Hen Oviduct Signal Peptidase Is an Integral Membrane Protein*  

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Membrane preparations from rough endoplasmic reticulum of hen oviduct resemble those of dog pancreas in their capacity to translocate nascent secretory proteins into membrane vesicles present during cell-free protein synthesis. As with the dog membranes, the precursor form of human placental lactogen is transported into the vesicles and processed to the native secretory form by an associated “signal peptidase.” The oviduct microsomal membranes glycosylate nascent ovomucoid and ovalbumin in vitro.

Attempts to extract the signal peptidase from these membrane vesicles revealed that it is one of the least easily solubilized proteins. A protocol for enrichment of signal peptidase was developed that took advantage of its tight association with these vesicles. These studies indicate that the enzyme has the characteristics of an integral membrane protein which remains active in membrane vesicles even after extraction with low concentrations of detergent that do not dissolve the lipid bilayer or after disruption of membrane vesicles in ice-cold 0.1 M Na2CO3, pH 11.5 (Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) J. Cell Biol. 93, 97–102), which releases the majority of membrane-associated proteins. Solubilization of non-denaturing detergents that totally dissolve the lipid bilayer. The detergent-solubilized enzyme retains the activity and the characteristic specificity of the membrane-bound form.

The transport of secretory proteins across, as well as integration of membrane proteins into, the lipid bilayer of the ER1 is usually accompanied by limited proteolysis of the nascent polypeptide chain (Kreil, 1981; Sabatini et al., 1982; Wickner, 1979). Such proteins are synthesized by membrane-bound polysomes as precursors with from 15 to 30 pre-dominantly hydrophobic amino acid residues on the NH2 terminus and these “signal peptide” extensions function in the recognition and binding of the ribosomal complex to the ER (Blobel and Dobberstein, 1975a). Once the ribosome is bound to the ER the polypeptide chain is translocated across the lipid bilayer and the signal peptide is removed from the nascent protein by a membrane-associated “signal peptidase” (Blobel and Dobberstein, 1975b).

The proteinase activity was first recognized in intracellular membranes from a myeloma tissue culture line (Milstein et al., 1972). It has since been demonstrated in membranes prepared from dog pancreas (Blobel and Dobberstein, 1975b; Shields and Blobel, 1978), ascites tumor cells (Szczesna and Boime, 1976), plasmacytomas (Green, 1979), ovine mammary glands (Gaye et al., 1979), rat liver (Kaschnitz and Kreil, 1978), Drosophila melanogaster embryos (Brennan et al., 1980), Escherichia coli (Zwizinski and Wickner, 1980), and Bacillus licheniformis (Chang et al., 1982). In spite of this wide range of sources, little is known about these enzymes. The E. coli “leader peptidase” has been purified (Zwizinski and Wickner, 1980) and has been partially characterized (Wolfe et al., 1982). A recent report describes another signal peptidase activity in E. coli membranes that is distinct from the leader peptidase activity (Tokunaga et al., 1982).

No eukaryotic signal peptidase has been purified. Dog pancreas signal peptidase has been solubilized using the detergent deoxycholate (Jackson and Blobel, 1977). The only known substrates for detergent-solubilized signal peptidase are fully synthesized precursor proteins prepared by cell-free translation of appropriate mRNAs in vitro. Deoxycholate-solubilized dog pancreas signal peptidase cleaves full length pre-prolactin molecules at the correct site but fewer than 50% of the substrate molecules react with the enzyme (Jackson and Blobel, 1977). This enzyme, in apparent contrast to the E. coli leader peptidase, depends upon phospholipid for activity (Jackson and White, 1981). Attempts to inhibit the enzyme with a range of inhibitors of proteolytic enzymes have failed to identify even one reagent capable of specifically inhibiting the solubilized enzyme (Jackson and Blobel, 1980). Consequently, it is not known if this protease belongs to any of the described mechanistic classes of proteolytic enzymes.

In an effort to learn more about signal peptidase, we have chosen to study its activity in hen oviduct. The oviducts of the laying hen is a tubular organ with four sequential distinct regions that synthesize and secrete proteins, carbohydrates, and ions around the descending yolk (Aitken, 1971). The magnum region secretes about 4 g of egg white protein/day, principally ovovomucin, conalbumin, ovomucoid, and lysozyme (Palmiter, 1972). The principal cells of the magnum, the tubular gland cells, have extensive granular endoplasmic reticular structures (Wyburn et al., 1970) and provide a good source of intracellular membranes. Although ovomucin is not synthesized with a cleavable signal sequence (Palmiter et al., 1978), the remaining three major magnum proteins do require proteolysis to remove signal peptides (Palmiter et al., 1977; Thibodeau et al., 1978a; Thibodeau et al., 1978b). Previous results from this laboratory have shown that oviduct membranes, treated with Triton X-100, remove the signal peptide from fully synthesized human preplacental lactogen (Thibodeau and Walsh, 1980).
Here we report the isolation of hen oviduct membranes that resemble membranes of dog pancreas in their capacity to translocate nascent secretory proteins into membrane vesicles during cell-free protein synthesis. We show that the signal peptidase activity in these membranes is not easily extracted and has the properties of an integral membrane protein. A protocol for the enrichment of signal peptidase activity has been developed that takes advantage of the tight association with membrane vesicles during removal of extrinsic and more loosely bound proteins.

**EXPERIMENTAL PROCEDURES**

**Preparation of Oviduct Membranes**—The isolation of rough endoplasmic membranes from hen oviduct was based on a previously described method (Hanover and Lennarz, 1978). All steps were performed at 4°C. Oviduct magnum tissue was rinsed in ice-cold buffer A (50 mM triethanolamine, 50 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, pH 7.6), cut into small pieces, suspended in 2 ml of buffer A/g of tissue, and homogenized using an Ultra-Turrax Tissuizer (Tekmar, Cincinnati, OH). The tissue homogenate was further homogenized with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) and then centrifuged for 10 min at 12,000 × g. The pelleted material was resuspended in the original volume of buffer A, homogenized with a motor-driven Potter-Elvehjem homogenizer, and centrifuged for 5 min at 3,000 × g. The supernatants including the upper tan-colored fluffy layers of the pellets were pooled and the membranes were collected by centrifugation for 60 min at 300,000 × g. The pelleted membranes were resuspended in buffer A using the Dounce homogenizer, layered over 5 ml of 1.0 M sucrose in buffer A, and centrifuged for 18 h at 125,000 × g. The pellets of rough endoplasmic membranes were stored at −80°C. A typical preparation yielded 45–60 mg of membrane protein/g of tissue.

**Membrane Purification**—Pelleted rough membranes were suspended in buffer A to a concentration of 75–100 A₂₈₀ units/ml and treated with 15 mM EDTA as described (Blobel and Dobberstein, 1975b). The EDTA-stripped membranes were collected by centrifugation for 60 min at 151,000 × g and then treated with a high concentration of KCl by resuspending them in twice the original volume of buffer A containing 0.5 M KCl. The pelleted EDTA- and KCl-stripped membranes were next treated with 14 μg/ml of bovine ribonuclease A in the original volume of buffer A for 30 min at 15°C. Ribonuclease A in the original volume of buffer A for 30 min at 15°C. The ribonuclease A was removed by centrifugation for 60 min at 39,000 × g. Pellets were carefully rinsed with small volumes of ice-cold buffer A and then stored at −80°C.

For electron microscopy, pellets of crude rough ER and carbonate-stripped membranes were treated at high pH as described (Fujiki et al., 1972) and translated at a concentration of 40 μg/ml. The translation products were separated by precipitation with ice-cold 5% trichloroacetic acid, and the precipitated reaction products were separated by SDS-PAGE. Dried SDS-PAGE gels were subjected to autoradiography using Kodak X-Omat AR film and the conversion of preHPL to HPL was quantified by microdensitometry using a Joyce-Loebel and Co. double beam recording microdensitometer equipped with an automatic film changer. The conversion of preHPL to HPL was calculated after correction for the loss of one Met residue when the signal peptide is removed. PreHPL contains 7 Met residues (Sherwood et al., 1979) and HPL contains 6 (Li et al., 1973).

**Partial NH₂-terminal Sequence Determination**—Plasmid mRNA was translated in separate 1-ml reaction mixtures containing 0.8 M[³⁵S]Met and 0.5 M[³⁵H]Leu. The translation products were combined and desalted on Sephadex G-25 and then incubated with 4 ml of freshly solubilized oviduct signal peptidase for 90 min at 26°C. After preparative SDS-PAGE, gels were dried without fixation. Bands corresponding to the processed and unprocessed forms of preHPL were located by autoradiography, excised, and extracted from the gel by agitating the crushed gel in 0.1% SDS containing 1% Triton X-100 for 2 h. The bands were then removed by filtration and the filtrate was lyophilized. The uncleaved preHPL was subjected to sequential Edman degradation in the presence of 5 mM of sperm whale aprotinin and 6 mg of Polybrene (Fisher Chemical Co.) using a Beckman model 890C automated protein Sequenceer as described (Palmiter et al., 1977).

The cleavage product purified by SDS-PAGE was further purified by high performance liquid chromatography on a Bondapak C₈ column (Waters Associates). The mobile phase was 0.1% trifluoroacetic acid and the mobile phase modifier was acetonitrile containing 0.07% trifluoroacetic acid. The flow rate was 2.0 ml/min and the concentration of the mobile phase modifier was increased linearly from 0% to 30% over 40 min. Native HPL (NHPL) and processed preHPL (preHPL) eluted at 32.5 min in the presence or absence of SDS and approximately 60% of the radioactivity eluted from the SDS-PAGE gel conigrated with native HPL. This peak fraction was lyophilized and subjected to sequence analysis as described above.

**Other Analytical Technique**—SDS-PAGE was performed according to Laemmli (1970). The molecular weight standards used were phosphorylase (Mₑ = 94,000), bovine serum albumin (Mₑ = 66,000), ovalbumin (Mₑ = 43,000), carbonic anhydrase (Mₑ = 30,000), soybean trypsin inhibitor (Mₑ = 20,000), and α-lactalbumin (Mₑ = 14,000).

**Protein** was estimated by the Lowry method as modified by Peterson (1977). Phospholipid was extracted from aqueous solution as described (Foich et al., 1957) and assayed in the presence of Mg(NO₃)₂ (Ames, 1966) by heating for 1 h in a muffine furnace at 700°C. The phosphate content of the ash was determined as described (Itaya and Michio, 1966). The RNA concentrations in membrane preparations were estimated by absorbance at 260 nm after correction for absorbance due to protein (Layne, 1957).

**Materials**—[³⁵S]Met (1000 Ci/mmol) and [³⁵H]Leu (130 Ci/mmol) were from Amerham Corp. Oxy-β-D-glucopyranoside was purchased from Calbiochem-Behring. Triton X-100, sodium dodecyl sulfate, and bovine pancreatic trypsin inhibitor were from Sigma. Protamine was obtained from the product of EM Biochemicals, Darmstadt, Germany. Worthington supplied the staphylococcal nuclease. The γ-globulin fraction of anti-human plasmaclactogen serum (rabbit) was prepared by ammonium sulfate precipitation and was kindly provided by Dr. Rick Meek, University of Washington. Anti-hen ovomucoid (rabbit) was supplied by Dr. Richard Palmiter, University of Washington.
RESULTS

Cleavage of Nascent preHPL by Hen Oviduct Microsomal Membranes in Vitro—Hen oviduct microsomal membranes were isolated and tested for the ability to translocate and proteolytically process nascent HPL molecules in vitro. Human polyadenylated mRNA was translated in a rabbit reticulocyte cell-free protein synthesis system (Palmiter et al., 1977), and the major product observed by SDS-PAGE was a protein of $M_r = 25,000$ (Fig. 1, lane 4). It has been shown that this protein is the secretory precursor form of HPL, termed preHPL (Szczesna and Boime, 1976), which is synthesized with a hydrophobic NH$_2$-terminal extension peptide 25 residues in length (Sherwood et al., 1979). Translation of placental mRNA in the presence of oviduct microsomal membranes resulted in conversion of preHPL to a protein with $M_r = 22,000$ (Fig. 1, lane 1) as shown before with microsomal membranes from Krebs ascites tumor cells (Birken et al., 1977). We, therefore, concluded that a “signal peptidase” activity present in hen oviduct membranes had correctly cleaved preHPL ($M_r = 25,000$) to HPL ($M_r = 22,000$).

The conversion of preHPL to HPL is directly dependent on the concentration of oviduct membranes present during protein synthesis (Fig. 2, closed circles) such that the precursor is quantitatively converted to HPL when excess membrane-binding sites are available. The addition of oviduct membranes to the cell-free translation system causes some inhibition of protein synthesis as judged by the decreased incorporation of [35S]Met into hot acid-precipitable protein (Fig. 2, open circles).

Transport of HPL into Hen Oviduct Microsomal Vesicles—The removal of signal peptides by intact microsomal vesicles in vitro is dependent upon transport of the nascent polypeptide across the lipid bilayer during protein synthesis (Milstein et al., 1972; Blobel and Dobberstein, 1975b). If the membranes are added after the completion of protein synthesis, transport does not initiate, the precursor remains outside of the vesicles, and signal peptidase is unable to cleave the signal peptide.

The localization of proteins synthesized in these systems can be demonstrated because proteins that have been transported into membrane vesicles are not readily degraded by proteolytic enzymes added to the external solution (Blobel and Dobberstein, 1975a; Scheele et al., 1980).

We used proteinase K, a powerful fungal proteinase with broad substrate specificity (Ebeling et al., 1974), to determine if HPL produced in the presence of hen oviduct membranes had been inserted into the vesicles (Fig. 1). Proteinase K degrades the preHPL molecules synthesized in the presence (lane 2) and absence (lane 5) of membranes. However, HPL produced in the presence of membranes was resistant to proteolysis (lane 2). When the vesicle bilayers were disrupted by detergent both preHPL and HPL were degraded (lanes 3 and 6). If oviduct membranes were added after the completion of protein synthesis, preHPL was the only product and it was readily degraded by exogenous proteinase K in the absence of detergent (not shown).

Glycosylation of Nascent Ovomucoid by Hen Oviduct Membranes in Vitro—Hen oviduct membranes have the enzyme systems necessary for the attachment of core oligosaccharides to glycoproteins (Czichi and Lennarz, 1977; Hanover and Lennarz, 1978). Fig. 3 shows the result of an experiment in which partially purified hen ovomucoid mRNA was translated in the absence (lane 2) and presence (lane 3) of EDTA-stripped oviduct membranes. A single immunoprecipitable ovomucoid species is produced in the absence of membranes whereas five additional forms appear as the result of synthesis in the presence of membranes. When ovomucoid mRNA was translated in the presence of dog pancreas microsomal membranes, these same bands were observed and the four of lower mobility were shown to bind to concanavalin A-Sepharose (Palmiter et al., 1980). Therefore, these four bands [MU(CHO)$_n$] must be partially glycosylated forms of the protein on the four known glycosylation sites (Kato et al., 1977). The band with faster mobility (MU) is nonglycosylated ovomucoid with the signal sequence removed (Thibodeau et al., 1979a). The result of a similar experiment using ovalbumin mRNA (not shown) showed that oviduct membranes also
Polyadenylated hen oviduct mRNA was partially enriched for ovomucoid mRNA by sucrose density gradient centrifugation (Palmiter and Smith, 1973) and translated in the cell-free protein synthesis system in the absence (lane 2) and presence (lane 3) of 0.5 A_{260} unit/ml of EDTA-stripped hen oviduct microsomal membranes. The reaction products were isolated by direct immunoprecipitation using rabbit anti-ovomucoid. The glycosylated preovomucoid was solubilized in the cell-free protein synthesis system in the absence of glycosylated ovomucoid. The immunoprecipitated reaction products were separated by SDS-PAGE and visualized by autoradiography. preMU, pre-ovomucoid; MU, nonglycosylated ovomucoid; MU(CHO), partially glycosylated ovomucoids.

Thus, hen oviduct microsomal membranes isolated by this technique appear to have the important processing systems of ER membranes. They proteolytically process glycosylate, and translocate nascent secretory proteins into membrane vesicles during cell-free synthesis. The precursor form of HPL is processed to its native size by a signal peptidase associated with membrane proteins without release of the peptidase, in order to take advantage of the immobilized state of the enzyme in the sedimentable membrane vesicles.

Table I summarizes the results obtained when oviduct membranes were treated sequentially with a variety of conditions designed to release proteins associated with the membranes without disruption of the lipid bilayer. The distributions of protein, enzyme, phospholipid, and RNA were determined after each stage. Aliquots of membrane pellets were solubilized with OG as described under "Experimental Procedures" and the 100,000 × g_{max} supernatants were assayed for protein concentration and signal peptidase activity. Enzyme activity at each stage of membrane purification is reported as the percentage of pre-HPL molecules cleaved by 50 μl of solubilized signal peptidase prepared and assayed under standard conditions. It is important to note that differences in the percentage of cleavage at each stage do not necessarily reflect differences in the level of signal peptide present because catalysis is influenced by the relative concentrations of detergent, phospholipid, and protein (see below).

**Detergent Solubilization of Hen Oviduct Signal Peptidase**

The concentration of detergent required to release signal peptidase from oviduct membranes was determined by treating a constant amount of membrane vesicles (about 1 μmol of phospholipid phosphorus) with a range of concentrations of Triton X-100, sodium deoxycholate, or OG, each of which solubilized the enzyme in an active state. OG was chosen for further studies because of its high critical micelle concentration and mild interaction with proteins (Stubbs et al., 1976).

**Purification of Oviduct Membranes**—Having demonstrated that signal peptidase is not released from intact membranes, we explored various techniques which would remove extrinsic membrane proteins without release of the peptidase, in order to take advantage of the immobilized state of the enzyme in the sedimentable membrane vesicles.

A detergent concentration that totally dissolves the lipid bilayer. Twelve 90-μl aliquots of partially purified hen oviduct membranes (25 mg/ml of protein, 1 mM phospholipid phosphorus) were mixed with equal volumes of buffer A containing increasing concentrations of octyl glucoside. The solutions were sonicated in a bath sonicator for 4 min at 23 °C and then centrifuged for 30 min at 100,000 × g_{max}. The supernatants were removed and the pellet material was sonicated in 180 μl of buffer A containing 50 mM octyl glucoside to extract any remaining detergent-soluble proteins. The resuspended pellets were centrifuged in the Airfuge at 100,000 × g_{max} for 60 min at 26 °C. The reaction products were separated by SDS-PAGE and visualized by autoradiography. A shows the fluorogram of the reaction products produced by signal peptidase activity released during the first centrifugation. Lanes 1 through 12 correspond to final concentrations of 0, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 15, 20, 25, 50, and 75 mM octyl glucoside, respectively. The fluorogram in the lower panel shows the reaction products produced by signal peptidase activity remaining in the corresponding pellets.

**Fig. 3.** Hen oviduct microsomal membranes cleave and glycosylate preovomucoid in vitro. Polyadenylated hen oviduct mRNA was partially enriched for ovomucoid mRNA by sucrose density gradient centrifugation (Palmiter and Smith, 1973) and translated in the cell-free protein synthesis system in the absence (lane 2) and presence (lane 3) of 0.5 A_{260} unit/ml of EDTA-stripped hen oviduct microsomal membranes. The reaction products were isolated by direct immunoprecipitation using rabbit anti-ovomucoid. The glycosylated preovomucoid was solubilized in the cell-free protein synthesis system in the absence of glycosylated ovomucoid. The immunoprecipitated reaction products were separated by SDS-PAGE and visualized by autoradiography. preMU, pre-ovomucoid; MU, nonglycosylated ovomucoid; MU(CHO), partially glycosylated ovomucoids.

**Fig. 4.** Solubilization of signal peptidase activity requires a detergent concentration that totally dissolves the lipid bilayer. Twelve 90-μl aliquots of partially purified hen oviduct membranes (25 mg/ml of protein, 1 mM phospholipid phosphorus) were mixed with equal volumes of buffer A containing increasing concentrations of octyl glucoside. The solutions were sonicated in a bath sonicator for 4 min at 23 °C and then centrifuged for 30 min at 100,000 × g_{max}. The supernatants were removed and the pelleted material was sonicated in 180 μl of buffer A containing 50 mM octyl glucoside to extract any remaining detergent-soluble proteins. The resuspended pellets were centrifuged in the Airfuge at 100,000 × g_{max} for 60 min at 26 °C. The reaction products were separated by SDS-PAGE and visualized by autoradiography. A shows the fluorogram of the reaction products produced by signal peptidase activity released during the first centrifugation. Lanes 1 through 12 correspond to final concentrations of 0, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 15, 20, 25, 50, and 75 mM octyl glucoside, respectively. The fluorogram in the lower panel shows the reaction products produced by signal peptidase activity remaining in the corresponding pellets.
**Table I**

Purification of oviduct rough microsomal membranes

Pelleted microsomal membranes were resuspended in buffer after each extraction step and aliquots were removed for determination of protein, RNA, phospholipid phosphorus, and detergent-solubilized signal peptidase activity as described under "Experimental Procedures." Signal peptidase activity is reported as the maximum cleavage obtained from 60 μl of detergent-solubilized pellet incubated with substrate for 90 min under the standard assay conditions and does not provide a precise measure of the amount of enzyme present (see text).

<table>
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<tr>
<th>Membrane purification stage</th>
<th>Total protein</th>
<th>Total RNA</th>
<th>Protein per phospholipid phosphorus</th>
<th>Detergent-solubilized protein</th>
<th>Signal peptidase activity (%) cleavage</th>
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</thead>
<tbody>
<tr>
<td>Crude rough ER</td>
<td>2300</td>
<td>600</td>
<td>160</td>
<td>710</td>
<td>46</td>
</tr>
<tr>
<td>EDTA/KCl-stripped</td>
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<td>260</td>
<td>82</td>
<td>550</td>
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<td>140</td>
<td>59</td>
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<td>48</td>
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<td>49</td>
<td>240</td>
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<tr>
<td>Carbonate-treated</td>
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<td>17</td>
<td>29</td>
<td>33</td>
<td>30</td>
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</tbody>
</table>

*See Fig. 5A.

**Fig. 5. Procedures that release extrinsic membrane proteins leave signal peptidase associated with microsomal membranes.** Aliquots of microsomal membranes removed after each sequential stage of the purification procedure (Table I) were solubilized with octyl glucoside and centrifuged for 1 h at 100,000 χ gmax. The supernatant was assayed for signal peptidase activity using the translocation-independent assay. The proteins of each supernatant were separated by SDS-PAGE in a linear 10 to 15% acrylamide gradient gel. The samples were loaded with a constant level of membrane phospholipid phosphorus (1 μg) in order to estimate the relative protein content of the solubilized supernatant at each stage of the purification.

**Fig. 6. Electron micrographs of (A) crude rough ER membranes and (B) pH 11.5 carbonate-treated purified ER membranes.** × 39,500.
preHPL molecules appears to be dependent on the state of the substrate as well as the relative concentrations of protein, detergent, and phospholipid in the enzyme solution. Excessive detergent inhibits the enzyme in a way that can be partially overcome by the addition of purified phosphatidylcholine. It has been shown that delipidated dog pancreas signal peptidase is inactive but can be reactivated by the addition of certain phospholipids and detergents (Jackson and White, 1981). However, optimum conditions for the activation of oviduct signal peptidase have not been determined.

The assay is complicated by the fact that the cleavage reaction fails to go to completion. Post-translational cleavage of fully synthesized preHPL by solubilized signal peptidase is very inefficient and as much as 90% of the substrate remains uncleaved after prolonged incubation. Fully synthesized preproteins are also poor substrates for dog pancreas signal peptidase (Jackson and Blobel, 1977). We have determined that the extent of cleavage of preHPL is increased when it is complexed with an antibody specific for HPL (Lively and Walsh, 1981). Placental mRNA was translated in the presence of a range of concentrations of partially purified anti-HPL immunoglobulins. When the translation products, presumably antibody-bound preHPL, were incubated with solubilized signal peptidase, the maximum cleavage was improved from less than 10% to as much as 60% when the concentration of antibody was in the range of 15 to 30 μg/ml in the cell-free translation. At antibody concentrations above or below this range cleavage was reduced. The antibody must be present during translation for the effect to be observed just as the inclusion of anti-lysozyme during translation of lysozyme mRNA was shown to improve the yield of prelysozyme synthesized in vitro (Palmiter et al., 1977).

The maximum cleavage obtained also depends upon the level of enzyme present and the time period of the reaction. Fig. 7 shows the time course of cleavage of antibody-bound preHPL by two different concentrations of signal peptidase. After 60 min, each reaction had reached a different level of maximum activity and these levels were not exceeded by further incubation for up to 16 h. Higher concentrations of enzyme will cleave more of the preHPL but 100% cleavage is not obtained (Fig. 8). The preHPL remaining after 16 h of incubation is even resistant to a second addition of enzyme (not shown). These results are in contrast to the complete conversion possible by co-translational processing in vitro (Fig. 2). These same properties are exhibited by the enzyme solubilized from crude rough membranes and are not the result of the membrane purification procedures.

**Specificity of Partially Purified Signal Peptidase**—The M, = 22,000 protein band produced upon incubation of antibody-bound [3H]Leu, [35S]Met-preHPL with solubilized signal peptidase was purified by preparative SDS-PAGE followed by high performance liquid chromatography as described under “Experimental Procedures.” The signal peptidase cleavage product comigrated with native HPL when chromatographed on a reverse-phase μBondapak C18 column. The partial NH2-terminal sequence of this material is consistent with the sequence of native HPL (Li et al., 1973) with Leu at positions 6, 9, and 15 and Met at position 14 (Fig. 9, A and B). The uncleaved M, = 25,000 band, purified by SDS-PAGE only, had the sequence of preHPL (Sherwood et al., 1979) with Leu at positions 8, 9, 10, 14, and 15 as well as Met at position 1 (Fig. 9, C and D). These data demonstrate that the signal peptidase cleavage product is native HPL and that the solubilized signal peptidase retains the characteristic specificity of the membrane-bound form.

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* M. O. Lively, and K. A. Walsh, manuscript in preparation.
purified cleavage product Met, respectively, released by each cycle of Edman degradation of the corresponding data for uncleaved preHPL to duct signal peptidase produces a product with the NH2-terminal sequence of native HPL.

merely separated the majority of these prematurely terminated preproteins from the processed form of preHPL (Fig. 9A and B).

DISCUSSION

The present results offer new insight into the nature of the association of signal peptidase with the ER membrane. Our data are consistent with previous conclusions that the enzyme active site is not exposed on the cytoplasmic surface: intact microsomal membranes failed to remove signal peptides from fully synthesized preproteins in vitro (Blobel and Dobberstein, 1975b); signal peptidase was not inactivated by proteolysis of intact vesicles (Walter et al., 1979); and signal peptidase activity was latent in dog pancreas membranes and required sodium deoxycholate for its release (Jackson and Blobel, 1977). However, those data did not distinguish between the possibilities that the dog pancreatic signal peptidase was an integral membrane protein, an extrinsic membrane protein bound to the luminal surface of the ER, or a soluble protein merely trapped within the ER lumen. The results reported here indicate that hen oviduct signal peptidase has the properties of an integral membrane protein.

Perhaps the strongest evidence to support the claim that the peptidase is a true integral membrane protein is its retention with the membrane after treatment in carbonate at pH 11.5. This treatment converts closed membrane vesicles into open membrane sheets where only integral proteins should remain bound to the sedimentable lipid bilayers (Fujiki et al., 1982a, 1982b). Similar conclusions have been drawn after analogous treatments of other membranes at high pH (Barrantes, 1980; Elliot et al., 1980; Neubig et al., 1979; Steck and Yu, 1973). Treatment at high pH is a highly effective technique for preparation of intact oviduct membranes (Fig. 6B) containing only integral membrane proteins and maintaining the enzymatic activity of signal peptidase (Figs. 7 and 8). Detergent treatment is required to release signal peptidase from carbonate-treated oviduct membranes. The integral association of the enzyme with the lipid bilayer is consistent with the dependence of the dog pancreas enzyme on phospholipid for activity in vitro (Jackson and White, 1981).

The optimum concentration of anti-HPL present during translation may stabilize a larger proportion of the precursor molecules in a conformation that facilitates recognition by signal peptidase. When antibody is not present, or is present at suboptimal concentrations, cleavage may be prevented by aggregation or folding of preHPL molecules in ways that preclude recognition by the peptidase. The effect of the antibody may be analogous to the way in which signal peptides are recognized in vitro. The true substrate could be a complex of the nascent precursor protein and a signal peptide receptor, e.g. the signal recognition protein of Walter and Blobel (1982).

The receptor may bind the signal peptide and hold it in a conformation that exposes the scissile peptide bond to the active site of the peptidase allowing proper recognition and cleavage. Antibody-bound preHPL in vitro may mimic the signal peptide-receptor complex.

The magnitude of the hen oviduct is a convenient source for preparation of microsomal ER membranes that contain active systems for transport and processing of secretory proteins. Ovalbumin, a protein secreted without cleavage of a signal peptide (Palmiter et al., 1978), is a major oviduct product and models of the process of protein transport across membrane barriers must be able to account for this apparent exception. With oviduct membranes, the transport of ovalbumin can be compared directly with the processing and transport of proteins with cleavable signal sequences. Differences in the mechanism of transport of ovalbumin are more likely to be observed in this homologous system. Purification of oviduct membranes as herein described provides a good source of signal peptidase for further studies of its nature as a proteinase and its role in the protein transport process.

Acknowledgments—We thank Dr. Rick Meek for providing the data presented in Fig. 3, Dr. Richard Palmiter for many helpful suggestions, and Professor Hans Neurath for his critical reading of the manuscript. We also wish to thank Dr. Jon Lewis at the Electron Microscopy Laboratory at Bowman Gray School of Medicine for preparation of the electron micrographs.
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