Mechanism of Compartmentation of Secretory Proteins: Transport of Exocrine Pancreatic Proteins across the Microsomal Membrane

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ABSTRACT The mechanism by which secretory proteins are segregated within the cisternal space of microsomal vesicles was studied using dog pancreas mRNA which directs the synthesis of 14 well-characterized nonglycosylated pancreatic exocrine proteins. In the absence of microsomal membranes, each of the proteins was synthesized as larger polypeptide chains (presecretory proteins), 1,000–2,000 daltons larger than their authentic counterparts as judged by polyacrylamide gel electrophoresis in SDS. Conditions optimal for the study of reconstituted rough microsomes in the reticulocyte lysate system were examined in detail using mRNA and microsomal membranes isolated from dog pancreas. Functional reconstitution of rough microsomes was considerably more efficient in the presence of micrococcal nuclease-treated membranes than in the presence of EDTA-treated membranes. Analysis for segregation of nascent secretory proteins by microsomal vesicles, using post-translational incubation in the presence of trypsin and chymotrypsin, 50 μg/ml each, was shown to be inadequate, because of the disruption of vesicles by protease activity. Addition of 1–3 mM tetracaine or 1 mM dibucaine stabilized microsomal membranes incubated in the presence of trypsin and chymotrypsin at either 0° or 22°C. Each of the pancreatic presecretory proteins studied was correctly processed to authentic secretory proteins by nuclease-treated microsomal membranes, as judged by both one-dimensional and two-dimensional gel electrophoresis. Post-translational addition of membranes did not result in either segregation or processing of nascent polypeptide chains. Post-translational proteolysis, carried out in the presence of 3 mM tetracaine, indicated that each of the 14 characterized dog pancreas secretory proteins was quantitatively segregated by nuclease-treated microsomal vesicles. Segregation of nascent secretory proteins was irreversible, since radioactive amylase, as well as the other labeled secretory proteins, remained quantitatively sequestered in microsomal vesicles during a 90-min incubation at 22°C after the cessation of protein synthesis. Studies employing synchronized protein synthesis and delayed addition of membranes indicated that all pancreatic presecretory proteins contain amino terminal peptide extensions. These peptide extensions are shown to mediate the cotranslational binding of presecretory proteins to microsomal membranes and the transport of nascent secretory proteins to the vesicular space. The maximum chain lengths which, during synthesis, allow segregation of nascent polypeptide chains varied between 61 (pretrypsinogen 2 + 3) and 88 (preprocarboxypeptidase A1) amino acid residues among dog pancreas presecretory proteins. Reconstitution studies using homologous and heterologous mixtures of mRNA (dog, guinea pig, and rat pancreas; rat liver) and micrococcal nuclease-treated microsomal membranes (dog, guinea pig, and rat liver; dog pancreas), in the presence of placental ribonuclease inhibitor, suggest that the translocation mechanism described is common to the rough endoplasmic reticulum of all mammalian tissues.

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In vitro translation studies using mRNA isolated from secretory tissues have provided information that has contributed to an understanding of the biochemical events involved in the synthesis and segregation of secretory proteins. Analysis of translation products according to size has indicated that a number of secretory proteins are synthesized as larger polypeptide chains, termed presecretory proteins. Amino terminal sequencing studies have indicated that the peptide extensions reside at the amino terminus and have additionally provided the amino acid sequence for these transport peptides (1). Reconstitution of rough microsomes using heterologous components (mRNA, stripped microsomal membranes, ribosomes, and synthesis factors) have been carried out to determine the function of the peptide extensions (2–9). These studies have clearly indicated that translation of mRNA in the presence of microsomal membranes can result in the cotranslational processing and segregation of nascent polypeptide chains. These data have been used to support the Transport Peptide Hypothesis,[1] which attempts to explain in molecular terms the mechanism by which secretory proteins are transported across the membrane of the rough endoplasmic reticulum (RER).[2] Examination of the data in each case, however, reveals that both proteolytic processing and segregation of polypeptide chains are incomplete. Proteolytic processing ranges from 50–75% while segregation of polypeptide chains, as judged by post-translational proteolysis, ranges from 10–43%. These findings are insufficient to exclude other interpretations of the data that support alternative models of cellular protein secretion (10, 11).

Detailed studies of the individual assays used in these reconstitution studies (proteolytic processing, vesicular segregation, and distribution of translation products) have not appeared. We have now performed such studies and demonstrate, after significant modification of the existing techniques, that highly efficient reconstitution studies can be carried out using either homologous or heterologous components, including mRNA and membranes from a variety of animal tissues. These techniques allow us to monitor simultaneously the biochemical events involved in the synthesis and segregation of 14 well-defined dog pancreas secretory proteins. In addition to the comprehensive nature of our study, our improved methods now provide considerably more reliable information than has been obtained from the studies of others, and the degree of reliability in our data now allows us to detail with certainty the biochemical events involved in the segregation of nascent secretory proteins into the cisternal space of the RER.

[1] This hypothesis is referred to here and elsewhere (1, 69) as the Transport Peptide Hypothesis in an attempt to describe in greater detail the structure which is responsible for the functional binding of presecretory proteins to the microsomal membrane and the transport of the corresponding secretory protein across the lipid bilayer. This designation is appropriate for both presecretory proteins that contain external transport peptides (e.g., the pancreatic presecretory proteins described here) and secretory proteins that contain internal transport peptides, as has been described for ovalbumin (68). Transport peptides, as described here, represent only one type of signal among the multiplicity of signals (recognition units formed by proteins, nucleotides, lipids and carbohydrates) which exist throughout nature.

[2] Abbreviations used in this paper: DFP, diisopropylfluorophosphate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; IEF, isoelectric focusing; IEP, isoelectric point; KIU, kallikrein inhibitor units; KRB, Krebs-Ringer bicarbonate; ^{7}mGp 7-methylguanosine-5′-phosphate; M_r, apparent molecular weight; PPO, 2,5-diphenyloxazole; RER, rough endoplasmic reticulum; STI, soybean trypsin inhibitor.

MATERIALS AND METHODS

Materials

Sucrose (sucrose density grade, ribonuclease free), urea (ultra pure) and sodium deoxycholate were from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). Diisopropylfluorophosphate (DFP), polyvinylsulfate (practical grade), bovine liver tRNA, dibucaine hydrochloride, and 7-methylguanosine-5′-phosphate (^{7}mGp), phenyl hydrazine (recrystallized) and guanidinium hydrochloride (ultra pure) were supplied by Sigma Chemical Co. (St. Louis, Mo.). Soybean trypsin inhibitor (STI), bovine trypsin (TRL 3 — 229 U/mg), bovine α-chymotrypsin (CDI, 68 U/mg), and micrococcal nuclease were products of Worthington Biochemical Corp. (Freehold, N. J.). Trasylol was supplied by FBA Pharmaceuticals (New York), and benzamidine was supplied by Aldrich Chemical Co., Inc. (Milwaukee, Wis.), as the hydrochloride hydrate. Sodium perchlorate (Analar), sodium SDS (specially pure), acrylamide (electrophoresis grade), and *N,N′*-methylenebisacrylamide (electrophoresis grade) were products of BDH Chemicals, (Poole, England). Dimethyl sulfoxide (DMSO) (practical grade) and 2,5-diphenyloxazole (PPO) (scintillation grade) were supplied by Eastman Organic Chemicals Div., Eastman Kodak Co. (Rochester, N. Y.), and Packard Instrument Co., Inc. (Downers Grove, Ill.), respectively. Ampholytes were products of LKB (Bromma, Sweden). Oligo(dT) cellulose was purchased from Collaborative Research Inc. (Waltham, Mass.). Guanidinium thiocyanate (purum grade) was from Fluka AG, (Basel, Switzerland). Proteinase K (fungal) was a product of E. Merck (Darmstadt, W. Germany). Wheat germ was a gift from General Mills, Inc. (Minneapolis, Minn.). [^{35}S]Methionine (~1,000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.). A mixture of ^{14}C-labeled amino acids was supplied as an algal hydrolysate by Schwarz/Mann. Cronex 2D medical x-ray film was supplied by E. I. Dupont de Nemours & Co. (Wilmington, Del.). Edeine was from Calbiochemical-Behring Corp., American Hoechst Corp. (San Diego, Calif.). Protein molecular weight standards were from Bio-Rad, Laboratories (Richmond, Calif.).

Preparation of Radioactively Labeled Dog Pancreas Secretory Protein

Dog exocrine pancreatic proteins were labeled as follows: The gland was immersed in ice-cold Krebs-Ringer's bicarbonate (KRB) solution containing 125 mM NaCl, 4.6 mM KCl, 1.16 mM $MgSO_4$, 2.4 mM KH_2PO_4, 2.53 mM $CaCl_2$, 25 mM $NaHCO_3$, and supplemented with glucose (1 mg/ml) and bovine serum albumin (100 μg/ml). The pH of this solution was maintained at 7.4 by exposure to a gaseous mixture of 95% O_2 and 5% CO_2. Lobules (1.5 × 1.0 × 0.6 cm) were defined by injecting KRB throughout the gland using a 50 ml syringe and number 18 needle. Individual lobules were removed by excision and five to seven slices per lobule were made using a Stadie-Riggs tissue slicing blade (Arthur H. Thomas Co., Philadelphia, Pa.). Pancreatic slices were ≤1 mm in thickness. Two tissue slices were incubated in 1 ml of KRB containing 0.1 mCi of [^{35}S]methionine, 19 cold amino acids each at 0.2 mM, 20 μg/ml soybean trypsin inhibitor and 100 Kallikrein inhibitor units (KIU)/ml Trasylol. Pancreatic slices were continuously labeled for 3 h at 37°C. Radioactive-labeled secretory proteins were obtained by three methods: (*a*) *Secreted proteins*: Pancreatic slices were incubated in the presence of [^{35}S]methionine and 10^{-5} M carbamylcholine for 3 h. After this incubation period, the medium was decanted and supplemented with DFP (1 mM) and benzamidine (1 mM). Contaminating cells and loose debris were removed by sedimentation for 30 min at 3°C and 105,000 *g*. (*b*) *Zymogen granule lysate*: After incubation in the presence of [^{35}S]methionine for 3 h, a crude zymogen granule fraction was prepared from pancreatic slices by a modification of the procedure of Jamieson and Palade (12). Briefly, six pancreatic slices were homogenized in 5.0 ml of 0.3 M sucrose containing 20 μg/ml soybean trypsin inhibitor, 100 KIU/ml Trasylol, 1 mM DFP, and 1 mM benzamidine. Nuclei and cell debris were removed by sedimentation for 10 min at 3°C and 600 *g*. A crude zymogen granule pellet was obtained from the postnuclear supernate by sedimentation for 10 min at 3°C and 1,000 *g*. The granule pellet was lysed in a 1.0-ml solution containing 1% Triton X-100, 25 mM Tris-HCl, pH 9.0, 20 μg/ml STI, 100 KIU/ml Trasylol, 1 mM DFP, and 1 mM benzamidine, and the majority of membranes were removed by sedimentation in a microcentrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.) for 15 min at 3°C and 8,000 *g*. (*c*) *Cell homogenate*: After incubation in the presence of [^{35}S]methionine for 3 h, two pancreatic slices were homogenized in a 1.0-ml solution containing 1% Triton X-100, 25 mM Tris-HCl, pH 9.0, 20 μg/ml STI, 100 KIU/ml Trasylol, 1 mM DFP, and 1 mM benzamidine. Unlysed particles (nuclei and some mitochondria) and membranes were removed by sedimentation in a Brinkmann microcentrifuge for 15 min at 8,000 *g* (3°C). Each of the three preparations of secretory protein described above were aliquoted in samples of 50–100 μl and stored at −80°C after rapid freezing in liquid N_2. Samples were thawed and used only once, as repeated freezing and thawing resulted in autoactivation and degradation of the samples.

Radioactive proteins from guinea pig or rat exocrine pancreas were labeled using pancreatic lobules as described by Scheele and Palade (13). The radioactive labeling medium was identical to that described above for the dog pancreas. Pancreatic lobules incorporate 95% of radioactivity into secretory proteins under these conditions (14). After the 3-h incubation, six lobules were homogenized in 1.0 ml of medium as described above under "Cell homogenate."

Cell Fractionation

Rough microsomes were prepared from dog pancreas, dog liver, guinea pig pancreas, guinea pig liver, and rat liver by the following procedure: Tissue was homogenized with a Brendler tissue grinder (Arthur H. Thomas Co., type C) at 3°C in 3 vol of a solution containing 0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 2 mM dithiothreitol (DTT). The thiol reagent was added to preserve the endogenous ribonuclease inhibitor present in these tissues (15, 16). All subsequent steps in the isolation of cell fractions were carried out at 3°C. Cell debris, nuclei, and mitochondria were removed by sedimentation for 10 min at 10,000 *g*. Visible lipid was removed by aspiration, and the postmitochondrial supernate was layered over a discontinuous sucrose gradient containing 1.25 ml of 1.3 M sucrose and 0.5 ml of 2.25 M sucrose both in the presence of 20 mM HEPES-KOH, pH 7.5, 1 mM $MgCl_2$, and 2 mM DTT. Sucrose gradients contained in 9-ml polycarbonate tubes were centrifuged in an angle rotor for 1 h at 105,000 *g*. Rough microsomes that sedimented at the 1.3/2.25 M interface were pooled, aliquoted in 100-μl samples, and stored at −80°C after rapid freezing in liquid N_2. Samples were thawed for use only once.

Preparation of Stripped Microsomal Membranes

Rough microsomes were stripped of their endogenous mRNA by the following two procedures.

EDTA TREATMENT: Rough microsomes were treated with EDTA according to the procedure of Blobel and Dobberstein (2) or Shields and Blobel (4). Briefly, a solution of 0.2 M EDTA-KOH, pH 7.0, was added at a final concentration of 3 or 1.5 μmol/10 A_{260} U of rough microsomes. This mixture which contained ~40–45 mM EDTA was incubated at 3°C for 10 min. Treated membranes were then diluted eightfold with a solution of 20 mM HEPES, 2 mM DTT, and layered over a discontinuous sucrose gradient containing 1.25 ml of 0.3 M sucrose and 0.5 ml of 2.25 M sucrose, both containing 20 mM HEPES-KOH, 7.5, and 2 mM DTT. Centrifugation was carried out in a fixed angle rotor for 1 h at 105,000 *g*. Stripped membranes that accumulated at the 0.3/2.25 M sucrose interface were pooled, aliquoted into 100-μl samples, and stored at −80°C after rapid freezing in liquid N_2.

MICROCCOCAL NUCLEASE TREATMENT: Rough microsomes were treated with nuclease just before their use in reconstitution experiments. Rough microsomes (~200 A_{260} U/ml) were treated with 1,500 U/ml micrococcal nuclease in the presence of 0.5 mM $CaCl_2$ for 30 min at 22°C. Control experiments using the rabbit reticulocyte lysate and the wheat germ extract indicated that this elevated dose of micrococcal nuclease did not result in the functional degradation of ribosomes or tRNA (P. Blackburn and G. Scheele, unpublished observations). Nuclease activity was terminated by the addition of 2.0 mM EGTA. Addition of nuclease-treated membranes to translation mixtures resulted in a 10-fold dilution, such that the final concentration of micrococcal nuclease during in vitro translation was 150 U/ml (Pelham and Jackson [17] used 80 U/ml micrococcal nuclease for the degradation of globin mRNA in the rabbit reticulocyte lysate).

Isolation of mRNA

Dog pancreata were obtained from 20- to 50-lb. mongrel dogs as previously described (3). Glands were wrapped in aluminum foil, frozen in liquid N_2, and stored at −80°C until used for extraction of RNA. One half of a pancreas (~15 g) was homogenized with 5 vol of a solution containing 0.25 M sucrose, 2 mM DTT, and 40 μg/ml of polyvinyl sulfate (a ribonuclease inhibitor). Cell debris and nuclei were sedimented at 600 *g* for 10 min (3°C). RNA was extracted from the postnuclear supernate by the ethanol/sodium perchlorate procedure of Wilcockson (18) as modified by Lizardi and Engelberg (19) for use with mammalian tissues. The resulting postnuclear supernate (72 ml) was added dropwise (during constant swirling) to a solution containing 72 ml of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM DTT, 4% SDS, 40 μg/ml polyvinyl sulfate, and 0.5 mg/ml of proteinase K (added fresh). This mixture was incubated at 37°C for 15 min with intermittent swirling. 96 ml of 3.5 M sodium perchlorate was added, mixed thoroughly, and allowed to incubate at 37°C for 5 min. 30 ml of this solution was then distributed into each of eight 150 ml Corex bottles (No. 1265, Corning Glass Works, Science Products Div., Corning, N. Y.). 120 ml of a solution of ethanol saturated with sodium perchlorate (200 ml of solution A, [250 g of sodium perchlorate in 200 ml of water] mixed with 800 ml of solution B [11 g of sodium perchlorate in 800 ml of 100% ethanol]) was added to each of the Corex bottles, and the resulting mixture was incubated at 10°C for 12 min. The resulting RNA precipitate was sedimented at 3,300 *g* for 10 min at 10°C in a swinging bucket rotor and the supernate discarded. Each pellet (containing RNA) was resuspended in a 40-ml solution of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.2% SDS, incubated at 37°C until clear (~5 min), adjusted to 0.2 M NaCl, mixed with 0.6 vol of isopropanol, and placed at −20°C for 16 h in order to precipitate the RNA a second time. Following sedimentation and resuspension of the RNA as described above, a third precipitation was carried out after adjustment to 0.2 M NaCl and mixture with 2.4 vol of 100% ethanol at −20°C for 16 h. This procedure was successful in the isolation of intact RNA, as dog pancreas contains relatively small quantities of ribonuclease (20) and a 40-fold molar excess of endogenous ribonuclease inhibitor (P. Blackburn and G. Scheele, unpublished observations).

Polyadenylated mRNA was extracted from total RNA by chromatography on oligo(dT) cellulose as described by Aviv and Leder (21). After chromatography, polyadenylated mRNA was recovered by adjusting the eluate to 0.2 M NaCl and precipitation of the mRNA with 2.4 vol of 100% ethanol at −20°C for 16 h. The final mRNA precipitate, after sedimentation as described above, was dissolved in autoclaved, deionized, distilled water at a concentration of ~10 A_{260} U/ml, separated into aliquots containing 10–50 μl, frozen in liquid N_2, and stored at −80°C. Samples were generally thawed and used only once. mRNA samples could be refrozen without loss of activity if 1 μg/ml of purified ribonuclease inhibitor (15) was added.

By use of the saturated perchlorate-ethanol procedure, it was possible to recover 283 A_{260} U of total RNA and 3.33 A_{260} U of polyadenylated mRNA/g of pancreas. By comparison, phenol extraction yielded 100 A_{260} U and 0.32 A_{260} U, respectively. In addition, the perchlorate-ethanol procedure yielded RNA with an A_{260}/A_{280} ratio of 2.10, as compared to 1.95 with the phenol method.

Rat and guinea pig pancreas contain relatively large amounts of ribonuclease (20) and therefore RNA from these tissues was isolated by the procedure of Chirgwin et al. (22). Using this procedure, we homogenized pancreatic tissue in 4 M guanidinium thiocyanate using a polytron homogenizer and successively precipitated RNA with ethanol, redissolving it twice in 7.5 M guanidinium HCl and finally in autoclaved deionized distilled water. Selection of polyadenylated mRNA and storage were as described for dog pancreas mRNA.

Purification of the Human Placental Ribonuclease Inhibitor

The ribonuclease inhibitor from the full term human placenta ($K_i = 3 \times 10^{-10}$ M for bovine pancreatic RNase A) was isolated and purified to homogeneiety as described by Blackburn (15). The inhibitor was prepared for in vitro translation studies as described by Scheele and Blackburn (16).

Rabbit Reticulocyte Lysate Translation System

PREPARATION OF THE RETICULOCYTE LYSATE: Circulating reticulocytes were stimulated in pathogen-free New Zealand White rabbits by injection of phenylhydrazine according to the dosage schedule of Ernst et al. (23). Reticulocytes were collected, washed, and lysed, and cell membranes were sedimented (24). DTT at 2 mM was added to the lysis solution to prolong the stability of the endogenous ribonuclease inhibitor (16). Endogenous globin mRNA was degraded with micrococcal nuclease (80 U/ml) according to the procedure of Pelham and Jackson (17). To remove endogenous amino acids as previously described by Palmiter et al. (25), the nuclease-treated reticulocyte lysate was passed over a G-25 Sephadex column equilibrated and eluted with 50 mM KCl, 20 mM HEPES-KOH, pH 7.5, 1.0 mM $MgCl_2$, 0.5 mM EGTA, and 2 mM DTT. Fractions were taken which contained concentrations of hemoglobin (absorbance at 415 nm) >60% of the loaded sample. 0.5-ml aliquots, which were frozen in liquid N_2 and stored at −80°C, were thawed for use only once.

Freshly prepared reticulocyte lysate contains an excess of endogenous ribonuclease inhibitor which will inhibit the ribonuclease activity associated with translation components (16). However, we have observed that reticulocyte lysate preparations stored for a number of months at −80°C, no longer inactivated these exogenous sources of ribonuclease, and showed decreased incorporation of [^{35}S]methionine into proteins, particularly those of larger molecular weight. Addition of the purified ribonuclease inhibitor to these preparations of reticulocyte lysate, before the addition of mRNA, increased the level of protein synthesis and improved the distribution of translation products.

IN VITRO TRANSLATION ASSAY: Assays were carried out in a final volume of 25–100 μl with 40% (vol/vol) nuclease-treated and gel-filtered reticulocyte lysate and the following translation components: 1 mM ATP, 0.2 mM GTP, 6 mM creatine phosphate, 80 μg/ml creatine phosphokinase, 70 mM KCl, 28 mM HEPES-KOH, pH 7.5, 2 mM DTT, 0.5 mM glucose-6-phosphate, 10 μg/ml placental ribonuclease inhibitor, 100 μM each of 19 unlabeled amino acids and 100 μCi/ml of [^{35}S]methionine. The optimum for magnesium varied between 0.95 and 1.45 mM, depending upon the individual lysate preparation and the optimal

concentration of spermine, which varied between 50 and 100 μM. In some experiments, bovine liver transfer RNA at 100 μg/ml was added to the translation mixture. In reconstitution experiments, micrococcal nuclease-treated microsomal membranes were added at a final concentration of 20 A_{260} U/ml. In vitro translation was initiated with the addition of 0.2 A_{260} U/ml polyadenylated mRNA and a temperature shift from 3° to 22°C and incubations were carried out at 22°C for 90 min in the absence of added hemin. Incorporation of [^{35}S]methionine into protein was determined in 10-μl samples using Whatman 3-mm filter disks processed for hot TCA-insoluble radioactivity (26).

Wheat Germ Cell-free Translation System

Wheat germ extract was prepared according to the procedure of Marcus (27). In vitro translation assays using this extract were conducted as previously described (16).

Post-translational Analysis for Segregation of Polypeptide Chains

After in vitro translation, the location of nascent polypeptide chains, whether intracisternal or extracisternal with respect to microsomal vesicles, was determined by their sensitivity to added proteases. Cycloheximide at 10 μg/ml and tetracaine at 3 mM were added to the incubation mixture, and the resulting mixture was divided into two equal aliquots. Samples were incubated for 5 min at room temperature before shifting to 0°C. A fresh solution of 500 μg/ml trypsin and 500 μg/ml chymotrypsin (0°C) was added to one aliquot to achieve a final concentration of 50 μg/ml each (28) and post-translational proteolysis was carried out generally for 1 h at 0°C. The other aliquot served as a control. Both tetracaine and trypsin/chymotrypsin stock solutions contained 70 mM KCl and 1 mM $MgCl_2$ and the pH of the stock solution of tetracaine was adjusted to 7.5 with HCl. Post-translational proteolysis was terminated by the addition of Trasylol at 1,500 KIU/ml. Samples were incubated at 0°C for 5 min before further processing. Using the procedure described above, we could successfully carry out post-translational proteolysis for 1 h at either 3° or 22°C.

Separation of Translation Products by One-dimensional Gel Electrophoresis and Analysis of Radioactive Proteins by Fluorography or Autoradiography

Translation products were separated according to size (apparent molecular weight [M_r]) using one-dimensional polyacrylamide gel electrophoresis in SDS. 10-μl samples of the translation mixture were prepared for electrophoresis in a final volume of 40 μl containing 5% SDS, 15% sucrose, 0.0025% bromphenol blue, Tris and glycine in concentrations identical to that in electrode buffer, and 50 mM dithiothreitol. Samples were heated in boiling water for 3 min and maintained at 37°C for 30 min. Freshly prepared iodoacetamide was added to a final concentration of 100 mM and samples were further incubated at 22°C for 30 min. 40 μl was applied to individual gel slots measuring 13 mm in width. The 1-mm slab electrophoresis gel, 20 cm in length, contained a 12–17% acrylamide gradient serving as a resolving gel and a 5% acrylamide stacking gel, both in 0.1% SDS and buffers as described by Maizel (29). Gels were aged for ≥2 d at 3°C before use. Samples were electrophoresed at 22°C for 16 h initially at 1 mA constant current per 13-mm slot. After 2 h the current was increased to 2 mA per slot. After electrophoresis, gels were stained in 50% methanol, 10% acetic acid, and 0.06% Coomassie Blue for 1 h and destained in 40% methanol, 10% acetic acid.

Gels were prepared for fluorography using DMSO and PPO as described by Bonner and Laskey (30) except that 3% glycerol was added to both the 20% PPO in DMSO solution and the final solution of water, and 5 min after the gel was immersed in water, methanol was added to a concentration of 20%. Methanol in this concentration served to restrict the gel's expansion in water without resolubilizing the PPO; glycerol served as a stabilizer, and the combination prevented the gels from cracking during the vacuum drying procedure at 22°C on Whatman 3 MM paper. Gels containing impregnated PPO and covered with Saran Wrap were dried and then exposed for either fluorography (−80°C) or autoradiography (22°C) using medical X-ray film. Fluorograms of one-dimensional gels were scanned for radioactivity at 500 nm using a Zeiss PM6 spectrophotometer with a scanning attachment. Measurements were made in the range where optical density at 500 nm was linear with respect to radioactivity.

Separation of Translation Products by Two-dimensional IEF/SDS Gel Electrophoresis and Analysis by Fluorography

Translation products were separated according to both size (apparent M_r) and isoelectric point (IEP) using the two-dimensional isoelectric focusing/SDS gel electrophoresis procedures developed by Scheele (31) and modified by Bieger and Scheele.[3] These procedures utilize slab gel isoelectric focusing (IEF) in the absence (31) or presence[3] of 8 M urea in the first dimension and SDS/slab gel electrophoresis in a polyacrylamide gradient (10–20%) in the second dimension. Translation samples containing membranes were treated with 1% Triton X-100, 25 mM Tris-HCl, pH 9.0, 100 KIU/ml Trasylol, and 1 mM DFP before application on the surface of the IEF gel. When nonradioactive dog pancreas secretory proteins (either proteins secreted into the incubation medium, or a crude zymogen granule fraction) were added as markers, samples were made 4–6 M in urea to prevent autoactivation of potential proteases.[3] IEF gels contained, in addition to 8 M urea, 10 KIU/ml of Trasylol and 0.1 mM DFP. IEF and SDS gel electrophoresis were carried out as described by Scheele (31). Second-dimensional gels were stained with Coomassie Blue R, destained, impregnated with PPO, dried on Whatman 3 MM paper, and exposed for fluorography as described above for one-dimensional gels.

RESULTS

Characterization of Dog Pancreas Secretory Proteins by Two-dimensional IEF/SDS Gel Electrophoresis and One-dimensional Polyacrylamide Gel Electrophoresis in SDS

Secretory proteins contained in a zymogen granule fraction isolated from dog pancreas were separated in two dimensions, using isoelectric focusing in the first dimension and gradient polyacrylamide gel electrophoresis in the presence of SDS in the second dimension as originally described by Scheele (31). 17 Discrete proteins were identified using this technique (cf. Table I). Proteins were identified in the first dimension according to actual and potential enzyme activity and in the second dimension according to the molecular weights of commercially available pancreatic proteins.[4] In Fig. 6 two-dimensional gel spots are labeled according to their activity by abbreviations described in Table I. The physical characteristics of these proteins are also indicated in the table. Finally, glycoproteins containing terminal sialic acid residues were identified by both periodic acid-Schiff staining (32) and neuraminidase-sensitive labeling with NaB^3H_4 after oxidation with 10 mM $NaIO_4$ (33). With this procedure, G1, G2, and lipase were shown to be glycoproteins. Both amylase and lipase were identified as clusters of spots, exhibiting heterogeneity of charge expression. While these two proteins migrate closely in the two-dimensional gel, amylase can be identified by precipitation with shellfish glycogen (35) and lipase can be identified by labeling with NaB^3H_4 after oxidation with $NaIO_4$.[4]

While the two-dimensional gel technique is the procedure of choice for the analysis of the complexity of secretory products synthesized by the dog pancreas and monitors unreduced proteins accurately by their charge and size, analysis of small differences in size that might exist between precursors and authentic products is more accurately carried out on reduced and alkylated proteins using one-dimensional gradient polyacrylamide gel electrophoresis in the presence of SDS. Fig. 1 shows, after fluorography, the pattern of dog pancreas secretory proteins, reduced, alkylated, and separated by one-dimensional SDS gel electrophoresis in a polyacrylamide gradient (12–17%). Radioactive methionine-labeled proteins are characterized by molecular weight and identified by actual or potential enzyme

[3] Bieger, W., and G. Scheele. 1980. Two-dimensional isoelectric focusing/sodium dodecyl sulfate gel electrophoresis of protein mixtures containing active or potentially active proteases. Analysis of human exocrine pancreatic proteins. *Anal. Biochem.* 107:369–376.

[4] Scheele, G. A. Characterization of canine exocrine pancreatic proteins by two-dimensional isoelectric focusing/SDS gel electrophoresis. Manuscript in preparation.

TABLE I

Characteristics of Dog Exocrine Pancreatic Proteins

	Abbreviation	IEP_n*	M_r‡	Distribution of radioactivity		PAS‖
				^{14}C-amino acid mix§	[^{35}S]methionine	
				%		
1. Glycoprotein 1	G1	3.9–4.2¶	97,000	0.6	6.2	+
2. Glycoprotein 2	G2	3.9–4.2¶	92,000	0.7	5.6	+
3. Amylase	A	6.0	53,000–54,500	35.4	24.0	–
4. Lipase	L	5.9	55,000	1.4	7.6	+
5. (Pro)carboxypeptidase A1	PA1	5.0	47,300	0.6	2.3	–
6. (Pro)carboxypeptidase A2	PA2	5.5	45,500	2.2	2.0	–
7. (Pro)carboxypeptidase A3	PA3	5.7	45,500		0.8	–
8. (Pro)carboxypeptidase B	PB	6.3	46,700	1.8	2.0	–
9. (Pro)elastase 1	PE1	5.0	29,800	1.6	4.4	–
10. (Pro)elastase 2	PE2	5.3	30,000	3.9	2.8	–
11. Chymotrypsin(ogen) 1	C1	5.5	27,500	2.4	4.2	–
12. Chymotrypsin(ogen) 2	C2	7.1	27,500	4.7	7.6	–
13. Chymotrypsin(ogen) 3	C3	9.5	—	0.4	0.2	–
14. Trypsin(ogen) 1	T1	4.7	28,000	35.5	16.7	–
15. Trypsin(ogen) 2	T2	8.05	26,400	5.6	9.6	–
16. Trypsin(ogen) 3	T3	8.15	26,400			–
17. (Pro)phospholipase A_2	PPL	7.3	14,600	0.5	3.3	–

Note: Enzymes and potential enzymes are numbered consecutively from anode to cathode following the recommendations of the IUPAC-IUB Commission on the biochemical nomenclature of multiple forms of enzymes separated by polyacrylamide gel electrophoresis (34). Two glycoproteins with unknown function are numbered arbitrarily.

* IEPs of undenatured (native) proteins (cf. footnote 4).

‡ M_r of carboxymethylated proteins as determined by gradient (12–17%) polyacrylamide gel electrophoresis in SDS.

§ The distribution of individual radiolabeled proteins as a percent of total incorporated radioactivity obtained with the mixture of 15 ^{14}C-amino acids gives a measure of relative mass among secretory proteins (31).

‖ Glycoproteins have been identified by periodic acid-Schiff reactivity (32) of two-dimensional gel spots and neuraminidase-sensitive radioactivity associated with proteins after oxidation with $NaIO_4$ and reduction with NaB^3H_4 (33).

¶ IEPs for G1 and G2 in 8 M urea (Fig. 5) are shifted to pH 6.5–7.5. IEPs of other proteins are shifted in urea only to a small extent (cf. footnote 3).

activity as described by Scheele.[4] The highest resolution gels separate proteins into the pattern observed in Fig. 1. Occasionally, procarboxypeptidases A2 and A3 separate into two distinct bands. In lower resolution gels procarboxypeptidase A1 and B migrate as a single band, and three serine proteases, trypsinogen 1 and chymotrypsinogens 1 and 2, migrate in a single band. Because of this slight variation in the migration of polypeptide chains from gel to gel, an individual experiment including all controls was analyzed on a single gel.

In Vitro Translation of Cell Fractions Isolated from Dog Pancreas; Analysis of Translation Products According to Size

Polyadenylated mRNA isolated from dog pancreas was translated both in the reticulocyte lysate system and in the wheat germ system, and the resulting translation products were compared to authentic radiolabeled secretory proteins by SDS/polyacrylamide gel electrophoresis and fluorography (Fig. 1). mRNA-directed translation products were identical according to M_r whether synthesized with rabbit reticulocyte lysate or wheat germ extract. Similarly, authentic secretory proteins were identical by M_r whether a tissue homogenate or an isolated zymogen granule fraction was analyzed. The banding pattern obtained with mRNA-directed translation products was, however, not congruent with that obtained from authentic secretory proteins. The primary translation products for the nonglycosylated secretory proteins appeared in every case 1,000–2,000 daltons larger than their counterparts among authentic secretory proteins. This was the case for amylase, 54 kdaltons; the group of four procarboxypeptidases, 45–47 kdaltons; the group of eight serine proteases, 26–30 kdaltons, and prophospholipase, 14.6 kdaltons. For the two high molecular weight secretory glycoproteins, G1 and G2, the differences observed in migration of polypeptide chains was just the reverse: their primary translation products were smaller than the authentic secretory glycoproteins.

Immunoreplication studies (36), carried out on pancreatic presecretory and secretory proteins separated by polyacrylamide gel electrophoresis in SDS, were conducted to identify individual precursor product pairs and to confirm that the apparent size difference between these related forms was no greater than 1,000–2,000 daltons as described above. Monospecific antibodies directed against six dog pancreas secretory proteins (amylase, procarboxypeptidase A1, chymotrypsinogen 1, chymotrypsinogen 2, trypsinogen 1, trypsinogen 2 + 3) were used in these studies. In every case a single polypeptide chain was identified among the products translated with dog pancreas mRNA and a single chain was identified among authentic secretory proteins. In every case, the primary translation product was larger by 1,000–2,000 daltons than its counterpart among authentic secretory proteins (data not shown).

Functional Reconstitution of Dog Pancreas Rough Microsomes

PREPARATION OF MICROSOMAL MEMBRANES STRIPPED OF ENDOGENOUS mRNA; COMPARISON OF TREATMENT WITH MICROCOCCAL NUCLEASE TO TREATMENT WITH EDTA: Data contained in Fig. 2 show

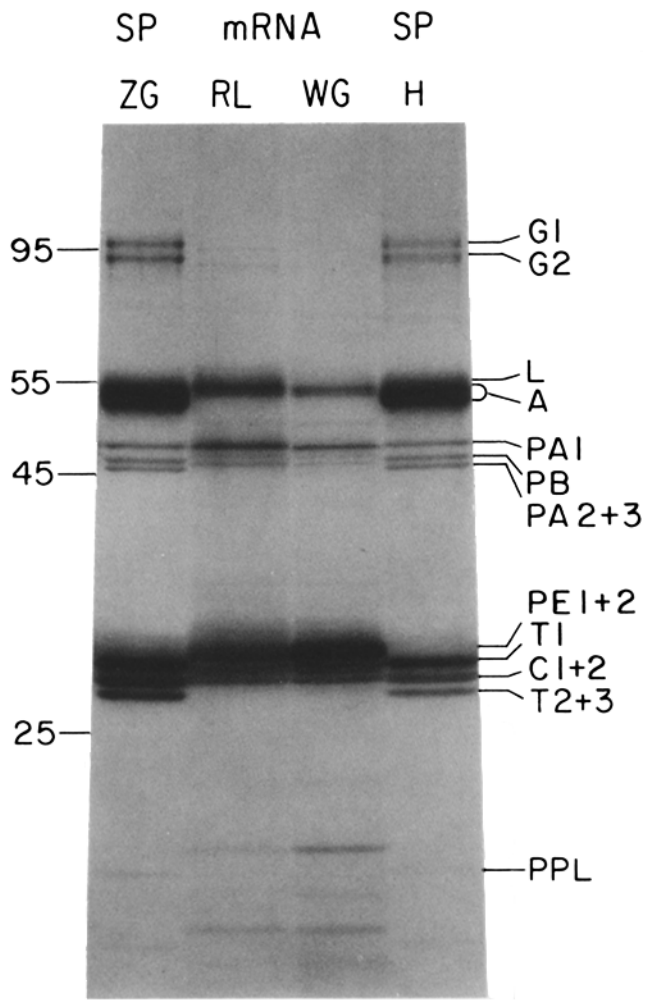

FIGURE 1 Comparison of translation products directed by dog pancreas mRNA in the reticulocyte lysate (*RL*) and wheat germ (*WG*) systems with authentic secretory proteins from dog pancreas. Secretory proteins (*SP*) were labeled in dog pancreas tissue slices with [^{35}S]methionine during a 3-h incubation and prepared for analysis as either a tissue homogenate (*H*) or a crude zymogen granule lysate (*ZG*). [^{35}S]methionine-labeled translation products and secretory proteins were separated by polyacrylamide gel electrophoresis in SDS and analyzed by fluorography. Numbers to the left indicate molecular weight markers. Secretory proteins are identified according to actual or potential enzyme activity by abbreviations given on the right. These abbreviations are described in Table I.

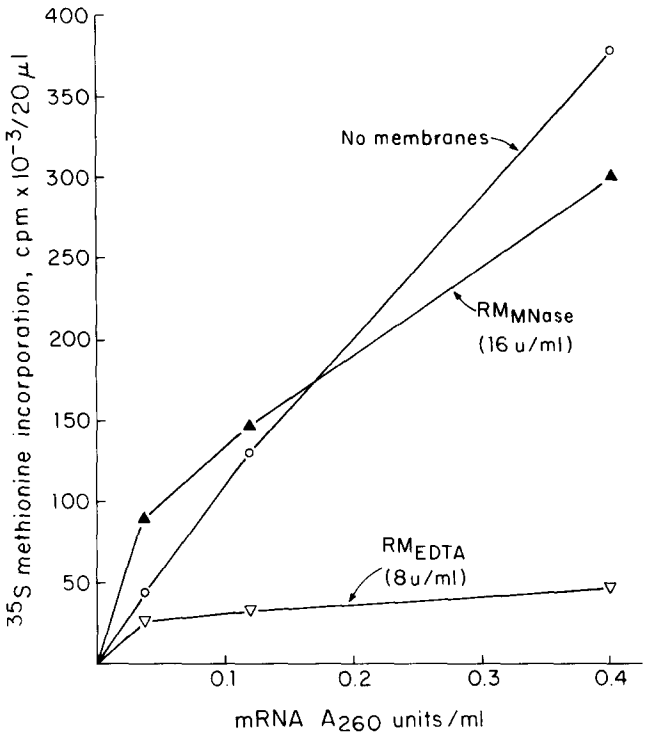

FIGURE 2 Comparison of the effect of micrococcal nuclease-stripped and EDTA-stripped microsomal membranes on mRNA-directed incorporation of [^{35}S]methionine into protein. Dog pancreas rough microsomes were treated either with 1.5 µmol EDTA/10 A_{260} U of membranes at 0°C or 1,500 U/ml micrococcal nuclease for 30 min at 22°C. Values are given for the incorporation of [^{35}S]methionine into protein with increasing concentrations of dog pancreas mRNA in the absence of microsomal membranes (○), or in the presence of EDTA-treated microsomal membranes, 8 A_{260} U/ml, (▽), or micrococcal nuclease-treated microsomal membranes, 16 A_{260} U/ml (▲). 10 A_{260} U/ml of EDTA-treated and nuclease-treated rough microsomes contained 1.15 mM and 0.8 mM phospholipid, respectively, as measured by phosphate assay after ashing in the presence of $Mg(NO_3)_2$ (37).

the effect of dog pancreatic microsomal membranes, treated either with EDTA or micrococcal nuclease, on the incorporation of [^{35}S]methionine into protein as a function of the concentration of mRNA. Concentrations of mRNA up to 0.4 A_{260} U/ml, in the absence of microsomal membranes, show a linear increase in incorporation of [^{35}S]methionine into protein. mRNA translation in the presence of rough microsomes treated with EDTA, in a manner similar to that described by Blobel and Dobberstein (2), and Shields and Blobel (4) was markedly inhibited, and the inhibition increased dramatically with increasing concentrations of mRNA. Addition of increasing concentrations of magnesium to the translation mixtures containing EDTA-treated membranes did not alter the inhibitory effect of these membranes, indicating that residual EDTA was not the cause of this inhibition. mRNA translation in the presence of rough microsomes treated with micrococcal nuclease was stimulated at lower concentrations of mRNA and partially inhibited at higher concentrations of mRNA.

Fig. 3 shows a fluorogram of the translation products synthesized by EDTA-treated microsomal membranes in the absence and presence of dog pancreas mRNA and compares these to translation products synthesized by nuclease-treated microsomal membranes, also in the absence and presence of mRNA. In the absence of added mRNA, EDTA-treated membranes directed the synthesis of identifiable dog pancreas secretory proteins. Micrococcal nuclease-treated membranes did not synthesize detectable levels of proteins. In the presence of added dog pancreas mRNA, the comparative effect of EDTA and nuclease-treated membranes on the distribution of nascent secretory proteins was marked. Fewer dog pancreas secretory proteins were synthesized in the presence of EDTA-treated membranes, which agrees with the inhibitory effect of EDTA-treated membranes seen in Fig. 2. In addition, the distribution of dog pancreas secretory proteins synthesized by the two membrane preparations was markedly different. Secretory proteins synthesized by dog pancreas mRNA in the presence of nuclease-treated microsomal membranes were similar to that synthesized in vivo (cf. Table III and Fig. 5). Secretory proteins synthesized in the presence of EDTA-treated membranes

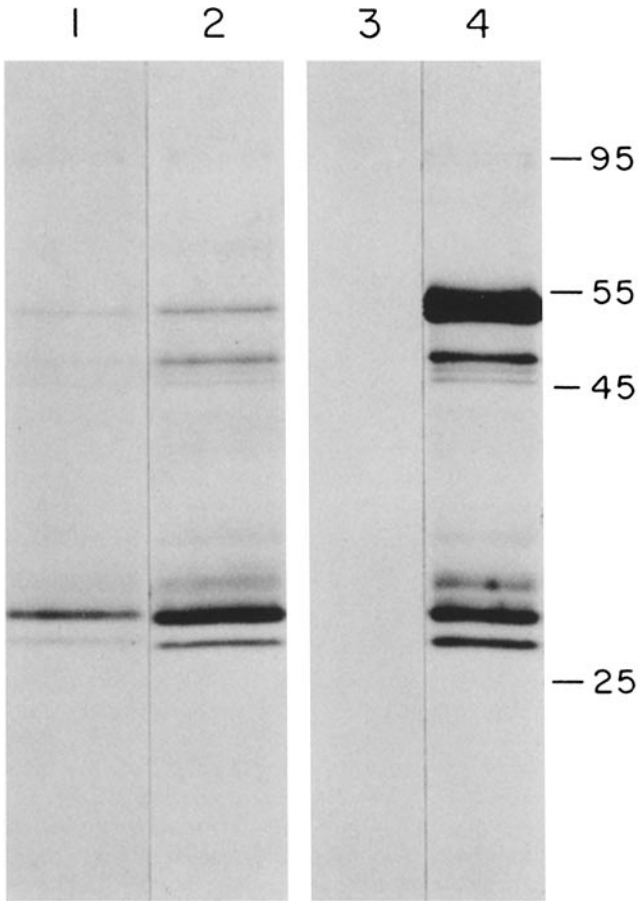

FIGURE 3 Comparison of in vitro translation products directed by EDTA-treated vs. micrococcal nuclease-treated microsomal membranes in the absence and presence of dog pancreas mRNA. Dog pancreas rough microsomal membranes were treated with either EDTA or micrococcal nuclease as described in Materials and Methods. In vitro translation was carried out using the nuclease-treated reticulocyte lysate system and translation products were separated by polyacrylamide gel electrophoresis in SDS and analyzed by fluorography. Tracks *1* and *2* give the translation products derived from 20 A_{260} U/ml EDTA-treated membranes without and with the addition of 0.2 A_{260} U/ml dog pancreas mRNA, respectively. Tracks *3* and *4* give the translation products derived from 20 A_{260} U/ml nuclease-treated membranes without and with the addition of 0.2 A_{260} U/ml dog pancreas mRNA, respectively. Numbers to the right indicate molecular weight values × 10^{-3}. Radioactive secretory proteins are identified as indicated in Fig. 1.

showed a progressive diminution in the larger molecular weight products, suggesting a defect in polypeptide chain elongation introduced by these membranes. Synthesis of amylase with added mRNA was 9.1 times greater in the presence of nuclease-treated membranes than EDTA-treated membranes. In vitro translation studies, utilizing dog pancreas mRNA and synchronized with respect to protein synthesis, showed lower average rates of polypeptide chain elongation in the presence of EDTA-treated microsomal membranes (<3 amino acid residues/min) than in the presence of nuclease-treated microsomal membranes (9.7 amino acid residues/min).

The ribosomes that remain attached to microsomal membranes after micrococcal nuclease treatment are judged to be functional, because they effect the translation of dog pancreas mRNA in the presence of a reticulosyte lysate devoid of ribosomes or ribosomal subunits, obtained by sedimentation of

TABLE II

Effect of Ribonuclease Inhibitor on the Stability of mRNA Associated with Rough Microsomes *

Rough microsomes	Incorporation of [^{35}S]methione into protein		
	−I	+I	% Stimulation
	cpm × 10^{-3}/10 μl		
Dog pancreas	540	598	10.7
Dog pancreas‡	432	410	0
Dog liver	62	129	108.1
Rat liver	292	452	54.8
Guinea pig liver	210	376	79.0

* Rough microsomes isolated as described in Materials and Methods were incubated with and without human placental ribonuclease inhibitor (I) at 10 μg/ml for 30 min at 22°C. An equivalent amount of inhibitor was then added to the experimental mixture and residual mRNA was monitored by the incorporation of [^{35}S]methionine into protein 90 min after the addition of reticulocyte lysate and the energy mix.

‡ Rough microsomes washed with 500 mM KCl.

the lysate at 100,000 *g* for 2.5 h at 3°C (38). These ribosomes are presumed to be functionally detached from the membrane shortly after in vitro peptide synthesis is activated and degraded messenger oligonucleotides are read out. Ribosomes introduced with nuclease-treated dog pancreas microsomal membranes can be expected, therefore, to be functionally equivalent with ribosomes contained in the reticulocyte lysate system.

ASSOCIATION OF RIBONUCLEASE ACTIVITY WITH MICROSOMAL MEMBRANES: INHIBITION WITH PLACENTAL RIBONUCLEASE INHIBITOR: Ribonuclease associated with rough microsomes isolated from the livers of dog, rat, and guinea pig, and the pancreas of the dog was measured using an assay procedure developed by Scheele and Blackburn (16). Rough microsomes were incubated without (experimental) and with (control) human placental ribonuclease inhibitor at 10 μg/ml for 30 min at 22°C. After that time, an equivalent amount of inhibitor was added to the experimental mixture and residual functional mRNA in both experimental and control mixtures was measured by the incorporation of [^{35}S]methionine into protein 90 min after the addition of reticulocyte lysate and the energy mix. The data contained in Table II indicate that rough microsomes prepared from dog pancreas contain relatively small quantities of ribonuclease activity. When washed in 500 mM KCl, dog pancreas rough microsomes showed no ribonuclease activity. Rough microsomes prepared from rat liver, guinea pig liver, and dog liver showed increasing amounts of ribonuclease activity that could be partially inhibited by the dose of placental inhibitor used.

FIDELITY OF IN VITRO mRNA TRANSLATION IN THE PRESENCE OF STRIPPED MICROSOMAL MEMBRANES: COMPARISON OF EDTA AND MICROCOCCAL NUCLEASE TREATMENT: Table III gives the distribution of dog pancreas secretory products synthesized in vitro in the absence and presence of stripped microsomal membranes, without and with bovine liver tRNA, and compares them to the distribution of authentic secretory proteins synthesized in pancreatic tissue slices. Distributions of radioactivity were determined between amylase (53–54.5 kdaltons), the group of procarboxypeptidases A and B (45–47 kdaltons), and the group of serine proteases (26.4–30 kdaltons). Thus, the data represent the percent synthesis of completed secretory proteins as analyzed for three molecular weight classes. All translations were carried out with 10 μg/ml ribonuclease inhibitor (16), which, in the presence of

TABLE III

Percent Distribution of Radioactivity among Dog Pancreas Secretory Proteins: Comparison of Cell-free and Cellular Synthesis

	mRNA		mRNA + EDTA-stripped membranes		mRNA + nuclease-stripped membranes		SP
	−tRNA	+tRNA	−tRNA	+tRNA	−tRNA	+tRNA	−
Amylase	6.7	24.2	14.5	17.5	35.9	37.1	41.7
Procarboxypeptidases A and B	12.5	22.9	16.5	18.7	15.4	15.0	6.8
Serine proteases	80.8	52.8	69.0	63.8	48.7	47.9	51.5

Translation products or authentic secretory products contained in zymogen granule lysates (*SP*) were separated by one-dimensional polyacrylamide gel electrophoresis in SDS, and analyzed by gel scanning after PPO fluorography. Peaks representing the groups of secretory proteins indicated were quantitated by gravimetric analysis.

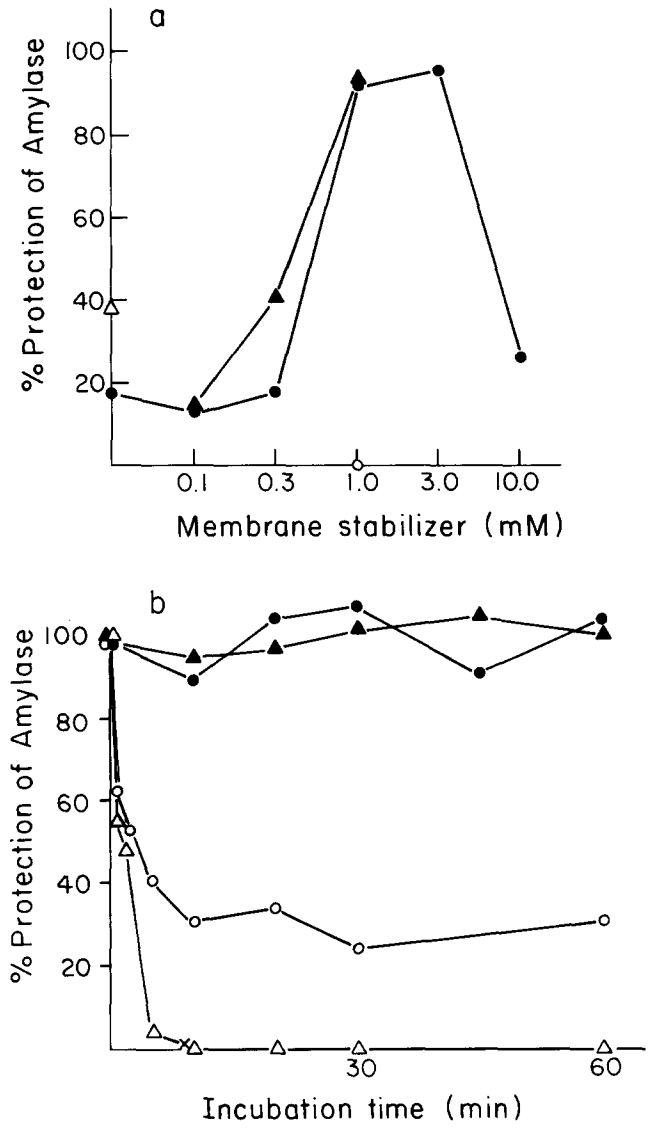

FIGURE 4 (*a*) Translation of dog pancreas mRNA in the presence of microsomal membranes; effect of tetracaine (●) and dibucaine (▲) on the protection of radioactive amylase during post-translational proteolysis. Radioactive methionine-labeled products were synthesized in the reticulocyte lysate system in the presence of 0.2 A_{260} U/ml dog pancreas mRNA, and 20 A_{260} U/ml micrococcal nuclease-treated dog pancreas microsomal membranes. Segregation of radioactive-labeled polypeptide chains was monitored by the addition of trypsin and chymotrypsin (50 μg/ml each) for 1 h at 0°C. Cycloheximide was not added during the proteolysis process. Post-translational proteolysis was terminated by the addition of 1,500 KIU/ml of Trasylol. Translation products were separated by polyacrylamide gel electrophoresis in the presence of SDS and radioactivity contained in protein bands was analyzed by optical density scanning of fluorograms. Scanning peaks representing amylase were cut out and weighed. Data are expressed as percent amylase observed after incubation with proteases compared to that observed after a control incubation. Tetracaine and dibucaine were added at 22°C, 5 min before a shift in temperature to 0°C and the addition of trypsin and chymotrypsin. The protective effect of 30 mM $CaCl_2$ is shown on the ordinate for comparison (△). The protective effect of 1 mM tetracaine in the presence of 1% sodium deoxycholate is indicated (○). (*b*) Protection of amylase during post-translational incubation with trypsin-chymotrypsin as a function of time, temperature, and the presence or absence of 3 mM tetracaine. Dog pancreas mRNA was translated in the presence of nuclease-treated microsomal membranes. After the completion of protein synthesis, at 90 min, translation mixtures were incubated in the presence of trypsin and chymotrypsin (50 μg/ml each), cycloheximide (10 μg/ml) with and without 3 mM tetracaine, at 0° and 22°C. At the indicated times, aliquots were removed from the incubation mixtures and prepared immediately for gel electrophoresis in SDS after the addition of Trasylol. Radioactive proteins separated by gel electrophoresis were analyzed by scanning at 500 nm after fluorography with PPO/DMSO. Scanning peaks were cut out with scissors and weighed. Percent amylase, resistant to the added proteases, is given for proteolysis at 0°C (circles) or 22°C (triangles) in the presence (closed symbols) or absence (open symbols) of 3 mM tetracaine. Amylase protection after 10 min of trypsin-chymotrypsin treatment at 0°C in the presence of 3 mM tetracaine and 1% deoxycholate is indicated (×).

dog pancreas microsomal membranes, completely inhibited ribonuclease activity associated with the translation components. In the case of translation of dog pancreas mRNA in the absence of membranes, tRNA addition resulted in an increase in incorporation of ^{35}S into protein, 1.3- to 2.2-fold, and a change in the distribution of translation products. In the presence of tRNA, there was a stimulation in the synthesis of the larger molecular weight products (Table III), such that the resulting distribution more closely resembled that observed during in vivo synthesis. In the presence of mRNA and EDTA-treated microsomal membranes, the addition of tRNA increased the incorporation of radioactivity into protein (~30%) and marginally improved the distribution of dog pancreas translation products. tRNA addition did not significantly increase the incorporation of radioactivity or change the distribution of products synthesized in the presence of dog pancreas mRNA and nuclease-treated microsomal membranes. With or without tRNA, fidelity in translation was most closely achieved using mRNA and nuclease-treated microsomal membranes.

TRANSLATION OF mRNA IN THE PRESENCE OF NUCLEASE-TREATED MICROSOMAL MEMBRANES: TRANSLATION WITH RETICULOCYTE LYSATE COMPARED TO

TRANSLATION WITH WHEAT GERM EXTRACT: While the translation of mRNA in the presence of nuclease-treated microsomal membranes proceeds efficiently in the reticulocyte lysate system, the addition of these same membranes to the wheat germ system markedly inhibited (75–95%) translation of mRNA. While the cause of this decrease in efficiency is not known, it indicates that translation components from plants and animals cannot be intermixed without loss of efficiency in the translation process. In agreement with this conclusion is the finding that detached polysomes and rough microsomes, isolated from the dog pancreas, synthesize proteins at a reduced efficiency in the wheat germ system (87 and 9%, respectively) compared to the reticulocyte lysate system (G. Scheele and R. Jacoby, unpublished observations). Although the presence of nuclease-treated membranes inhibits the translation of mRNA in the wheat germ system, polypeptide chains that are synthesized and completed appear to be correctly processed and segregated.

Analysis of Segregation of Polypeptide Chains by Microsomal Vesicles: Post-translational Proteolysis Studies

Translation of 0.2 A_{260} U/ml dog pancreas mRNA in the reticulocyte translation system, in the presence of 20 A_{260} U/ml nuclease-treated microsomal membranes, resulted in complete processing of all secretory polypeptide chains. (cf. Figs. 5 and 6). According to the transport peptide hypothesis (1, 39, 40), this finding would suggest that all nascent chains are transported across the microsomal membrane and segregated within the cisternal space. However, analysis for segregated polypeptide chains by post-translational proteolysis as described by Blobel and Dobberstein (2) or Shields and Blobel (4) indicated that only 17–30% of radiolabeled amylase was resistant to a 1-h incubation with 50 μg/ml trypsin and 50 μg/ml chymotrypsin at 0°C (Fig. 4). Amylase resistance to post-translational proteolysis for 1 h at 22°C was zero. The addition of calcium or magnesium during post-translational proteolysis at 0°C increased the amount of radioactive amylase protected from degradation to between 39 and 54%. Tetracaine at 3 mM and dibucaine at 1 mM resulted in a dramatic increase in protection of radioactive amylase, >95%. A number of other exogenous agents, including La^{3+}, Co^{2+}, Ni^{2+}, and Mn^{2+}, gave results similar to those with calcium and magnesium. Benzocaine, chlorpromazine, cholesterol, tetramethyl and tetraethyl ammonium chloride, sphingomyelin, and EGTA had no effect on protection of radioactive amylase.

Tetracaine showed a biphasic effect on the protection of radiolabeled amylase (Fig. 4*a*). At 1–3 mM it dramatically increased the resistance of amylase to the action of proteases. At 10 mM, however, the protective effect of tetracaine was markedly decreased. The protective effect of dibucaine as a function of concentration was similar to tetracaine, although higher concentrations could not be studied because of the solubility characteristics of this compound. In the presence of 1% sodium deoxycholate, which solubilizes the lipid bilayer of microsomal vesicles, radioactive amylase was completely degraded in the presence of trypsin, chymotrypsin, and 1 mM tetracaine (Fig. 4*a*).

Fig. 4*b* shows a kinetic analysis of the protection of amylase by microsomal membranes during a 60-min incubation in the presence of trypsin and chymotrypsin as a function of time, temperature, and the presence or absence of tetracaine. Proteolysis at 0°C in the absence of tetracaine resulted in the degradation of 68% of radioactive amylase by 10 min. Further incubation through 60 min resulted in no further degradation of nascent amylase. Proteolysis at 22°C in the absence of tetracaine resulted in the complete degradation of radioactive amylase at all time points tested. In contrast, proteolysis at either 0° or 22°C, carried out in the presence of 3 mM tetracaine, resulted in the complete protection (95–102%) of radioactive amylase by the microsomal membranes.

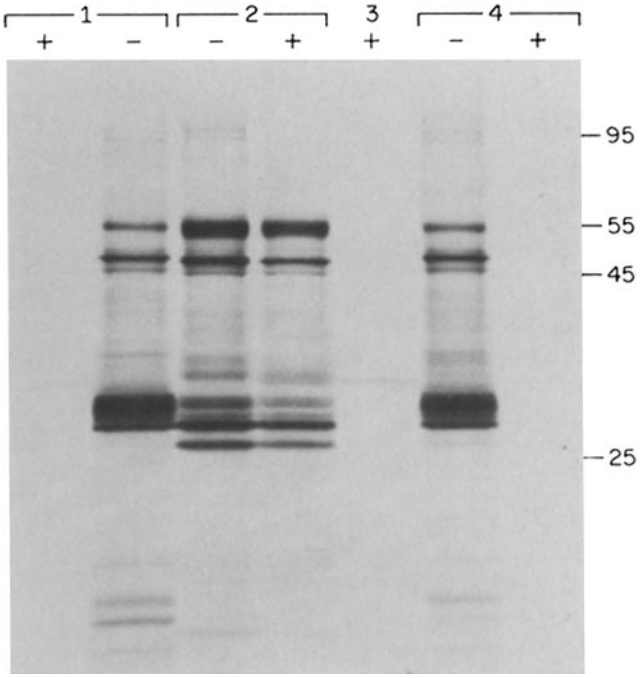

FIGURE 5 Analysis of translation products directed by dog pancreas mRNA in the absence and presence of micrococcal nuclease-treated dog pancreas microsomal membranes. Rough microsomal membranes were treated with 1,500 U/ml micrococcal nuclease and 0.5 mM $CaCl_2$ for 30 min at 22°C. Nuclease activity was terminated by the addition of 2.0 mM EGTA. Translation products, generated in the reticulocyte lysate system, are shown for 0.2 A_{260} U/ml mRNA (region *1*), 0.2 A_{260} U/ml mRNA plus 20 A_{260} U/ml nuclease-treated membranes (regions *2* and *3*) and 0.2 A_{260} U/ml mRNA for 1 h followed by the addition of 1 μM edeine and, after 10 min, the further addition of 20 A_{260} U/ml nuclease-treated membranes for an additional hour at 22°C (region *4*). Segregation of polypeptide chains was analyzed by the post-translational addition (+) of trypsin and chymotrypsin (50 μg/ml each) for 1 h at 22°C in the presence of 3 mM tetracaine. Cycloheximide was not included. Deoxycholate, 1%, was added during post-translational proteolysis in one sample (region *3*). Proteolysis was terminated by the addition of 1,500 KIU/ml Trasylol. Radioactive polypeptide chains were separated by polyacrylamide gel electrophoresis in SDS and analyzed by fluorography. Numbers to the right indicate molecular weight values × 10^{-3}. Radioactive secretory and presecretory proteins are identified as indicated in Fig. 1.

Effect of Cotranslational vs. Post-translational Addition of Nuclease-treated Microsomal Membranes on Segregation and Processing of Nascent Secretory Proteins

Translation of 0.2 A_{260} U/ml dog pancreas mRNA in the presence of 20 A_{260} U/ml of nuclease-treated microsomal mem-

branes resulted in the complete processing of nascent polypeptide chains which then comigrated with authentic secretory proteins (compare Fig. 5 to Fig. 1; see also Fig. 6). Translation products synthesized in the absence and presence of microsomal membranes were analyzed for their resistance to trypsin and chymotrypsin added post-translationally for 1 h at 22°C in the presence of 3 mM tetracaine. Translation products synthesized in the absence of membranes were completely degraded by this treatment. Those products synthesized in the presence of stripped membranes were nearly completely protected from protease activity. Post-translational proteolysis in the presence of 3 mM tetracaine and 1% deoxycholate resulted in complete degradation of processed polypeptide chains. Finally, translation products synthesized in the absence of microsomal membranes and then, after the addition of 1 μM edeine (an initiation inhibitor), incubated in the presence of membranes for 1 h at 22°C, showed no evidence of processing during this period of time. After this second incubation, the translation products were identical in size and distribution to those observed immediately after synthesis. These translation products were completely degraded upon addition of trypsin and chymotrypsin.

Processing of Nascent Secretory Proteins by Nuclease-treated Microsomal Membranes: Analysis by Two-dimensional IEF/SDS Gel Electrophoresis

Translation products were separated in two dimensions using IEF in the presence of 8 M urea in the first dimension and SDS gel electrophoresis in a polyacrylamide gradient (10–20%) in the second dimension as described by Bieger and Scheele.[3] Unlabeled dog pancreas secretory protein was added to each of the samples to provide Coomassie Blue markers of authentic secretory proteins in each gel. Translation products from dog pancreas mRNA in the absence of microsomal membranes separated into only a few discrete two-dimensional spots (Fig. 6*a*). None of these spots coincided with those of authentic secretory protein, although in several instances they appeared in the vicinity of secretory proteins. The majority of [^{35}S]methionine labeled translation products synthesized in the absence of microsomal membranes are observed to smear in both dimensions of the gel procedure. The two-dimensional gel spot in the lower right corner represents hemoglobin synthesized from a small amount of undegraded endogenous globin mRNA which survived the micrococcal nuclease treatment given to the rabbit reticulocyte lysate. The position of this translation product is unchanged in panels *a*, *b*, and *c*.

Dramatic changes were observed when dog pancreas mRNA was translated in the presence of stripped microsomal membranes. In this case translation products migrated as discrete spots, the majority of which coincided with authentic secretory proteins, located by Coomassie Blue stain. A comparison of the translation products synthesized by dog pancreas mRNA in the presence of stripped microsomal membranes with those synthesized by dog pancreas rough microsomes (Fig. 6*b* compared to 6*c*) reveals a remarkable degree of identity among the products. Qualitatively and quantitatively the patterns are very

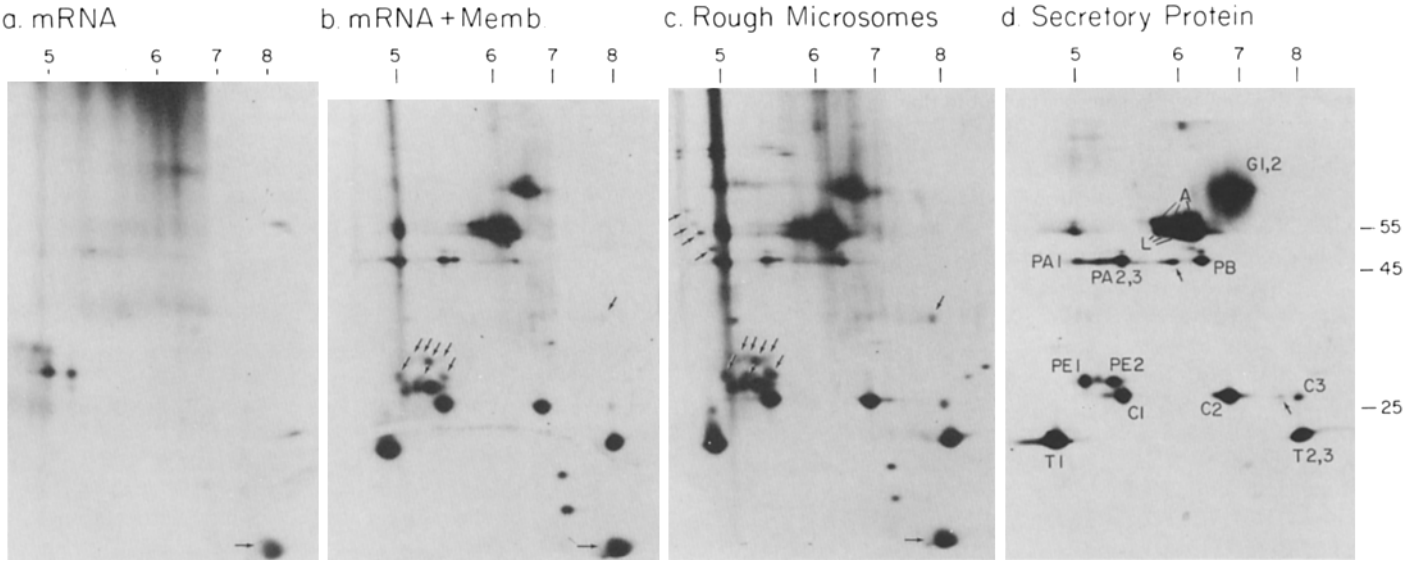

FIGURE 6 Two-dimensional gel analysis of translation products directed by dog pancreas mRNA in the absence (*a*) and presence (*b*) of micrococcal nuclease treated-dog pancreas microsomal membranes, dog pancreas rough microsomes (*c*) and authentic dog pancreas secretory proteins (*d*). In vitro protein synthesis was carried out in the reticulocyte lysate system in the presence of 3.3 mM dithiothreitol and 6 mM oxidized glutathione. Before analysis of the translation products by the two-dimensional gel technique, incubation mixtures were made 1% in Triton X-100 and 25 mM in Tris-HCl, pH 9.0, 100 KIU/ml in Trasylol, and 1 mM in DFP. Radioactive methionine-labeled translation products and secretory protein extracted from dog pancreas tissue slices were separated in two dimensions using IEF in the presence of 8 M urea in the first dimension and SDS gel electrophoresis in the second dimension as described by Bieger and Scheele (footnote 3). Equal quantities of TCA-insoluble radioactivity were applied to the gels. Radioactivity contained in the separated proteins was analyzed by fluorography. Numbers on the upper abscissa indicate IEPs. Numbers on the ordinate indicate molecular weight values × 10^{-3}. Secretory proteins separated by the two-dimensional gel technique (*d*) are labeled according to their actual or potential activity by abbreviations described in Table I. Two o'clock arrows indicate four proteins synthesized by rough microsomes but not observed among the translation products synthesized by mRNA and stripped microsomal membranes. Seven o'clock arrows indicate proteins synthesized by both rough microsomes and mRNA plus membranes, but not observed among authentic secretory proteins. Eleven o'clock arrows indicate proteins observed among authentic secretory product but not observed among the translation products of rough microsomes or mRNA plus stripped membranes. Three o'clock arrows indicate hemoglobin.

similar. Among the identified dog pancreas secretory proteins, the patterns are identical. Among the translation products of rough microsomes (Fig. 6 *c*) four two-dimensional gel spots, marked by the 2 o'clock arrows, are unique (not observed among the proteins in Fig. 6 *b*). They may represent proteins synthesized by mRNA which is associated with rough microsomes, but lost during mRNA purification, e.g., RNAs lacking poly A tracts at their 3′ termini. These proteins are also not found among authentic secretory proteins (Fig. 6 *d*). The translation products synthesized with mRNA in the presence of microsomal membranes, contained no unique proteins which were not seen when rough microsomes were translated.

A comparison of authentic secretory proteins (Fig. 6 *d*) with translation products common to rough microsomes (Fig. 6 *c*) and mRNA plus membranes (Fig. 6 *b*) also reveals a remarkable degree of similarity. Among the identified dog pancreas secretory proteins, labeled in Fig. 6 *d*, the patterns are identical. For these proteins, comigration of segregated proteins (mRNA and membranes) with authentic secreted proteins also occurred when two-dimensional gel electrophoresis was carried out in the absence of urea in the first dimension. As expected, radiolabeled hemoglobin is not present among authentic dog pancreas secretory proteins. Two proteins indicated by the 11 o'clock arrows are present among authentic secretion products, which are not observed among the translation products of either reconstituted or authentic dog pancreas rough microsomes. These proteins probably represent secretory proteins which have been modified by post-translational processing as has been demonstrated for guinea pig pancreatic proelastase (41). Six proteins, indicated by the 7 o'clock arrows, are observed among the translation products of authentic or reconstituted rough microsomes, but not among authentic secretory proteins. These proteins represent polypeptide chains which are synthesized by ribosomes attached to dog pancreas microsomal membranes but which, under in vivo conditions, are not exported by the cell.

Irreversible Segregation of Nascent Secretory Proteins by Microsomal Vesicles

Translation products synthesized in the presence of nuclease-treated microsomal membranes were incubated at 22°C for an additional 90 min after the cessation of protein synthesis. During the period of further incubation, from 90 to 180 min after the onset of translation, the location of nascent amylase was determined using a shortened proteolysis assay: 10-min treatment with trypsin and chymotrypsin, 50 μg/ml each, in the presence of 10 μg/ml cycloheximide and 3 mM tetracaine at 0°C. In the absence of protective membranes, amylase was completely degraded by this treatment (cf. Fig. 4 *b*). In the presence of intact microsomal membranes, protection of nascent amylase, as judged by scanning gel fluorograms, was 93.6, 102, 97.5, and 101.2% at 90, 120, 150, and 180 min of incubation, respectively. Fig. 7 shows the fluorogram of translation products that were resistant to proteolysis after 180 min of incubation at 22°C and compares them to a control incubation without proteolysis treatment. Under these conditions, all nascent secretory proteins, as well as amylase, were quantitatively protected from proteolytic attack.

Characterization of the RER Membrane Component(s) Responsible for the Translocation of Nascent Secretory Proteins

15-min treatments of dog pancreas rough microsomal membranes with either 5 μg/ml trypsin at 22°C, heat at 60°C, or *N*-ethylmaleimide at 10 mM, resulted in the complete inability of these treated membranes to translocate dog pancreas secretory proteins (data not shown). The trypsin added to membranes was inhibited by the addition of a twofold molar excess of Trasylol before the addition of mRNA. Membranes incubated at 60°C for 15 min were shifted to 22°C before the addition of mRNA. These findings, as described here and elsewhere (2), strongly suggest that the mechanism that transports these proteins across the microsomal membrane is mediated by protein components associated with the microsomal vesicles. In addition, the effect of *N*-ethylmaleimide, which confirms an earlier report (42), suggests that the translocase associated with the microsomal membrane requires an intact sulfhydryl group for translocation activity.

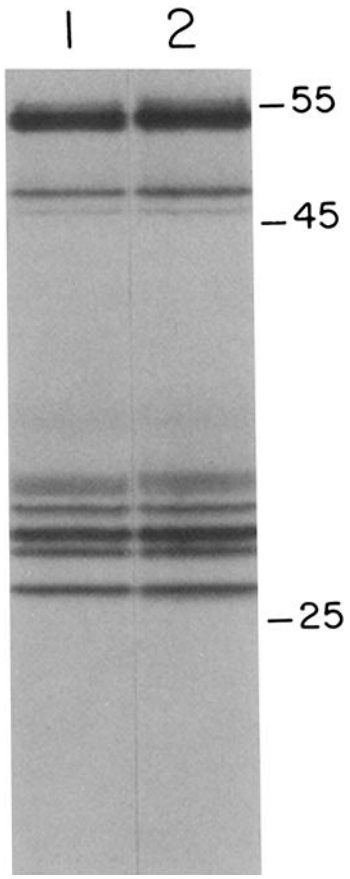

FIGURE 7 Analysis of the location of dog pancreas mRNA-directed translation products, after synthesis in the presence of nuclease-treated microsomal membranes and post-translational incubation for 90 min at 22°C. mRNA was translated at 22°C in the presence of microsomal membranes for 90 min. Translation mixtures were then incubated for an additional 90 min at 22°C. After the entire incubation period of 3 h, the location of nascent secretory proteins, whether intracisternal or extracisternal with respect to the microsomal vesicles, was determined by a 10-min incubation in the presence of 50 μg/ml trypsin, 50 μg/ml chymotrypsin, 10 μg/ml cycloheximide, and 3 mM tetracaine at 0°C. Proteolysis was then terminated by the addition of 1,500 KIU/ml Trasylol. Translation products were separated by polyacrylamide gel electrophoresis in SDS and analyzed by fluorography after gel impregnation with PPO. Track *2* indicates translation products that were resistant to proteolysis; track *1* gives the products after a control incubation without proteases. Numbers to the right indicate molecular weight values $\times 10^{-3}$. Radioactive secretory proteins are identified as indicated in Fig. 1.

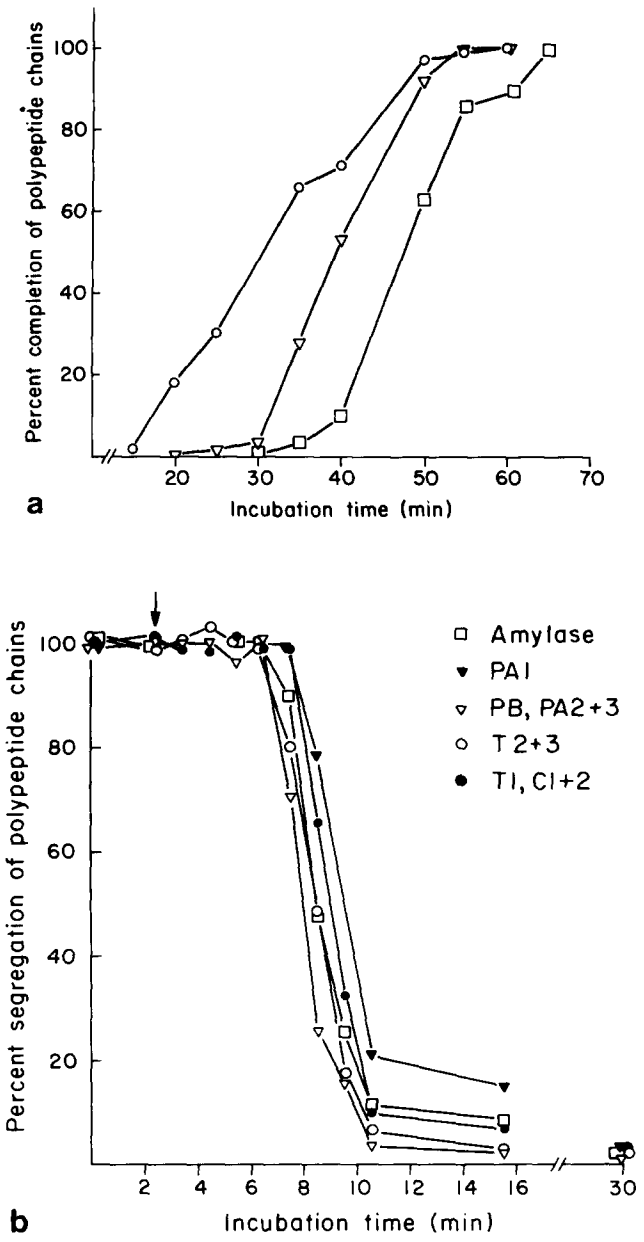

FIGURE 8 (*a*) Polypeptide chain elongation times for dog pancreas secretory proteins synthesized in vitro with rabbit reticulocyte lysate in the presence of dog pancreas mRNA and dog pancreas microsomal membranes stripped of endogenous mRNA by treatment with micrococcal nuclease. tRNA was not added to the translation mixture. Synthesis of polypeptide chains was synchronized by the addition of 1 μM edeine and 3 mM ^{7}mGp, both initiation inhibitors, at 2.5 min in a manner similar to that described by Rothman and Lodish (43). Aliquots of the incubation mixture were taken at timed intervals and processed immediately for electrophoresis on polyacrylamide gels containing SDS. Radioactivity contained in proteins separated by gel electrophoresis were analyzed by fluorography and quantitated by spectrophotometric scanning of fluorograms at 500 nm. Radioactivity observed in the group of serine protease zymogens is indicated (○); that observed in the group of procarboxypeptidases is indicated (▽), and that in amylase (□). (*b*) Segregation of nascent pancreatic secretory proteins during in vitro protein synthesis as a function of time of addition of dog pancreas microsomal membranes. tRNA was not added to the translation mixture. Synthesis of polypeptide chains was synchronized by the addition of 1 μM edeine and 3 mM ^{7}mGp at 2.5 min. Microsomal membranes, stripped of endogenous mRNA by treatment with micrococcal nuclease, were added at the indicated times on the abscissa. After protein synthesis was allowed to proceed to completion, the location of nascent polypeptide chains, whether cisternal or extracisternal with respect to the microsomal vesicles, was determined by treatment with trypsin and chymotrypsin in the presence of 3 mM tetracaine for 1 h at 0°C as described in the legend to Fig. 4. Segregated polypeptide chains labeled with[^{35}S]methionine were separated by one-dimensional polyacrylamide gel electrophoresis in SDS, analyzed by fluorography, and quantitated by spectrophotometric scanning of fluorograms at 500 nm. The radioactive bands analyzed represent amylase (□), procarboxypeptidase A1 (▼), procarboxypeptidase B and the procarboxypeptidases A2 and A3 (▽), trypsinogens 2 and 3 (○), and trysinogen 1 and the chymotrypsinogens 1 and 2 (●).

TABLE IV

Cotranslational Binding of Nascent Pancreatic Presecretory Protein to Microsomal Membranes: Maximal Chain Length Allowed for Segregation of Secretory Proteins

Secretory protein*	Polypeptide elongation rate‡	Maximum time of synthesis allowed for segregation§	Maximum polypeptide chain length allowed for segregation
	amino acid residues/min	*min*	*amino acid residues*
T2+3	8.6	7.15	61
T1; C1+2	8.6	7.75	67
PB; PA2+3	10.6	6.75	72
PA1	10.6	8.30	88
A	10.3	7.15	74

* T = trypsinogen; C = chymotrypsinogen; PB = procarboxypeptidase B; PA = procarboxypeptidase A, A = amylase.

‡ Calculated based on rates of polypeptide chain elongation determined in Fig. 8 *a*. M_r values of presecretory proteins were converted to amino acid residues using a mean amino acid molecular weight = 115.

§ Given by the time interval from the midpoint of polypeptide chain initiation to the point at which 50% of the polypeptide chains were segregated by microsomal membranes.

Cotranslational Binding of Nascent Polypeptide Chains to Stripped Microsomal Membranes: Analysis of Maximum Polypeptide Chain Lengths Which, during Synthesis, Allow for Segregation of Nascent Secretory Proteins

To study the critical time during which nascent secretory proteins can bind to the microsomal membrane and form a functional ribosomal-membrane junction, we simultaneously studied the rates of polypeptide chain elongation in our in vitro reconstitution system (mRNA plus membranes) and the effect of delayed addition of microsomal membranes on the segregation of nascent secretory proteins by microsomal vesicles. Syntheses of polypeptide chains were synchronized by the addition of 1 μM edeine and 3 mM ^{7}mGp, both initiation inhibitors, at 2.5 min after the start of in vitro protein synthesis. Completion of polypeptide chains was analyzed at periodic intervals throughout the duration of protein synthesis. Fig. 8 *a* gives the percent of polypeptide chains completed as a function of time for dog pancreas secretory proteins of three size classes, the serine protease zymogens (including trypsinogens 1–3, chymotrypsinogens 1–3, and proelastases 1 + 2) which range in size from 26,000–30,000 daltons; the procarboxypeptidases (including three forms of A and one form of B) which range from 45,000–47,000 daltons; and amylase and lipase, which range from 53,000–55,000 daltons. In the absence of added tRNA to the in vitro translation mixture, average polypeptide elongation

rates were 8.6 amino acid residues/min for the collection of serine protease zymogens, 10.6 residues/min for the collection of procarboxypeptidases, and 10.3 residues/min for the combination of amylase and lipase (cf. Table IV). Fig. 8 *b* gives the percent segregation of nascent secretory proteins as a function of the time of addition of dog pancreas rough microsomal membranes stripped of endogenous mRNA by micrococcal nuclease treatment. Again, synthesis of nascent presecretory proteins was synchronized by the addition of edeine and ^{7}mGp at 2.5 min after the start of in vitro protein synthesis. All dog pancreas secretory proteins were segregated when microsomal membranes were added during the first 6 min of incubation. Addition of membranes after 6 min resulted in a dramatic decrease in the segregation of polypeptide chains. The maximum time of synthesis beyond which segregation of polypeptide chains no longer occurred (Table IV) was estimated by the time interval from the midpoint of polypeptide chain initiation to the point at which the segregation of secretory proteins was reduced to 50% of the control. As indicated in Fig. 8 *b* and Table IV, this critical time varied among the proteins studied, from 7.15 min for trypsinogen 2 + 3 to 8.30 min for procarboxypeptidase A1. Table IV also gives the maximum chain lengths, in amino acid residues, beyond which functional binding of presecretory proteins to microsomal membranes no longer occurred, as judged by our analysis for segregation of nascent secretory proteins.

Translation of mRNA in the Presence of Micrococcal Nuclease-treated Microsomal Membranes; Heterologous Mixtures of mRNA and Membranes

Using the reconstitution system developed here, we were able to study the translation of dog pancreas mRNA in the presence of microsomal membranes derived from a number of tissue sources. Micrococcal nuclease-treated (rough) microsomal membranes isolated from dog liver, guinea pig liver, and rat liver were compared to nuclease-treated membranes isolated from dog pancreas. Placental ribonuclease inhibitor at a final concentration of 10 μg/ml was added 5 min before the addition of mRNA. Post-translational proteolysis at 0°C with trypsin and chymotrypsin was carried out in the presence of 3 mM tetracaine. Fig. 9 shows a fluorogram of the translation products derived from such studies. Polypeptide chains synthesized in the presence of dog pancreas mRNA and dog pancreas membranes were completely segregated within microsomal vesicles as judged by their resistance to post-translational proteolysis. Microsomal vesicles from dog liver, guinea pig liver, and rat liver were able to segregate polypeptide chains synthesized from dog pancreas mRNA. Furthermore, translation products segregated by each of the three liver membrane preparations were correctly processed. Segregation and correct processing was observed for all the major dog pancreas secretory products, including the group of serine proteases, the group of procarboxypeptidases A and B, and amylase.

mRNA isolated from the pancreas and liver of the rat and the pancreas of the guinea pig were translated in the presence of dog pancreas microsomal membranes. Fig. 10 shows the fluorograms of translation products resulting from these studies. The major translation products synthesized with rat and guinea pig pancreas mRNA were segregated and correctly processed as judged by their post-translational resistance to proteolysis and their comigration with authentic secretory proteins, respectively. The distribution of translation products synthesized by rat pancreas mRNA in the presence of dog pancreas microsomal membranes is similar to that observed in vivo. The distribution of products observed with translation of guinea pig pancreas mRNA in the presence of dog pancreas membranes was not similar to that observed after in vivo synthesis. The in vitro translation products showed a progressive diminution in the larger molecular weight proteins suggesting that guinea pig pancreas mRNA was partially degraded during isolation by the guanidinium thiocyanate procedure. Rat liver mRNA directed the synthesis of many polypeptide chains, including one ~70,000 daltons, judged to be rat liver preproalbumin. Dog pancreas membranes segregated and processed this secretory protein, as well as a number of other smaller molecular weight products, which remain unidentified.

DISCUSSION

The exocrine pancreas synthesizes and secretes a number of enzymes and zymogens which, when secreted into the intestinal

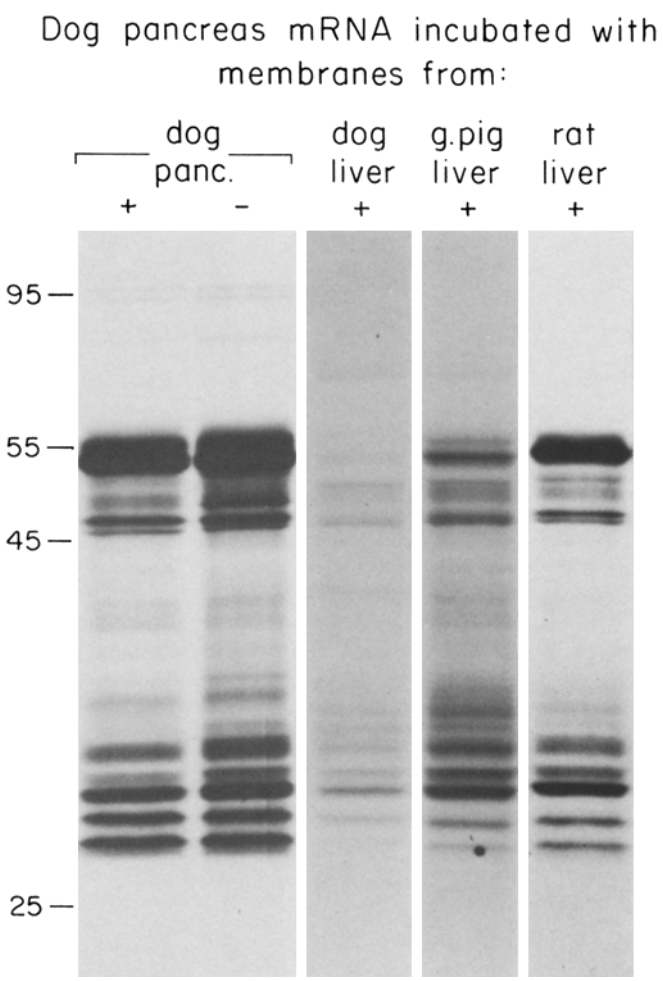

FIGURE 9 Translation of dog pancreas mRNA (0.2 A_{260} U/ml) in the presence of micrococcal nuclease-treated membranes (20 A_{260} U/ml) from homologous and heterologous tissue sources. Translation products were incubated in the absence (−) and presence (+) of proteases at 0°C as described in the legend to Fig. 5. Homologous and heterologous sources of rough microsomes are as indicated above the figure and micrococcal nuclease treatment of these membranes was as described in Materials and Methods. Numbers to the left indicate molecular weight values × 10^{-3}. Radioactive secretory proteins are indentified as indicated in Fig. 1.

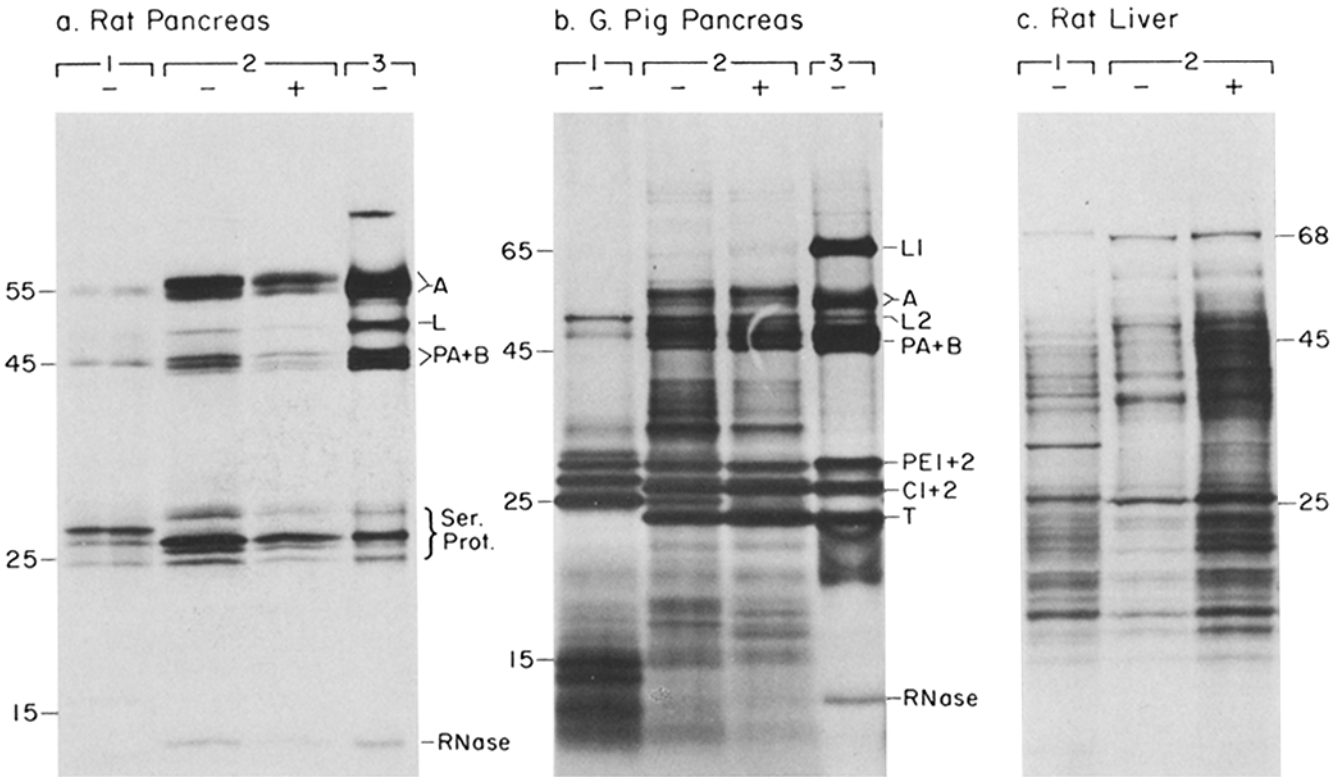

FIGURE 10 Translation of mRNAs from guinea pig pancreas, rat pancreas, and rat liver with micrococcal nuclease-treated microsomal membranes from dog pancreas. Fig. 10 *a* gives the products derived from translation of rat pancreas mRNA in the absence (region *1*) and presence (region *2*) of dog pancreas nuclease-treated microsomal membranes and compares these to authentic secretory proteins synthesized in vivo (region *3*). Segregation of radioactive proteins was monitored by post-translational incubations in the absence (−) and presence (+) of proteases at 0°C as described in the legend to Fig. 5. Molecular weight values $\times 10^{-3}$ are given on the left. Identifications are given on the right as follows: *A*, amylase; *L*, lipase; *PA+B*, procarboxypeptidases A and B; *Ser. Prot.*, serine proteases. Fig. 10 *b* gives the products derived from translation of guinea pig pancreas mRNA in the absence (region *1*) and presence (region *2*) of dog pancreas microsomal membranes and compares these to authentic secretory products synthesized in vivo (region *3*). Segregation of radioactive proteins was monitored by post-translational incubations in the absence (−) and presence (+) of proteases at 0°C as described in the legend to Fig. 5. The numbers to the left indicate molecular weight values $\times 10^{-3}$. Authentic secretory proteins are labeled to the right as identified by Scheele (31). The abbreviations are: *L*, lipase; *A*, amylase; *PA+B*, procarboxypeptidases A and B; *PE*, proelastase; *C*, chymotrypsinogen; *T*, trypsinogen. Fig. 10 *c* gives the products derived from translation of rat liver mRNA in the absence (region *1*) and presence (region *2*) of dog pancreas nuclease-treated microsomal membranes. Post-translational incubations in the absence (−) and presence (+) of proteases was carried out at 0°C as described in the legend to Fig. 5. Numbers to the right indicate molecular weight values $\times 10^{-3}$. Translation products in Fig. 10 *a,b,* and *c* were separated by polyacrylamide gel electrophoresis in SDS and analyzed by fluorography.

tract, are involved in the digestion of macromolecular food products in the diet (44). In the dog pancreas, 17 discrete secretory proteins have been identified and characterized using two-dimensional IEF/SDS gel electrophoresis as developed by Scheele.[4] As indicated in Table I and Fig. 6, these include three forms each for trypsinogen, chymotrypsinogen, and procarboxypeptidase A, two forms for proelastase, and single forms for amylase, lipase, procarboxypeptidase B, and prophospholipase A_2. Based on the incorporation of radioactivity into proteins with relatively short periods of pulse, 5 min,[4] the majority of these proteins appear to be products of separate genes. Our information does not allow us to determine if trypsinogens 2 and 3 are separate gene products, as these forms comigrate during electrophoresis in both one-dimensional and two-dimensional SDS gels (hence the designation trypsinogen 2 + 3). 14 of the identified dog pancreas secretion products represent proteins without attached carbohydrate, and these form the primary focus of the analysis reported here. Lipase and two high molecular weight acidic proteins have been identified as glycoproteins and their charge heterogeneity is presumed to be secondary to heterogeneity in the saccharide moieties of these proteins. Using methods that provide high resolution in the separation of these proteins, both by size (29) and by a combination of size and charge (31), we were able to study simultaneously the biogenesis of this relatively large group of well-defined secretory proteins. This approach allowed us not only to make internal comparisons among individual secretory proteins, but also to monitor closely the fidelity of their synthesis in vitro as compared to their synthesis in vivo. This latter comparison provided us with a sensitive assay for our success in reproducing in vitro the biochemical events that occur in vivo.

Translation of dog pancreas polyadenylated mRNA in either the reticulocyte lysate translation system or the wheat germ translation system gave similar products as analyzed by one-dimensional SDS/polyacrylamide gel electrophoresis. The molecular weights of the products generated in the two systems were identical. For each of the bands representing nonglycosylated proteins, the mRNA translation products appeared to be 1,000–2,000 daltons larger than their counterparts among secreted proteins. Thus, these larger mRNA-directed forms represent precursor molecules, designated as presecretory pro-

teins. Immunoreplication studies, using six monospecific antibodies, directed against amylase, procarboxypeptidase A1, trypsinogen 1, trypsinogen 2 + 3, chymotrypsinogen 1, and chymotrypsinogen 2, identified individual pairs of precursors and products and verified the size differences between individual pairs as uniformly 1,000–2,000 daltons.

For the group of nonglycosylated proteins, translation of mRNA associated with dog pancreas rough microsomes, as shown previously (3), resulted in translation products that comigrated with their authentic counterparts among secreted proteins. This finding indicated that microsomal membranes associated with rough microsomes are responsible for the proteolytic reduction of presecretory proteins to authentic secretory products and suggested that the protease involved is associated with the microsomal membrane. These findings further indicated that for dog pancreas secretory proteins, there is no further reduction in the size of polypeptide chains from the site of segregation within the cisternal space of the RER to the site of discharge to the extracellular space. Further processing can occur, however, for a number of these proteins in the intestinal luminal space, where enterokinase initiates a cascade of activation events, which involves the proteolytic conversion of inactive zymogens (prosecretory proteins) to active proteolytic enzymes (trypsin, chymotrypsin, elastase, and carboxypeptidases A and B) and phospholipase A_2 (45).

In contrast to the group of nonglycosylated secretory proteins identified by enzyme and potential enzyme activity, the two high molecular weight secretory glycoproteins, G1 and G2, showed different findings. Translation products directed by either dog pancreas mRNA or dog pancreas rough microsomes appeared smaller by SDS/polyacrylamide gel electrophoresis than their authentic counterparts among secreted proteins. This finding was not surprising, as the carbohydrate attached to these proteins in the RER and Golgi complex can be expected to retard the mobility of these proteins during electrophoresis and to obscure the demonstration of the proteolytic processing event, as has been demonstrated for several other secretory glycoproteins (6, 46).

Functional reconstitution studies were carried out using isolated mRNA and stripped microsomal membranes obtained from dog pancreas to determine the function of these presecretory proteins in the secretory process. To achieve the efficiency necessary to analyze the translocation of 17 individual nascent secretory proteins across the microsomal membrane, we found it necessary to modify both the in vitro reconstitution system and the post-translational assay for the location of nascent polypeptide chains. Our studies yielded the following results: (*a*) Dog pancreas rough microsomes stripped of endogenous functional mRNA with micrococcal nuclease proved to be considerably more efficient in reconstitution studies than rough microsomes stripped with EDTA as previously described (2, 4). (*b*) Rough microsomes treated with EDTA were found to be incompletely stripped of ribosomes and endogenous functional mRNA because they incorporated [^{35}S]methionine into identifiable dog pancreas secretory proteins. Micrococcal nuclease treatment of microsomal membranes reduced functional mRNA to undetectable levels. (*c*) The distribution of radioactivity observed among the translation products synthesized in the presence of micrococcal nuclease-treated rough microsomes closely mimicked that observed after synthesis in vivo by dog pancreas tissue slices. Using EDTA-treated microsomal membranes, in the absence or presence of dog pancreas mRNA, we observed a marked diminution in the translation of the larger molecular weight mRNAs secondary to a polypeptide chain elongation defect introduced by these membranes. (*d*) Addition of bovine liver tRNA to the translation mixtures indicated that the reticulocyte lysate translation system is deficient in tRNAs necessary for the translation of dog pancreas secretory proteins. Nuclease-treated microsomal membranes, but not EDTA-treated membranes, were associated with adequate quantities of dog pancreas tRNA. Addition of tRNA to EDTA-treated microsomal membranes did not correct the apparent elongation defect observed in the use of these membranes. (*e*) Ribonuclease activity was found associated with microsomal membranes obtained from a variety of sources (dog liver > guinea pig liver > rat liver > dog pancreas). Ribonuclease inhibitor completely inactivated the ribonuclease associated with microsomal membranes isolated from the dog pancreas. Using microsomal membranes associated with larger quantities of ribonuclease, we were not able to completely inactivate nuclease activity by the addition of the placental inhibitor at 10 μg/ml. However, we markedly improved the extent of protein synthesis directed by mRNA in the presence of these membranes, and this improvement has allowed us to conduct meaningful reconstitution studies with dog pancreas mRNA and nuclease-treated microsomal membranes from the livers of rat, guinea pig, and dog (cf. Fig. 9). (*f*) Tetracaine at 3 mM and dibucaine at 1 mM, when added to the post-translational proteolysis assay increased the protection of radioactive amylase from 18 to between 90 and 100%. In the proteolysis assay, we observed that the control mixture, incubated in the absence of proteases, continued to synthesize and segregate small quantities of [^{35}S]methionine-labeled proteins during the 1-h incubation at 0°C. When cycloheximide was added to inhibit further incorporation of [^{35}S]methionine during the incubation period at 0°C, protection of nascent amylase routinely achieved experimental values of 97–102%. Furthermore, the use of tetracaine during treatment with trypsin and chymotrypsin resulted in an increase in the protection of the other pancreatic secretory proteins similar to that seen for amylase, as judged by both fluorograph and radioautograph scanning after separation of translation products by SDS polyacrylamide gel electrophoresis. Tetracaine did not confer protease resistance to the proteins themselves, as judged by their complete degradation when protease treatment was carried out in the presence of 3 mM tetracaine and 1% sodium deoxycholate. These findings indicate that microsomal membranes isolated from dog pancreas and added to the reticulocyte lysate system are unstable in the presence of 50 μg/ml each of trypsin and chymotrypsin. By contrast, in the presence of 3 mM tetracaine, microsomal vesicles were resistant to protease treatment at either 0° or 22°C for the time studied, 60 min. Tetracaine and dibucaine, both positively charged amphiphilic molecules, may stabilize microsomal membranes by two possible mechanisms. The insertion of such agents into the lipid bilayer may inhibit, by charge repulsion, the degradative effect of trypsin and chymotrypsin on integral membrane proteins, and/or the insertion of these agents into the membrane may stabilize the lipid bilayer itself.

Using the methods developed here for the functional reconstitution of rough microsomes, including the assay for location of nascent polypeptide chains, we have been able to determine that transport of the entire group of pancreatic secretory proteins across the microsomal membrane is a cotranslational event. Translation of dog pancreas mRNA in the presence of nuclease-treated microsomal membranes resulted in complete

processing and segregation of nascent secretory polypeptide chains. Translation of dog pancreas mRNA in the absence of membranes followed by the post-translational addition of nuclease-treated microsomal membranes for 1 h (22°C) did not result in translocation of radioactive polypeptide chains across the microsomal membrane as judged by the complete absence of processing of nascent chains and the complete degradation observed during the subsequent treatment with trypsin and chymotrypsin. Taken together, our findings not only indicate that pancreatic secretory proteins are quantitatively transported across the microsomal membrane, but show for the first time absolute coupling between segregation and processing of these nascent secretory polypeptide chains.

Because nascent amylase, without protection of microsomal membranes, was completely degraded by trypsin-chymotrypsin at 10 min, we were able to shorten our post-translational segregation assay. This rapid (10 min) proteolysis assay allowed us to determine the fate of sequestered amylase during a further 90-min incubation at 22°C. During this period of time there was no movement of radioactive amylase into the extravesicular space, as judged by its complete resistance to degradation by the added proteases. Over the entire 3-h period of the study, nascent amylase remained segregated within microsomal vesicles. Fluorographic analysis indicated that the findings for amylase were representative for all dog pancreas secretory proteins (cf. Fig. 7). These findings indicate that dog pancreas microsomal vesicles are stable under in vitro conditions in the absence of added proteases for periods up to 3 h. Transport of secretory proteins into these microsomal vesicles is irreversible and therefore represents a vectorial process. For the pancreatic secretory proteins studied, with molecular weights ranging from 15,000 to 97,000, redistribution across the microsomal membranes did not occur, contrary to that postulated by Rothman and co-workers (cf. reference 10), and by Tabe et al. (11). Further transport of secretory proteins through the cell interior, therefore, can be expected to occur through fusion of interconnectable membrane-bound compartments, as previously postulated (47, 48).

Analysis of translation products by two-dimensional gel electrophoresis in the presence[3] or absence (31) of 8 M urea in the first-dimensional gel allowed us to monitor both the size and charge of newly synthesized polypeptide chains and to gain insight into the physical properties (conformational state and solubility properties) of presecretory and secretory proteins. Translation products directed by dog pancreas mRNA in the presence of nuclease-treated microsomal membranes comigrated with authentic secreted proteins under these separation conditions, indicating identity in size, charge, and conformation between corresponding proteins. Conformational changes alter the migration of proteins during both IEF[3] and SDS gel electrophoresis (31). As segregated proteins were observed to comigrate with authentic secreted proteins in these procedures, we conclude that the folding characteristics of the respective proteins are identical, including formation of the correct set of disulfide bridges. We can thus hypothesize that these segregated forms are biologically active molecules, containing the actual or potential enzyme activities expected. In contrast, translation products directed by dog pancreas mRNA in the absence of stripped microsomal membranes were, in large majority, not separated into discrete spots by the two-dimensional gel procedures, but were observed to smear in both dimensions of the analyses. These presecretory forms, which are known to differ from corresponding secreted proteins in size and charge, because of their peptide extensions, are observed to aggregate under either nondenaturing conditions or denaturing conditions (8 M urea) during IEF. We conclude that presecretory proteins exhibit conformations distinctly different from those of the corresponding authentic secretory proteins, and, coupled with their marked tendency to aggregate, we hypothesize that they will possess little, if any, enzyme activity (or potential activity in the case of pancreatic zymogens).

In vitro translation studies with synchronized synthesis of dog pancreas presecretory proteins and delayed addition of nuclease-treated microsomal membranes have indicated that all pancreatic presecretory proteins studied contain their peptide extensions at the amino terminus. The maximum polypeptide chain lengths that allowed segregation of these proteins varied between 61 (pretrypsinogen 2 + 3) and 88 (preprocarboxypeptidase A1) amino acid residues among pancreatic presecretory proteins. Because 39 amino acid residues are required to span the channel in the large ribosomal subunit (49), the maximal extraribosomal chain lengths that allow binding vary between 22 and 49 residues.

Based on the amino terminal sequence of pretrypsinogen 2 + 3 (50), we can predict that synthesis of 59 amino acid residues will result in the emergence of an aspartic acid residue from the channel in the large ribosomal subunit. Further synthesis will result in the appearance of three additional aspartic acid residues followed by a lysine residue. The emergence of this peptide domain containing five highly charged amino acid residues can be expected to interfere with functional binding of the nascent polypeptide chain to the microsomal membrane.

From these studies we can also conclude that presecretory protein synthesis may begin on free ribosomes which, during translation, become bound to microsomal membranes, establishing functional ribosome-membrane junctions. Polypeptide elongation and chain completion, then, results in the tranlocation of the secretory proteins across the microsomal membrane and its segregation within the cisternal space of the microsomal vesicle. Should functional binding not be established, we can predict that the nascent presecretory protein will be neither segregated nor processed. Such nontranslocated presecretory proteins can be expected to aggregate (in a manner similar to that observed during two-dimensional gel electrophoresis) which would lead to their probable uptake and degradation by lysosomes.

Studies from a number of laboratories have provided supporting evidence for the transport peptide hypothesis.[1] With one exception (51), presecretory proteins studied to date do contain amino terminal peptide extensions with core regions (signals) containing extraordinarily high concentrations of hydrophobic amino acid residues, and in particular, four with bulky side chains, Leu, Ile, Phe, Val (25, 50, 52–64). In vitro translation studies with mRNA from a number of secretory tissues in the presence of EDTA-stripped microsomal membranes, have indicated that secretory polypeptide chains *can* be cotranslationally processed and segregated within microsomal vesicles (2–9, 46, 65–68). However, in the studies reported to date, incomplete processing and relatively low efficiency of segregation of radioactive polypeptide chains have been observed. In the most efficient quantitative study published to date (4), processing of preprolactin occurred with an efficiency of ~75% and segregation of prolactin, as judged by its protection during post-translational proteolysis, occurred with an efficiency of ~43%. In other studies the efficiencies of process-

ing and segregation have been even less, with representative values for proteolytic processing ranging from 40 to 75% and representative values for segregation ranging from 10 to 43%. From these previously published data, it can be argued that a significant number of secretory polypeptide chains are neither processed nor segregated within the RER. Further, the consistently lower efficiency of segregation, compared to processing, suggests that a significant number of polypeptide chains are processed, but not segregated, events that are claimed to be coupled in the transport peptide hypothesis. Neither of these interpretations is consistent with the hypothesis as proposed and, in fact, they provide circumstantial supporting evidence for alternative models of secretion, which claim direct secretion of exportable proteins from the cytoplasmic space (10). Furthermore, in none of the cases cited above have the investigators demonstrated that the processed and segregated polypeptide chain is biologically active. Without such data, it can be argued that the mechanism for the translocation of proteins is nonphysiological.

In the in vitro translation study reported here, using dog pancreas mRNA that directs the synthesis of 17 discrete and 15 well-characterized secretion products, we have demonstrated the following:

(*a*) Polypeptide chain synthesis, initiated on free ribosomes, will result in the functional attachment of associated ribosomes to the microsomal membrane and the subsequent translocation of the secretory polypeptide chains across the microsomal membrane and into the cisternal space of the microsomal vesicle.

(*b*) Each of the 14 nonglycosylated secretory proteins contains, during translation, an amino terminal peptide extension, 1,000–2,000 daltons in size, which interacts with a specific protein receptor in the microsomal membrane and therefore is responsible for the establishment of a functional ribosome-membrane junction.

(*c*) Segregation and processing of nascent secretory proteins are cotranslational events. The maximum extra-ribosomal polypeptide chain lengths which, during synthesis, allow functional binding of nascent chains to microsomal membranes vary from 22 to 49 amino acid residues among 10 pancreatic presecretory proteins. At levels of mRNA that do not saturate binding sites in the membrane, processing and segregation of nascent secretory proteins are complete.

(*d*) Segregated and processed polypeptide chains are folded in a manner indistinguishable from authentic secreted proteins, as judged by two-dimensional IEF/SDS gel electrophoresis.

(*e*) The distribution of translocation products (proportions of radiolabeled secretory proteins) directed by mRNA in the presence of nuclease-treated microsomal membranes closely mimicks that observed during in vivo synthesis.

Thus, we have been able to reproduce in vitro, in its entirety, the first step in the secretory process (47) and have reproduced this step simultaneously for each of the 17 pancreatic exocrine proteins studied. Our success in this endeavor, which has depended on our development of highly efficient and dependable assays for processing, segregation, and folding of individual polypeptide chains, has allowed us to exclude, for the first time with certainty, other transport mechanisms which might account for the movement of these secretory proteins across the RER membrane. We are thus able to define, now with certainty, the sequence of biochemical events necessary to transport secretory proteins across the microsomal membrane. Functional reconstitution studies employing homologous and heterologous mixtures of mRNA and nuclease-treated microsomal membranes indicate that the translocation mechanism studied here is common to mammalian microsomal membranes of diverse tissues and species and suggests, in fact, that it is common to all mammalian microsomal membranes.

Further studies are necessary, however, to demonstrate that translation products directed by dog pancreas mRNA in the presence of stripped microsomal membranes are biologically active molecules. For the moment, the folding characteristics of segregated polypeptide chains, as determined by two-dimensional urea IEF/SDS gel electrophoresis, is indistinguishable from those of authentic secreted proteins, a finding that suggests that these proteins are biologically active molecules, containing the actual or potential enzyme activities expected.

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Note Added in Proof: Recent experiments have indicated that (*a*) rough microsomes isolated from dog pancreas are associated with significant quantities of secretory prophospholipase A_2, (*b*) trypsin treatment during post-translational proteolysis resulted in a sixfold increase in the activity of phospholipase A_2 and a 23-fold increase in the quantity of lysolecithin associated with membranes, and (*c*) 3 mM tetracaine inhibited the increase in lysolecithin observed in microsomal membranes incubated in the presence of trypsin.

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