak. However, when the 18-kDa protein contained in these fractions was gel-purified and subjected to automated Edman degradation, the same double sequence observed previously was obtained. Only after performing reverse-phase chromatography as described under "Experimental Procedures" was a single amino acid sequence obtained (Fig. 1). This sequence was similar but not identical to the deduced amino acid sequence of SPC 21 (Greenburg et al., 1989) between residues 12 and 43. By subtraction, the amino-terminal 15 amino acids of the second protein were also determined. This sequence demonstrated no similarity to proteins in the National Biomedical Research Foundation Protein Identification Resources Database including ConA.

Polymersize Chain Reaction—A pair of partially degenerate PCR oligonucleotides were synthesized based on the amino-terminal amino acid sequence of HPLC-purified SPC 18 (Fig. 1B, oligonucleotides I and II). Oligonucleotide II was used to prime cDNA synthesis on poly(A) selected canine liver RNA. The cDNA was then subjected to 25 rounds of PCR amplification (Saiki et al., 1988; Lee et al., 1988) using oligonucleotides I and II in combination (Fig. 1; see "Experimental Procedures"). The products were fractionated by gel electrophoresis, and DNA migrating in the expected size range of ~90 base pairs was eluted and cloned into plasmid vectors. Four PCR clones of the proper size were sequenced (two each from two independent PCR experiments) and found to encode the known amino-terminal amino acid sequence of SPC 18 (Fig. 4C).

dDNA Cloning and Characterization—A synthetic oligonucleotide corresponding to the sense strand of the SPC 18 PCR product (Fig. 1 D) was used to screen an unamplified canine liver cDNA library cloned in agt10. Two positive clones (XL50 and XL51) were identified and sequenced. Fig. 2 shows the sequence of an 847-nucleotide region of overlap between the clones. RNA blot analysis using a portion of the SPC 18 cDNA coding region as a probe (Fig. 3) revealed a single species of mRNA with an approximate size of 850 nucleotides. Assuming a 150-nucleotide poly(A) tail, SPC 18 mRNA appears to migrate slightly faster than predicted from the length of the cDNA. Nevertheless, this analysis indicates that the cDNA sequence shown in Fig. 2 probably represents most of the sequence contained within the mRNA.

The 5′-border of the SPC 18 cDNA open reading frame (coordinate +1) was identified by comparison with the amino-terminal amino acid sequence of the native protein derived by direct protein sequencing (Fig. 1A). This open reading frame gives rise to a polypeptide of 179 amino acids and a calculated molecular mass of 20,626 Da. This value is ~2,600 Da higher than that estimated by SDS-PAGE (Evans et al., 1986a) but is internally consistent with the gel migration of SPC 22/23, the differentially glycosylated SPC subunit. SPC 22/23 has a calculated molecular mass of 20,314 Da (Shelness et al., 1988) and, upon endoglycosidase H treatment, virtually co-migrates with SPC 18 (Evans et al., 1986a). In contrast, the gel mobility of SPC 21 is relatively consistent with its calculated molecular mass of 21,585 Da (Greenburg et al., 1989).

Further analysis of the cDNA sequence reveals that SPC 18 is probably a primary translation product rather than a processed precursor. The sequence upstream of nucleotide +1 is in good agreement with the consensus sequence (CCRCGAUG) found at most sites of translation initiation (Kozak, 1984). In addition, the cDNA would potentially encode 2 in-frame prolines at positions −9 and −11, a primary structure inconsistent with signal peptide function (von Heijne, 1986).

The DNA-derived amino acid sequences of SPC 18 and SPC 21 (Greenburg et al., 1989) were found to be strikingly similar (Fig. 4). Between residues 4 and 79, there are no amino acid insertions or deletions; identity exists at 143 of 176 positions (81%). Of the 33 mismatches in this region, 27 are conservative based on structural similarity or genetic interconvertibility (SG matrix values of 4 or 5) (Feng et al., 1979).

Fig. 1. Strategy for synthesis of SPC 18-specific PCR amplification probe. A, amino-terminal amino acid sequence of SPC 18 determined by Edman degradation; B, partially degenerate sense oligonucleotide (J) and antisense oligonucleotide (II) PCR primers; C, nucleotide sequence of SPC 18-specific PCR amplification product; D, sense strand oligonucleotide used to screen unamplified canine liver cDNA library. Lower-case letters in B and D indicate linker sequences.
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Fig. 3. Northern blot analysis. Four µg of Madin-Darby canine kidney cell (lane 1) and canine liver (lane 2) poly(A)-selected RNAs were fractionated on a formaldehyde-agarose (1%) gel and transferred to nitrocellulose (Thomas, 1980). The nitrocellulose membrane was probed with a 32P-labeled, randomly primed (Feinberg and Vogelstein, 1983) AccI-PvuII restriction fragment (coordinates 52-565; see Fig. 2). The same hybridization conditions used for X plaque hybridization (see “Experimental Procedures”) were employed; however, the final two washes were performed at 55 °C for 30 min each. The mobilities of RNA size standards (Bethesda Research Laboratories) are indicated on the left (in kilobases).

1985). The most dramatic primary structure differences are the substitution of 4 closely spaced acidic residues in SPC 18 with neutral or basic amino acids in SPC 21 (Asp to Gin, Asp to His, Glu to Ala and Glu to Gln). Since SPC 21 was previously identified as a mammalian homolog of Sec1lp (Greenburg et al., 1989), it appears that the canine SPC contains at least two yeast Sec1lp-like subunits. Despite the extensive amino acid sequence similarity, the cDNA sequences of SPC 18 and SPC 21 demonstrate considerable variability at degenerate codon positions and virtually complete divergence in the untranslated regions. Therefore, it appears that the 18- and 21-kDa SPC subunits are the products of separate genes. The fact that both proteins appear in all rough microsomal preparations examined to date also tends to eliminate the possibility that SPC 18 and SPC 21 are the products of codominant parental alleles.

As expected for two proteins with highly related amino acid sequences, the hydropathy plots for SPC 21 (Greenburg et al., 1989) and SPC 18 (Fig. 5) are very similar. Most notably, both the 18- and 21-kDa canine proteins and Sec1lp contain two distinctly hydrophobic domains. One stretch is located near the amino terminus (SPC 18 residues 17-57) and contains 40 predominantly hydrophobic residues. The amino terminal part (residues 17-36) of this hydrophobic domain is...
flanked by basic residues and is of sufficient length to serve as a transmembrane anchor. A second hydrophobic domain is located between SPC 18 residues 144 and 176. However, this segment is interrupted by 3 charged residues at positions 158, 161, and 163 and therefore is unlikely to function as a classical transmembrane anchor. Assuming that the amino-terminal hydrophobic domain spans the membrane once and that SPC 18 lacks a cleavable signal peptide, it is likely that the protein is oriented with a small amino-terminal domain facing the cytoplasm and the bulk of the remaining protein mass situated on the luminal side of the ER membrane. This topology is similar to what is predicted for SPC 22/23 (Shelness et al., 1988), the glycosylated signal peptidase subunit, and is consistent with the known ER luminal localization of the signal peptidase active site (Walter et al., 1979). The lack of glycosylation sites in SPC 18 to confirm localization of protein domains to the ER lumen, however, makes this prediction tentative. In addition, the length of the two hydrophobic segments as well as the distribution of charged and polar residues may indicate a more complex and dynamic interaction with the ER membrane than can be predicted from primary structure alone.

Both Sec11p-like Subunits of SPC Are Integral Membrane Proteins—Signal peptidase activity is an integral component of the ER membrane (Lively and Walsh, 1983). It is not known, however, whether all of the individual canine signal peptidase complex subunits are integral membrane proteins. This question has become critical in light of the apparent specificity of each antibody was established by reacting the post-immune sera with Western blot lanes containing either 4 µg of purified signal peptidase complex (B and C, lanes C) or 0.5 µg of gel-purified SPC 22/23 (A, lane O). The serum dilutions were 1:1000 (A), 1:250 (B), and 1:100 (C). Immunoblotting techniques were performed according to Watanabe and Blobel (1989).

homologous subunits identified in dog are removed by such treatment. To examine this and other questions concerning the structure of the canine SPC, antisynthetic peptide antibodies were raised against the signal peptidase glycoprotein and the 18- and 21-kDa Sec11p homologs. These antisera were separately reacted with canine SPC, antisynthetic peptide antibodies were raised with the known ER luminal localization of the signal peptide active site (Walter et al., 1979). The lack of glycosylation sites in SPC 18 to confirm localization of protein domains to the ER lumen, however, makes this prediction tentative. In addition, the length of the two hydrophobic segments as well as the distribution of charged and polar residues may indicate a more complex and dynamic interaction with the ER membrane than can be predicted from primary structure alone.

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Salt- and EDTA-washed rough microsomes were solubilized with 0.5 M sodium carbonate, pH 11.5, and pelleted. Thirty-five eq of pellet (lanes P) and supernatant (lanes S) fractions were subjected to sodium carbonate extraction of microsomes (lanes S), or purified signal peptidase complex (lanes C). Each reacted with the intended signal peptidase complex subunit, although the anti-SPC 18 serum also reacted with a second protein of ~27 kDa (Fig. 6C, post-immune lane P). Despite the overall similarity between SPC 18 and SPC 21, the antibodies directed against these two proteins demonstrated no detectable cross-reactivity. In the case of SPC 21, this is because the synthetic peptide antigen corresponds to an essentially unique amino-terminal domain of the protein (residues 2-17 of the primary translation product). The SPC 18 peptide, however, corresponds to residues 3-17 and shares 5 amino acids of identity with SPC 21 (SPC 21 residues 25-29). Despite this region of identity, anti-SPC 18 antisemur demonstrated no detectable cross-reactivity with the SPC 21 homolog. The analysis made possible by the availability of these specific antibodies revealed that each SPC antigen examined was quantitatively recovered in the membrane pellet following extraction with sodium carbonate. Therefore, in addition to the signal peptidase glycoprotein (SPC 22/23), both the 18- and 21-kDa Sec11p homologs are integral proteins of the ER membrane.

Both Sec11p-like Proteins Form Quantitative Complex with Glycoprotein Subunit of SPC—Evidence for a signal peptidase complex lies in the co-migration of SPC proteins during multiple rounds of purification including sucrose gradient sedimentation and gel filtration chromatography (Evans et al., 1986a). In addition, the SPC binds to ConA-Sepharose even though only one of the subunits is glycosylated (Evans, 1986; Evans et al., 1986b). From these previous analyses, it could not be ascertained whether the association between SPC subunits was quantitative or whether a steady-state pool of monomers may have been lost during initial membrane solubilization or subsequent purification steps. The availability of antibodies to the SPC 22/23 glycoprotein and the 18- and 21-kDa Sec11p homologs allowed us to examine this question.

Salt- and EDTA-washed rough microsomes were solubilized with Nikkol in the presence of 0.5 M KOAc and chromatographed on ConA-Sepharose (Shelness et al., 1988). The column fractions were subjected to 12% SDS-PAGE and electroblotted to nitrocellulose. Three separate nitrocellulose protein blots prepared from the ConA-Sepharose column fractions were individually probed with antipeptide antibodies specific for the 22/23-, 21-, and 18-kDa SPC subunits. The results of this analysis are shown in Fig. 7. As expected for a glycoprotein containing a high mannose oligosaccharide residue, SPC 22/23 is quantitatively bound and specifically eluted during ConA-Sepharose affinity chromatography (Fig. 7A). The 18- and 21-kDa SPC subunits, which contain no carbohydrate groups and no potential sites for asparagine-linked oligosaccharide addition, are also retained by and eluted from ConA-Sepharose (Fig. 7, B and C). No detectable levels of either protein are found in the ConA-Sepharose flow-through fraction. In addition, the elution profiles of the three proteins, as judged by SDS-PAGE and immunoblotting, as well as by signal peptidase activity (Fig. 7D), appear to be superimposable.

The affinity of the nonglycosylated Sec11p-like proteins for ConA-Sepharose is not due to an artifactual interaction with the column matrix since conditions which perturb the SPC cause various amounts of the Sec11p-like proteins to appear in the ConA-Sepharose flow-through fraction. For example, if salt- and EDTA-washed rough microsomes are solubilized at pH 11 and then salt-exchanged by gel filtration into a buffer which reflects the standard solubilization condition used above, ~85% of the 21-kDa Sec11p-like protein appears
in the ConA-Sepharose flow-through fraction (compare the load (L) and flow-through (FT) lanes of Fig. 8B, condition 2). In contrast to yeast signal peptidase, which demonstrated optimal post-translational processing activity between pH 8 and 12 (Yeadon and Blobel, 1989), the canine enzyme, under the conditions used here, appears to be alkali-sensitive. No activity is detected if the pH 11 membrane extract is assayed directly (data not shown). However, after the alkaline membrane extract is exchanged into neutral pH buffer, chromatographed on ConA-Sepharose, and analyzed as described above. The gel filtration of the membrane extract produced under condition 2 caused an ~4-fold dilution. Because the ConA-Sepharose column volume is small as compared to the sample size, flow-through fractions are essentially undiluted relative to the load. In C, 4 µl of each fraction were assayed for signal peptide activity (see “Experimental Procedures”).

DISCUSSION

The cDNA for the 18-kDa subunit of the canine SPC has been cloned and sequenced. This subunit was found to be a second canine signal peptidase complex homolog of Sec1lp, a gene product necessary for cell growth and signal peptide processing in yeast (Bohni et al., 1988). A variety of subunit proteins have evolved as a result of partial or complete gene duplication (Doolittle, 1979). Examples of membrane-protein complexes containing homologous subunits include the aceetylcholine receptor (Noda et al., 1983), the light-harvesting centers of photosynthetic bacteria (Rees et al., 1989), and the asialoglycoprotein receptor (Bischoff et al., 1988). In addition, the soluble mitochondrial matrix processing protease from both yeast (Yang et al., 1988) and Neurospora crassa (Hawlischem et al., 1988), while not a physical complex, requires two homologous gene products for
optimal activity. The analogous processing protease from rat liver mitochondria has, in contrast, been purified as a tight complex of two proteins (Ou et al., 1989). The relationship between the rat and the yeast and fungal processing proteases has not yet been established at the molecular level.

Based on the examples cited above, the existence of homologous subunits within the canine SPC is not unprecedented. Another possibility, however, is that the 18- and 21-kDa SPC proteins, while products of separate genes, are functionally identical and segregate at random to separate populations of the signal peptidase complex. The evolutionary pressure to maintain two separate genes which encode functionally equivalent proteins is not, however, easily rationalized.

The initial identification of the signal peptidase complex was based on the co-migration of SPC proteins during multiple rounds of purification including sucrose gradient sedimentation and a gel filtration chromatography (Evans et al., 1986a). It was subsequently demonstrated that the entire signal peptidase complex could be retained by ConA-Sepharose, although only the 22/23-kDa SPC subunit is glycosylated (Evans et al., 1986b; Evans, 1986). With the use of antisynthetic peptide antibodies, this latter result has now been extended to show that, in microsomal membrane detergent extracts, the 18- and 21-kDa Sec11p-like subunits exist quantitatively as a complex with SPC 22/23, the signal peptidase complex glycoprotein subunit; no steady-state monomer population of either canine Sec11p-like protein can be detected. Even when the SPC is partially dissociated by treatment with alkali, no activity is observed in ConA-Sepharose flow-through column fractions containing SPC 21 (and presumably other nonglycosylated SPC proteins). These results coupled with the confirmation that SPC 18 and SPC 21 are integral membrane proteins suggest that the canine Sec11p-like subunits probably function entirely as part of a hetero-oligomeric integral membrane-protein complex.

An apparent discrepancy exists in the literature as to the number of proteins which copurify with signal peptidase activity. Whereas Evans et al. (1986a) identified a complex of five proteins in canine pancreas, Baker and Lively (1987) have isolated active fractions of signal peptidase from hen oviduct which contain an apparent complex of only two proteins. One of these proteins, the hen oviduct signal peptidase glycoprotein, is the avian analog of canine SPC 22/23 (Shelness et al., 1988). The apparent discrepancy in the number of signal peptidase complex proteins in preparations from hen and dog could, among other things, be attributable to species differences or differences in purification procedures. As demonstrated in this report, the use of alkali-washed membranes (Fig. 8), which are used in the avian purification, could cause partial disruption of protein-protein interactions necessary for the integrity of the five-protein complex observed in the dog. The use of pH 5.8 buffer during cation-exchange chromatography (Baker et al., 1986) could further perturb the complex. These treatments could potentially result in the gradual loss of some members of the complex during subsequent manipulations. The presence of processing activity within these partially depleted fractions could indicate that only one or two SPC proteins are required for catalysis (Baker and Lively, 1987) and that the other proteins observed in the dog play different roles. Alternatively, it is possible that the processing activity within the final avian signal peptidase preparation has been depleted of one or more necessary subunit(s) and is roughly proportional to the amount of functionally intact complex still remaining. This situation may not be easily recognized if subunits of the avian signal peptidase complex were to co-migrate during SDS-PAGE.

The demonstration that two of the subunits within the canine SPC are homologous to yeast SEC11 protein indicates that they are probably valid components of the signal peptidase complex. SPC 22/23 also appears to be a key component of the complex since it is common to both the canine and avian systems. In addition, even under partially dissociating conditions, signal peptidase activity quantitatively binds to and specifically elutes from ConA-Sepharose. Nevertheless, none of the SPC proteins yet characterized has demonstrated detectable amino acid sequence similarity to the 36 kDa E. coli leader peptidase (signal peptidase I) (Wolfe et al., 1983), even though this enzyme and the canine signal peptidase activity demonstrate overlapping substrate specificities (Roggenkamp et al., 1981; Watts et al., 1983; Müller et al., 1982). Such primary structure similarity may still be detected in the as yet uncharacterized 12- or 25-kDa SPC protein and would provide a convincing identification of the catalytic subunit within the SPC. However, even if a leader peptidase analog is identified within the SPC, the biochemical and genetic evidence to date suggests that the molecular organization of the eukaryotic signal peptidase is distinct from that of E. coli and other known processing proteases. That eukaryotic signal peptidase activity is contained within an integral membrane-protein complex may be a reflection of a more general involvement in protein translocation and/or involvement in other related functions of the ER membrane.

Acknowledgments—We thank Donna Atherton and the staff of The Rockefeller University/Howard Hughes Medical Institute Biopolymer Facility for performing protein sequencing and oligonucleotide and peptide syntheses. We also acknowledge the helpful comments of Dr. Chris Nicchitta.

REFERENCES
Two subunits of the canine signal peptidase complex are homologous to yeast SEC11 protein.
G S Shelness and G Blobel


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