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The 70 Carboxyl-terminal Amino Acids of Nascent Secretory Proteins Are Protected from Proteolysis by the Ribosome and the Protein Translocation Apparatus of the Endoplasmic Reticulum Membrane*

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We have used proteolysis to examine the environment through which nascent secretory proteins are translocated across the membrane of the endoplasmic reticulum. After solubilization of rough microsomes with detergent, fragments comprised of the approximately 70 carboxyl-terminal amino acids of translocating nascent chains initiated and targeted *in vivo* were protected from digestion by added proteases. About 40 amino acids of nascent chains were protected from proteolysis by the ribosome; thus, membrane-derived components protect an additional 30 amino acids. Under conditions in which those 30 additional amino acids are protected, only a small set of integral membrane proteins remained associated with the ribosome. These proteins include the Sec61 complex previously identified as the core component of the membrane-bound protein translocation apparatus. These results support the concept of a translocation pore that makes intimate contact with the ribosome and thereby protects nascent chains from proteolytic digestion for an additional, constant length.

Considerable progress has been made recently toward determining the environment through which nascent secretory proteins traverse the membrane of the endoplasmic reticulum (ER)[1]. One of the fundamental questions about protein translocation, that of the participation of membrane proteins in the process, has been answered. Cross-linking studies have demonstrated the immediate proximity of several integral membrane proteins to the nascent chain (Krieg *et al.*, 1989; Wiedmann *et al.*, 1989; High *et al.*, 1991a, 1991b, 1993a, 1993b; Thrift *et al.*, 1991; Görlich *et al.*, 1992a; Musch *et al.*, 1992; Sanders *et al.*, 1992). One of these proteins, Sec61α, has been demonstrated to be adjacent to the nascent chain at all times during its translocation (Krieg *et al.*, 1989; High *et al.*, 1993b; Mothes *et al.*, 1994). Purification of the Sec61 complex followed by its reconstitution into lipid vesicles demonstrated it to be the sole component required for translocation once targeting of nascent chains with signal sequences to the membrane is complete (Görlich and Rapoport, 1993). While important, this result does not preclude the presence of other membrane proteins at the site of translocation. Indeed, several other integral membrane proteins or protein complexes (the translocating-chain associated membrane (TRAM) protein and the translocon-associated protein (TRAP) complex) can also be cross-linked to the nascent chain (Görlich *et al.*, 1992b). The TRAM protein can be cross-linked to the signal sequence, while the immediately following section of the nascent chain can be cross-linked to Sec61α (High *et al.*, 1993b; Mothes *et al.*, 1994), suggesting that these two proteins are very close to one another, and possibly in contact.

In addition to proteins that have been cross-linked to nascent chains, at least two other protein complexes (the signal peptidase and glycosyltransferase complexes) perform reactions on the nascent chain during its translocation (Evans *et al.*, 1986; Kelleher *et al.*, 1992). Each of them can perform its reaction before the nascent chain is long enough to have a significant lumenal domain, suggesting that the reactions may take place at the site of translocation (Glabe *et al.*, 1980). Because glycosylation sites are spread throughout secretory proteins, the glycosyltransferase could remain in proximity to the nascent chain throughout translocation. Consistent with these ideas is the finding by Görlich and colleagues (1992a) that the components of several of these complexes (Sec61, TRAP, and the ribophorin complex, which contains the glycosyltransferase activity) compose almost all of the integral membrane proteins that remain tightly associated with membrane-bound ribosomes after solubilization of the membrane with the detergent digitonin. Together, these results suggest that while the Sec61 complex plays the central catalytic role in translocation, it may do so as a member of a larger structure, termed the translocon, at the site of translocation.

Several independent approaches have provided evidence that the translocon performs its role by providing a continuous channel from one side of the membrane to the other through which the nascent chain passes during translocation. Simon and Blobel (1991), using electrophysiological techniques, observed the appearance of aqueous channels across the membrane when rough microsomes were treated with puromycin. This result suggested that those channels were previously occupied by translocating nascent chains, which prevented the passage of ions. Consistent with these observations are those of Crowley and colleagues (1993, 1994), who concluded that fluorophores incorporated into translocating nascent chains

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[1] The abbreviations used are: ER, endoplasmic reticulum; aa, amino acid; CTABr, cetyltrimethylammonium bromide; KOAc, potassium acetate; $Mg(OAc)_2$, magnesium acetate; RM, rough microsome; TEA, triethanolamine; TRAM, translocating-chain associated membrane protein; TRAP, translocon-associated protein; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

remain in an aqueous environment throughout translocation. Because water-soluble quenching agents can gain access to fluorophores present in nascent chains of about 70 amino acids or longer if they are supplied from the lumenal side of the membrane, the aqueous environment must extend completely across the membrane. Taken together, these observations are consistent with earlier evidence from Gilmore and Blobel (1985), who examined the conditions required to extract partially translocated nascent chains from membranes and concluded that translocation occurs in a microenvironment that is accessible to aqueous perturbants.

Detailed examination of the proximity of Sec61α to translocating nascent chains suggests the possibility that it may be a component of the channel wall. Sec61α makes continuous contact with about 40 amino acids of the nascent chain once it reaches a certain length (Mothes *et al.*, 1994). The region of contact is positioned between roughly 30 and 70 amino acids from the peptidyltransferase site, and this relative position does not change as the nascent chain grows longer, despite the fact that the sequence in contact with Sec61 does. Because the ribosome surrounds about the carboxyl-terminal 40 amino acids of a nascent chain, the length and position within the nascent chain of the contact region are consistent with it spanning the plane of the membrane. It is not clear from this data, however, whether Sec61α completely surrounds the nascent chain.

In addition to suggesting a channel through which translocation occurs, these observations also suggest the presence of a tight seal between the ribosome and the membrane. Quenching agents as small as iodide ions could not reach fluorophores in short nascent chains when delivered from the cytosolic side of the membrane under conditions in which those nascent chains can be cross-linked to Sec61α and the TRAM protein (Crowley *et al.*, 1993; Mothes *et al.*, 1994). Similarly, because the contact region between Sec61α and the nascent chain begins just beyond the point at which it could be expected to emerge from the ribosome, the ribosome must be very close to the translocon (Mothes *et al.*, 1994). This possibility is also suggested by the observed protection of Sec61α from proteolysis when ribosomes are bound to the membrane (Kalies *et al.*, 1994).

As an independent means of examining the geometry of the translocon with respect to the nascent chain and the proposed seal between the ribosome and the membrane we have used proteases to probe the disposition of translocating nascent chains in rough microsomes. This approach allowed us to assess directly the disposition of different translocating species in translocation events initiated *in vivo*. Our results support the model that translocation occurs through a protein pore that is tightly associated with the ribosome and allow us to estimate the number of amino acids of the nascent chain that are enclosed within it.

EXPERIMENTAL PROCEDURES

Materials—[^{35}S]Methionine (>1000 Ci/mmol) was from Amersham. The detergent Emulgen-913 was the kind gift of Dr. Yoshio Imai (University of Osaka, Japan), and is commercially available from the KAO Atlas Co. Ltd. of Tokyo, Japan. Emulgen-913 is a linear polyoxyethylenenonylphenyl ether, with an average of 13 ethoxy groups per molecule. Digitonin (receptor-grade) was from Gallard-Schlessinger Industries, Inc. of Carle Place, New York. Nuclease and protease-free sucrose (ICN Biomedical Inc.) was used to make the gradients for separation of ribosomes and polysomes. All other chemicals were of the highest grade available from common suppliers. Antibodies to the dog pancreas secretory proteins amylase, chymotrypsinogen 2, and proelastase were the kind gift of Dr. George Scheele (Harvard Medical School).

The molecular weight standards used were "Protein Molecular Weight Standards, Molecular Weight Range 3,000–43,000" from Bethesda Research Laboratories (Gaithersburg, MD). They are ovalbumin (43.0 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), bovine trypsin inhibitor (6.2 kDa), and the insulin B chain (3.4 kDa, 30 amino acids). SDS-polyacrylamide gel electrophoresis in 27.5% polyacrylamide gels was performed by the method of Maurer and Allen (Maurer and Allen, 1972).

Buffers and Solutions—A 1 M stock solution of triethanolamine (TEA) was adjusted to pH 7.5 at room temperature with acetic acid, as was a 4 M stock solution of potassium acetate (KOAc). The pH of buffers prepared from these stock solutions was not readjusted after mixing. Phenylmethylsulfonyl fluoride (PMSF) was prepared as a fresh stock solution (100 mM) in ethanol which was diluted to 5 mM PMSF in 5% ethanol immediately prior to use. Stock solutions of trypsin and chymotrypsin were prepared at 1 mg/ml in water and stored as aliquots at −80 °C.

Labeling of Nascent Chains in Rough Microsomal Membranes—Rough microsomal membranes (RMs) were prepared from canine pancreas by the method of Walter and Blobel (1983) and were stored at −80 °C. The endogenous nascent chains were labeled essentially as described by Blobel and Dobberstein (1975). In brief, the reaction mixture contained final concentrations of 10 A_{280} units/ml of RMs, 1 mM ATP, 1 mM GTP, 8 mM creatine phosphate, 8 μg/ml creatine phosphokinase, 25 μM of each amino acid except methionine, 200 μg/ml calf liver tRNA, and 100 units/ml human placental ribonuclease inhibitor. Final salt conditions were 20 mM TEA, 100 mM KOAc, 3 mM magnesium acetate ($Mg(OAc)_2$), and 1 mM dithiothreitol (DTT) (buffer A). Also included was [^{35}S]methionine, usually at 1 μCi/μl. Reactions were incubated at 30 °C for 3 min to label nascent chains without completing them. The time required to complete a significant amount of polypeptide chains was about 15 min under these conditions.

Quantitation of Nascent Chains in RMs—The endogenous methionine concentration in a standard labeling reaction was measured by including a trace amount of [^{35}S]methionine and determining the concentration of unlabeled methionine required to halve the rate of incorporation of radioisotope into trichloroacetic acid-precipitable material. Using this approach, the concentration of endogenous methionine in the reaction was estimated to be 0.72 μM. The rate of incorporation of total methionine per equivalent of RMs (as defined in Walter and Blobel, 1983) was determined by subtracting the rate in the presence of cycloheximide from that in a standard labeling reaction containing methionine of known concentration and specific activity. At intervals, an aliquot was removed, the trichloroacetic acid-precipitable material collected and washed, and the amount of radioisotope incorporated determined by liquid scintillation counting. As standards, known dilutions of the [^{35}S]methionine stock solution were counted similarly. Linear incorporation of methionine lasted between 12 and 15 min. After 4 min approximately 400 fmol of methionine had been incorporated per equivalent of RMs in the reaction. During that time, we estimate that each nascent chain was elongated by approximately 70 or fewer amino acids because at 4 min the combined label in the 40- and 70-amino acid bands (see "Results") is the same as the total label incorporated in an unproteolyzed reaction (data not shown). At all subsequent times it was less. As methionine constitutes between 1 and 2% of the amino acids in soluble proteins (Kuhn and Leigh, 1985), we estimate that approximately 1 methionine had been incorporated into each nascent chain. Therefore, about 400 fmol of nascent chains had been elongated per equivalent of RMs. Published values of the amount of SRP receptor per equivalent are 93 fmol (Tajima *et al.*, 1986) and approximately 170 fmol (Görlich and Rapoport, 1993), and the ratio of SRP receptor to the components of the Sec61 complex is estimated to be between 1:5 and 1:10 (Görlich and Rapoport, 1993) The number of bound ribosomes per equivalent is estimated to be in the range of 500 fmol (Gilmore *et al.*, 1982). Using these figures, we can calculate as extremes either that labeled nascent chains occupy 80% of the engaged translocation sites (400 fmol of labeled nascent chains per equivalent/500 fmol of bound ribosomes per equivalent), or that there is a ratio of approximately 1:5 of labeled nascent chains to Sec61 complex (400 fmol of labeled nascent chains per equivalent/1700 fmol of Sec61 complex per equivalent) in our reactions. The latter calculation gives a figure of approximately 20% as the lowest fraction of nascent chains that could be labeled.

Detergent Disruption of RMs and Proteolysis—Labeling reactions (42 μl) were incubated for 3 min and then placed in ice water. All subsequent operations were performed at 4 °C. To each reaction a 5x Emulgen-913 solution (11 μl) was added while the reaction mixture was gently vortexed. After a 30-min incubation, an aliquot (10 μl) was removed and mixed with 1 μl of a 5 mM PMSF solution. A solution of trypsin and chymotrypsin (300 μg/ml each; 7 μl) was added to the remainder of the reaction mixture. 10-μl aliquots were removed at intervals thereafter and mixed with 1 μl of a 5 mM PMSF solution to stop proteolysis. After 15 min those proteolytic fragments covalently

attached to tRNAs were precipitated with cetyltrimethylammonium bromide (CTABr). CTABr precipitations were performed by the successive addition to each 10-μl aliquot of 250 μl of 2% (w/v) CTABr and 250 μl of 500 mM sodium acetate, pH 5.4, containing 200 μg/ml yeast tRNA. After a 10-min incubation at 30 °C these mixtures were centrifuged for 5 min in a microcentrifuge (~12,000 × g_{av}), and the pellet washed with 1 ml of acetone (Gilmore and Blobel, 1985). The precipitate was resolved by SDS-gel electrophoresis (SDS-PAGE) in high percentage (27.5%) polyacrylamide gels and visualized by autoradiography. Sarkosyl permeabilizations were performed similarly, with a final concentration of 0.016% Sarkosyl.

To determine which products remained associated with the membranes after Sarkosyl permeabilization and proteolysis, a sample (12 μl) was loaded on top of a 50-μl cushion of 250 mM sucrose in buffer A containing 0.016% Sarkosyl, and centrifuged for 3 min at 20 p.s.i. in a Beckman A-100–30 Airfuge rotor. Under these conditions microsomal membranes sediment quantitatively, while monosomes and most polysomes remain in the supernatant layer. The top 22-μl fraction was removed ("supernatant fraction"). The remaining supernatant layer was discarded, and the pellet was resuspended in 22 μl of 5 mM PMSF. Both the supernatant and pellet fractions were then precipitated with CTABr and processed as described above.

Labeling Reaction with Reticulocyte Lysate and Proteolysis of Products—A reaction mixture (70 μl) composed of 30% (v/v) reticulocyte lysate was incubated for 3 min under the same conditions described above for labeling reactions with RMs. It was then placed in ice water, and all subsequent operations were performed at 4 °C. An aliquot (10 μl) was removed, and a solution of trypsin and chymotrypsin (350 μg/ml each; 1 μl) was added to the remaining reaction mixture. Aliquots (12 μl) were removed after various intervals and PMSF (2.5 mM; 2 μl) was added to each. After 15 min the samples were precipitated with CTABr and analyzed by SDS-PAGE as described above.

Nuclease Treatment and Gradient Fractionation—A solution of 3% (w/v) Emulgen-913 (25 μl) was added to a labeling reaction (120 μl) at 4 °C. The sample was incubated for 30 min with occasional mixing. Buffer A (7.6 μl) containing 20 mM $CaCl_2$ was added to give a final $CaCl_2$ concentration of 1 mM. Micrococcal nuclease was added to a final concentration of 1000 units/ml from a stock solution of 20,000 units/ml (kept frozen at −20 °C), and the sample was incubated for 1 h on ice. After this incubation, buffer A (8.5 μl) containing 40 mM EGTA was added to yield a final EGTA concentration of 2 mM.

The mixture was loaded onto 10–40% (w/v) sucrose gradients (~12 ml) prepared on top of a 1-ml sucrose cushion (1.8 M) to prevent membrane vesicles from pelleting. Gradients and cushions were prepared in buffer A containing 0.3% Emulgen-913 when the sample contained detergent, and 2 mM EGTA when the sample contained EGTA. The gradients were poured in Beckman Ultra-Clear tubes which had been coated with gelatin. Centrifugation was in a Beckman SW-40 rotor for 1.5 h at 40,000 rpm (202,000 × g_{av}) at 2 °C with slow acceleration and deceleration profiles.

Prior to fractionation a 1.1-ml fraction was removed from the top of each gradient to remove unincorporated radioactive label. The remainder of each gradient was then fractionated using an ISCO gradient fractionator equipped with a flow cell. Fractions of 1.35 ml were collected. Absorbance was monitored at 254 nm to visualize ribosomal peaks.

A 50-μl sample of each gradient fraction was precipitated with CTABr and the precipitated polypeptides resolved on 10–15% SDS-polyacrylamide gels. The gels were fluorographed with DuPont NEN Enhance. The pattern of labeled products was qualitatively identical in every fraction, differing only in total intensity. The amount of labeled nascent chain in each fraction was quantified by densitometry of prominent bands. After quantification the total recovery of nascent chains between gradients did not differ by more than 15%.

A solution of trypsin and chymotrypsin (300 μg/ml each in buffer A; 33 μl) was added to aliquots (200 μl) of gradient fractions at 0 °C. Aliquots (73 μl) were removed at various intervals thereafter and mixed with 15 μl of 2.5 mM PMSF. Each sample was precipitated with CTABr and the precipitates were analyzed by SDS-PAGE in 27.5% gels as described above.

Digitonin Extraction of RMs and Proteolysis—A chilled labeling reaction (40 μl) was adjusted to 100 or 800 mM KOAc and 6 mM $Mg(OAc)_2$. To this sample (60 μl) a digitonin solution (7.6% (w/v); 20 μl) was added to yield a final concentration of 1.9%. The mixture was incubated for 30 min. The salt concentration was then adjusted to 100 mM KOAc and 3 mM $Mg(OAc)_2$ to yield a final volume of 640 μl. A 160-μl aliquot was removed as a "before proteolysis" sample. A solution of trypsin and chymotrypsin (300 μg/ml each; 40 μl) was added to the remaining reaction mixture. Aliquots (173 μl) of this mixture were taken at 5 and 10 min and mixed with 20 μl of a 5 mM PMSF solution. After several minutes 387 μl of 3% (w/v) CTABr were added to each aliquot, followed by 400 μl of 500 mM sodium acetate, pH 5.4, containing 200 μg/ml yeast tRNA. The remainder of the precipitation procedure was as described above.

Cholate Extraction of RMs and Proteolysis—This was performed identically to digitonin extraction and proteolysis (above) except that the labeling reaction was brought to 300 mM KOAc and 2% (w/v) cholate.

Identification of Membrane Proteins Associated with Polysomes after Digitonin or Cholate Extraction—A 200-μl aliquot of RMs in 50 mM TEA, 250 mM sucrose, and 1 mM DTT was diluted with an equal volume of buffer to yield final salt conditions of 25 mM TEA, 800 mM KOAc, 6 mM $Mg(OAc)_2$, and 1 mM DTT. A solution (600 μl) of 3.33% (w/v) digitonin in the same buffer was added. A second 200-μl aliquot of RMs was treated identically except that the final KOAc concentration was 300 mM and a 3.33% (w/v) solution of cholate was added. After a 30-min extraction, each mixture was centrifuged for 52 min at 75,000 rpm (~200,000 × g_{av}) in a Beckman TL 100.2 rotor. The pellet was resuspended by repeated pipetting in 120 μl of a buffer containing 20 mM TEA, 50 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM DTT, and 1% (w/v) CHAPS. After resuspension the conditions were adjusted to 20 mM TEA, 1 M KOAc, 6 mM $Mg(OAc)_2$, 1 mM DTT, and 1% (w/v) CHAPS in a volume of 500 μl. After a 30-min incubation at 4 °C the mixture was centrifuged under the same conditions described above. The supernatant was removed and membrane proteins were isolated from it by Triton X-114 phase separation (Bordier, 1981) as follows. To each of three 140-μl aliquots of the supernatant was added a cold mixture of 720 μl of 50 mM Tris/HCl, pH 7.5, 500 mM KOAc, and 180 μl of Triton X-114 previously equilibrated with the same buffer. The reaction was then moved from ice to 37 °C, where it remained for 10 min. It was then centrifuged at room temperature at approximately 12,000 × g_{av} for 2 min. Two distinct phases resulted. After removal of the aqueous (upper) phase, the detergent phase was subjected to successive acetone washes (2 × 1 ml) followed by one wash with methanol (1 ml) (Görlich *et al.*, 1992a). The resulting protein pellet was resuspended and boiled in sample buffer, combined with the other two identically treated pellets, and the pool analyzed by SDS-PAGE on 10–15% gels.

Proteolysis in Digitonin Followed by Extraction and Proteolysis in Cholate—A chilled labeling reaction (15 μl) was adjusted to 300 mM KOAc and 6 mM $Mg(OAc)_2$ by the addition of 7.5 μl of the appropriate buffer. A digitonin solution (8% (w/v); 7.5 μl) was then added to give a final digitonin concentration of 2% (w/v). After 30 min at 4 °C the conditions were returned to 100 mM KOAc, 3 mM $Mg(OAc)_2$, and 1% digitonin by the addition of 60 μl of an appropriate buffer. An aliquot (30 μl) of the resulting mixture was removed as a before proteolysis sample. To the remaining 60 μl was added a solution of trypsin and chymotrypsin (6.7 μl; 400 μg/ml each), and 5 min were allowed for proteolysis. An aliquot of 33 μl was then removed and mixed with a PMSF solution (6 μl; 5 mM) to illustrate the pattern of proteolytic fragments at this stage of the procedure. The remaining 33-μl fraction was brought to 300 mM KOAc, 6 mM $Mg(OAc)_2$, 0.5% (w/v) digitonin, and 1.5% (w/v) cholate while doubling the volume. After a 10-min incubation this mixture was diluted three-fold to 100 mM KOAc, 3 mM $Mg(OAc)_2$, 0.17% (w/v) digitonin, and 1% (w/v) cholate. An additional 5 min were allowed before 7.5 μl of a solution of 5 mM PMSF was added. The three samples taken at various points in the procedure were then CTABr-precipitated and processed as described above.

Immunoprecipitation for Individual Secretory Proteins after Proteolysis of Digitonin-extracted RMs—A labeling reaction (480 μl) was divided into halves and placed on ice. One sample was subjected to proteolysis, and the other served as the control. To each half were added 120 μl of a buffer to bring the composition to 20 mM TEA, 100 mM KOAc, 6 mM $Mg(OAc)_2$, and 1 mM DTT. A solution containing 8% (w/v) digitonin in the same buffer (120 μl) was then added. This mixture was left on ice for 30 min to allow extraction. A solution of trypsin and chymotrypsin (450 μg/ml each; 40 μl) was then added to one tube, and the other received an equal volume of buffer. After 5 min on ice PMSF (5 mM; 60 μl) was added to each tube. To each of these mixtures were added 400 μl of 3% (w/v) CTABr and 250 μl of 500 mM NaOAc, pH 5.4, containing 200 μg/ml yeast tRNA. The resulting precipitate was left overnight in 50 μl of 50 mM Tris base, pH 10–11, and 2% (w/v) SDS, and then heated to 65 °C for 5 min. A 950-μl aliquot of a buffer was added to give final conditions of 50 mM TEA, 150 mM NaCl, 1% (v/v) Triton X-100, and 0.1% SDS. This mixture was split into thirds, and to each was added 10 μl of the appropriate antiserum. After 24 h of end-over-end mixing at 4 °C, 30 μl of packed Protein A-Sepharose beads were

added. After an additional 3–4 h of mixing, the beads were collected by centrifugation and washed three times with 1 ml of 50 mM TEA, 150 mM NaCl, 1% (v/v) Triton X-100, and 0.1% (w/v) SDS, and then once with this buffer lacking both detergents. The beads were collected and boiled in sample buffer. Immunoprecipitates were resolved by SDS-PAGE in 27.5% gels and autoradiographed.

RESULTS

Labeling of Translocating Nascent Chains—During translocation nascent polypeptide chains are thought to cross the ER membrane through an aqueous pore in the translocon. This mechanism predicts that the portion of a nascent chain that temporarily resides in the plane of the membrane is surrounded by proteins. Moreover, translocation is coupled to protein synthesis and the ribosome maintains a tight junction with the translocon. Thus, the same length portion of all nascent chains would be enclosed in the connected ribosome and translocon, regardless of the stage of completion of elongation of each chain. A different length portion of each individual nascent chain would be exposed in the lumen of the ER, however, that length depending on how far the synthesis of the particular nascent chain had proceeded.

To test these predictions experimentally, we probed the disposition of translocating nascent chains directly. We chose to analyze the nascent chains contained in RMs isolated from canine pancreas. RMs contain membrane-bound polysomes programmed with mRNAs that encode mostly pancreatic secretory proteins. Protein elongation was interrupted when the pancreatic tissue was chilled and homogenized to prepare the microsomal membrane fraction. Membrane-bound polysomes are therefore isolated with nascent chains that are at varying stages of completion. Analysis of this heterogeneous population of nascent chains should therefore allow us to draw conclusions about their disposition during ongoing translocation.

To allow detection of the nascent chains, RMs were incubated in a buffer containing a mixture of amino acids including [^{35}S]methionine, tRNA, ATP, GTP, and an energy regenerating system. Under such conditions, we observed the incorporation of [^{35}S]methionine into polypeptide chains. Presumably, sufficient amounts of tRNA synthetases and elongation factors are present in the RM preparation to support elongation of the polypeptide chains that were initiated but not completed *in vivo* prior to the disruption of the cells. The incorporation of [^{35}S]methionine continued for about 30 min and was roughly linear for the first 15 min (data not shown). The reaction was inhibited by cycloheximide, an inhibitor of elongation, but was not affected by 7-methylguanosine monophosphate and edeine, both inhibitors of initiation (data not shown). This indicates that the incorporation of label was exclusively due to the elongation of pre-existing polypeptide chains and that no significant new initiation of protein synthesis occurred under these conditions.

After prolonged incubation, a large number of protein species were labeled. The pattern of bands of the synthesized products analyzed on SDS-polyacrylamide gels resembled the previously described pattern of bands of dog pancreas secretory proteins (Scheele *et al.*, 1980). The labeled protein bands therefore correspond to the major secretory proteins produced by the exocrine pancreas. The labeled proteins were protected from digestion by added proteases, unless detergents were also added to disrupt the membrane vesicles. In the presence of detergents, the proteins were completely digested (data not shown). Additionally, when a labeling reaction was subjected to an equilibrium floatation that separated RMs from free ribosomes, all labeled products floated in the position of the membranes (data not shown). Taken together, these results support the premise that incomplete nascent polypeptide chains were isolated as part of the microsomal membrane fraction and were then completed and translocated into the lumen of the vesicles *in vitro*.

Quantitation of the number of nascent chains being elongated in our reactions revealed that at least 20% of the total nascent chains in RMs, and possibility as much as 80%, are being elongated and labeled in our reactions (see "Experimental Procedures"). We conclude that our experiments examine a representative population, if not the majority, of the translocating nascent chains.

Protection of Nascent Chains by Membranes and Ribosomes—To probe the disposition of nascent chains experimentally, we first labeled nascent chains in a brief *in vitro* elongation reaction and then added proteases. If a tight ribosome-membrane junction is maintained throughout translocation, all nascent chains should be protected from digestion from the site of peptide bond formation within the ribosome to the nascent chain exit site on the lumenal face of the ER membrane. We made the lumen of the RMs accessible to proteases by disrupting the membranes with the mild, non-ionic detergent Emulgen-913. This detergent was chosen because it was previously suggested to preserve the ribosome-membrane junction (Yoshida *et al.*, 1987). The membranes were disrupted to varying degrees by titration of the Emulgen-913 concentration and then incubated with a mixture of trypsin and chymotrypsin at 0 °C. After inactivation of the proteases, the proteolytic fragments of nascent chains still attached to tRNAs were selectively precipitated with CTABr and analyzed on high percentage SDS-polyacrylamide gels. This procedure restricted the analysis to those proteolytic fragments whose carboxyl-terminal ends were at the peptidyltransferase site of the ribosome.

The results of a representative experiment are shown in Fig. 1. At each detergent concentration a large fraction of carboxyl-terminal nascent chain fragments was detected after proteolysis as a broad band centered in length around 40 amino acids. We will refer to this as the "40-amino acid band" (Fig. 1, *40aa*). Most likely, these fragments were protected from digestion by ribosomes, consistent with the observations of Malkin and Rich (1967) and Blobel and Sabatini (1970), who showed that large ribosomal subunits protect about 40 carboxyl-terminal amino acids from proteolysis. Because the nascent chains analyzed here are made by membrane-bound ribosomes, the generation of the 40-aa band is likely to result from proteolytic cleavage of the nascent chain between the ribosome and the membrane.

Most importantly, in addition to the 40-aa band a population of larger carboxyl-terminal fragments was also generated. These bands are likely to result from protection of the nascent chain by membrane components. The most prominent of the larger fragments comprises a distribution centered around a length of approximately 70 amino acids, with extremes of roughly 55 and 85 amino acids. We will refer to this distribution as the "70-amino acid band" (Fig. 1, *70aa*). The intensity of the 70-aa band increased with the concentration of detergent used in the disruption (Fig. 1, *lanes 2–5*), presumably because access of the proteases to the lumenal face of the membrane was enhanced. At 0.5% detergent (Fig. 1, *lane 5*) the 70-aa band and the 40-aa band were about equally prominent. When the detergent concentration was increased further, the distinction between the 40-aa band and the 70-aa band became less pronounced (Fig. 1, *lanes 5–7*). Similar results were obtained when the experiment was repeated with different non-ionic detergents (*e.g.* Triton X-100, data not shown).

In addition to the major 40-aa and 70-aa bands, we reproducibly observed a series of minor bands migrating above the major bands (most clearly seen in Fig. 1, *lanes 4–6*). These bands appear to be about evenly spaced at approximately 10-

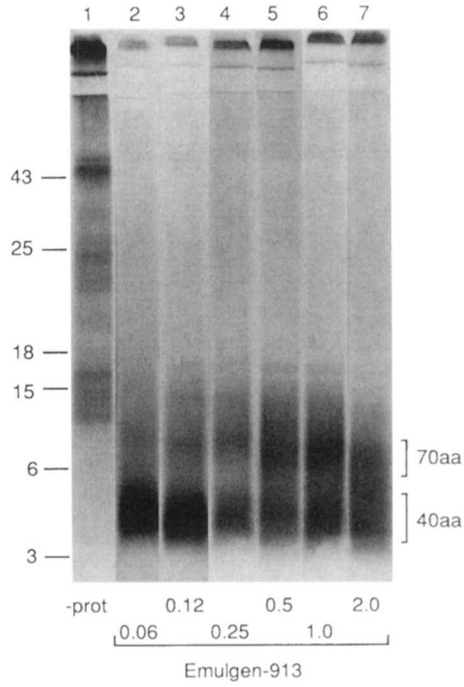

FIG. 1. **Proteolysis of labeled nascent chains after disruption of RMs with Emulgen-913.** Nascent chains contained in RMs were labeled and the RMs incubated with the indicated concentrations of Emulgen-913. The extracts were then digested with trypsin and chymotrypsin as described under "Experimental Procedures." After a 6-min digestion samples were precipitated with CTABr and the precipitates electrophoresed on 27.5% SDS-polyacrylamide gels (*lanes 2–7*). The gels were processed for autoradiography. *Lane 1* shows an aliquot removed before the addition of proteases. Molecular masses of standards are shown in kilodaltons; *brackets* indicate the distributions of proteolytic fragments around lengths of approximately 40 and 70 amino acids (*40aa* and *70aa* bands). These bands are not terminal digestion products because continued digestion at 37 °C results in their disappearance. Emulgen-913 concentrations used during extraction are expressed as percentages (w/v).

amino acid intervals, giving a ladder effect. The intensity of these bands increases as their size decreases, suggesting that they might be successive degradation products of nascent chains.

Detection of the 70-aa Band in Sarkosyl-permeabilized RMs—To verify these results under conditions where the membranes were less drastically perturbed, RMs were permeabilized with Sarkosyl. At low concentrations Sarkosyl forms holes in the membrane without extensive disruption of the membrane structure (Helenius and Simons, 1975). To determine the lowest concentration of Sarkosyl that would allow access of proteases to the lumen, RMs were loaded with radiolabeled secretory proteins in a prolonged labeling reaction. The sensitivity of these proteins to digestion with proteinase K was then tested in the presence of increasing amounts of Sarkosyl. A concentration of 0.016% Sarkosyl rendered most labeled proteins protease-sensitive, while they were completely insensitive in the absence of Sarkosyl (data not shown). Interestingly, little of either the labeled proteins or the lumenal content proteins was released into a supernatant fraction after permeabilization with 0.016% Sarkosyl followed by sedimentation of the vesicles (data not shown). These results indicated that the membranes had been only slightly permeabilized, so as to allow only a few protein molecules to pass. Since the protease acts catalytically, a relatively small number of molecules can account for the observed digestion of the lumenal contents.

The experiment shown in Fig. 1 was repeated under these conditions of minimal disruption of the membranes. Nascent chains were labeled as described above, and the vesicles were permeabilized with 0.016% Sarkosyl. After protease treatment for different times, CTABr-precipitable peptides were analyzed on SDS-polyacrylamide gels (Fig. 2*A*). Similar to the experiment shown in Fig. 1, a major 40-aa band and a clear, albeit less abundant, 70-aa band resulted. The 70-aa band did not appear when Sarkosyl was omitted (not shown, but see Fig. 3, *lane 2*, for a similar control). When the protease digestion step was followed by centrifugation to pellet RMs but not free ribosomes, the 70-aa band was recovered exclusively in the pellet fraction (Fig. 2*B*, *lane 3*). In contrast, most of the 40-aa band (presumed to be protected by the ribosome) was recovered in the supernatant (Fig. 2*B*, *lane 2*). When the protease was omitted in a control experiment, the nascent chains appeared only in the pellet fraction (data not shown).

As a further control, nascent chains on reticulocyte ribosomes were labeled. These ribosomes synthesize primarily globin (a cytoplasmic protein) and are not membrane associated. As expected, all of the labeled nascent chains in the reaction are less than 15 kilodaltons, the approximate molecular mass of the hemoglobin chains (Fig. 2*C*, *lane 1*). Digestion of this reaction with protease in either the presence or absence of Sarkosyl gave rise to only a 40-aa band (Fig. 2*C*, *lane 2*). This result supports the identification of the 40-aa band observed after proteolysis of the permeablized membranes as a ribosome-protected fragment and shows that the detection of a 70-aa band correlates with the membrane-bound state of the ribosomes that synthesize the nascent chains.

Taken together, the results presented in Figs. 1 and 2 indicate that components of the microsomal membrane confer protection on an additional 30 amino acids of the translocating nascent chain beyond that protected by the ribosome itself (the 40-aa band). To generate the protected 70-aa fragments, the protease must gain access to the lumenal face of the membrane. When the membranes are minimally disrupted, and hence the protease concentration is probably much lower in the lumen of a vesicle than on its exterior, proteolysis leads primarily to the release of ribosomes protecting the 40-aa fragments; as expected, however, the 70-aa fragments remain associated with the membranes.

Protection of the 70-aa Band in Detergent-solubilized Polysomes—To determine the nature of the membrane components protecting the nascent chain, we assessed the degree of solubilization of the RMs under conditions that maintain the protection of the 70-aa band. To do this, a labeling reaction was extracted with detergent under the same conditions used in Fig. 1, *lane 5*. The extract was then fractionated by sucrose gradient velocity sedimentation (Fig. 3*B*). The distribution of nascent chains within the gradient was similar to that of reticulocyte polysomes (Fig. 3*D*). In the absence of detergent, the nascent chains were recovered in the bottom fraction of the gradient (Fig. 3*A*). As observed in Fig. 1 for the unfractionated detergent extract, both the 40-aa and 70-aa bands were obtained when proteolysis was performed on individual gradient fractions (Fig. 3*C*). Note that proteolysis of fraction 2 (containing the monosome peak; see Fig. 3*D*) produced mostly the 40-aa band, whereas proteolysis of fractions sedimenting toward the bottom of the gradient (*e.g.* fraction 8) produced mostly the 70-aa band. From the middle fractions (fractions 4 and 6) the 40-aa and 70-aa bands were generated in about equal proportions. Thus, the protection of these nascent chain fragments remains intact upon solubilization of the polysomes from the membrane.

To confirm that the nascent chains were indeed attached to polysomes which conferred them the observed sedimentation properties, the detergent extract was digested with micrococcal nuclease prior to sedimentation. Nuclease treatment resulted

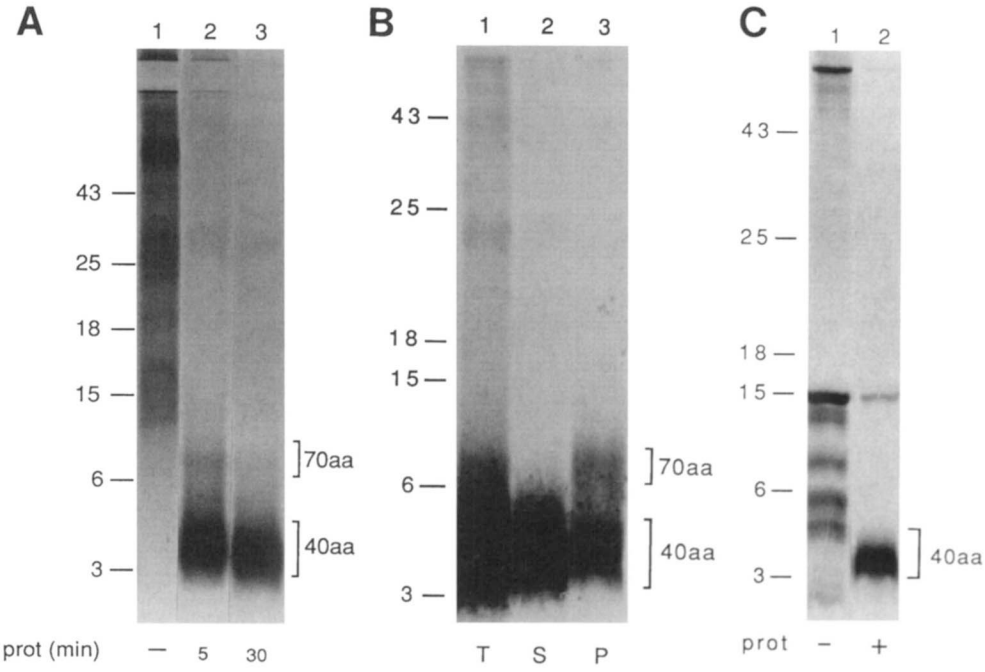

FIG. 2. *Panel A,* time course of proteolysis of labeled nascent chains in Sarkosyl-permeabilized rough microsomes. A labeling reaction was performed as described under "Experimental Procedures," placed on ice, and then brought to a final concentration of 0.016% (w/v) Sarkosyl. An aliquot was removed (*lane 1*) and the remainder digested for the indicated times with trypsin and chymotrypsin (*lanes 2* and *3*). Proteolytic fragments associated with tRNAs were precipitated with CTABr and fractionated by SDS-PAGE on a 27.5% gel. Molecular mass standards and *brackets* are as in Fig. 1. *Panel B,* distribution of proteolytic products between supernatant and pellet. A sample identical to that shown in *A, lane 2,* was fractionated by centrifugation into a supernatant and a pellet as described under "Experimental Procedures." Under these conditions membranes, but not free ribosomes, were pelleted. CTABr precipitations were then performed on each of the two fractions, and the precipitates electrophoresed on a 27.5% polyacrylamide gel. *Lane 1* shows the unfractionated sample, and *lanes 2* and *3* the supernatant and pellet fractions, respectively. *Panel C,* proteolysis of labeled nascent chains on free ribosomes. A labeling reaction was performed with reticulocyte lysate as described under "Experimental Procedures." The samples were treated identically to those described in *A*. CTABr-precipitable products without proteolysis or after 5 min of proteolysis are shown in *lanes 1* and *2*, respectively.

in a pronounced shift of the distribution of nascent chains toward the top of the gradient (Fig. 4, compare *panels A* and *B*). In control reactions, no significant shift was observed in mock treated detergent extracts (Fig. 4*C*), or when the micrococcal nuclease was added after the addition of the calcium chelator EGTA (Fig. 4*D*), indicating that the observed shift resulted from nucleolytic activity and not a fortuitously activated protease. Thus, we conclude that nascent chains sediment in the sucrose gradient because they are attached to polysomes, which upon degradation of the mRNA linking the individual ribosomes are converted into smaller units and monosomes.

Most importantly, nascent chains sedimenting at the monosome position after nuclease treatment (Fig. 4*B*, fraction 2) were still protected from protease digestion to yield the 70-aa band (Fig. 4*B, insert*). Thus, the component(s) that give rise to the protection of the 70-aa fragments remain attached to the ribosome upon detergent extraction. Furthermore, these results suggest that the component(s) conferring the protection must be small relative to the size of a ribosome, because protection is still observed on ribosomes sedimenting at the approximate position of monosomes.

Production of the 70-aa Band by Proteolysis after Digitonin Solubilization—While this work was in progress Görlich and Rapoport (1993) reported the co-isolation of ER membrane proteins with polysomes that were solubilized by digitonin (Görlich *et al.*, 1992a). Specifically, they found that after extraction of RMs with digitonin at 800 mM KOAc or higher a limited set of integral membrane proteins remained associated with the ribosomes. Among these proteins are the components of the Sec61 complex, which is likely to form the protein translocation pore in the translocon. Thus, it seemed plausible that these proteins might be responsible for the protection of nascent chains seen in our assays. To test this possibility directly, we extracted labeled RMs with digitonin at 800 mM KOAc. Proteolysis as in the experiments described above yielded a CTABr-precipitable 70-aa band (Fig. 5*A, lanes 5, 6, 8,* and *9*), identical to that seen under the solubilization conditions using Emulgen-913 at low KOAc concentrations (Fig. 5*A, lanes 2* and *3*). In contrast to Emulgen-913, however, only a small amount, if any, of a 40-aa band was produced in the digitonin extracts, indicating that the ribosome/translocon junction may be more stable upon digitonin solubilization, thus preventing protease access to the nascent chains between ribosome and translocon.

Proteolysis of labeled RMs extracted with cholate produced only a 40-aa band (Fig. 5*B, lanes 1–3*), in contrast to Emulgen-913 or digitonin, suggesting that the components of the translocon did not remain stably associated with the ribosome after extraction of RMs with cholate.

The difference in protease protection of nascent chains after extraction with digitonin and cholate suggested that comparison of the membrane components associated with ribosomes under the two conditions might identify those responsible for protection. For this comparison proteolysis was omitted in similar extraction reactions. Integral membrane proteins associated with the ribosomes under the different conditions were then isolated using Triton X-114 phase partitioning. The simple protein pattern resulting after extraction with digitonin (Fig. 5*B, lane 6*) was indistinguishable from that reported by Görlich and Rapoport (1993). The identity of several of the bands as components of the Sec61, TRAP, or glycosyltransferase complexes was verified by Western blotting with specific antibodies (Fig. 5*B, lane 9*). All the other prominent bands are of molecular masses that correspond to other characterized subunits of one of these three complexes (see legend to Fig. 5).

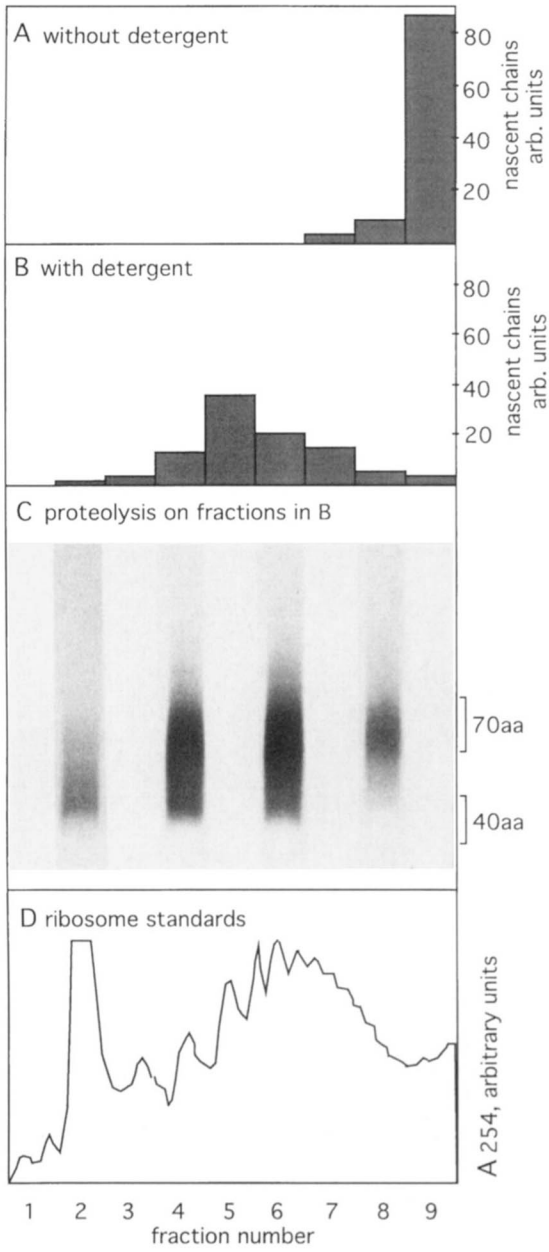

FIG. 3. **Sucrose gradient fractionation of protected nascent chains.** Labeling reactions were fractionated on sucrose gradients as described under "Experimental Procedures" either without (*Panel A*) or after extraction with 0.5% Emulgen-913 (*Panels B* and *C*). Nascent chains were quantified as described under "Experimental Procedures." The interface between the bottom of the gradient and the 1.8 M sucrose cushion was collected in fraction 9 in each case. *Panel C* shows the results of proteolysis of every other fraction of the gradient in *Panel B*. *Panel D*, a reticulocyte lysate polysome profile (recorded as absorbance at 254 nm) from an identical gradient as in *Panels A-C* is shown to provide size markers.

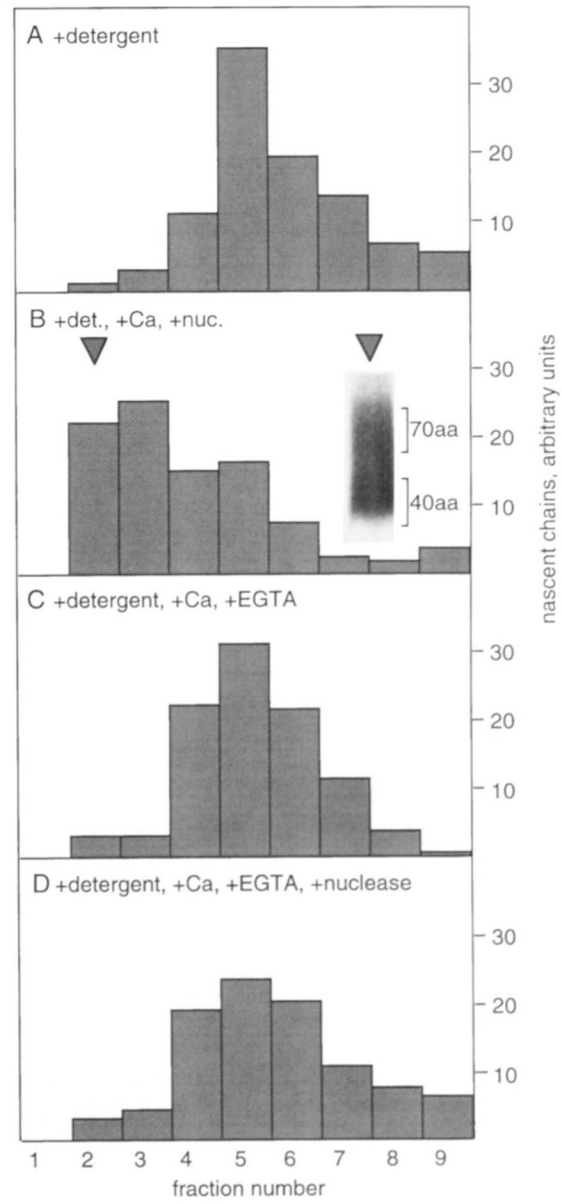

FIG. 4. **Alteration of the sedimentation properties of labeled nascent chains by nuclease digestion.** Labeling reactions were extracted with Emulgen-913 as in Fig. 3, *Panel B*. Nuclease digestions or control incubations and subsequent gradient fractionations were then performed as described under "Experimental Procedures." *Panel A*, control incubation with only mock buffers added. *Panel B*, 1 mM Ca^{2+} and 1000 units/ml micrococcal nuclease were added, and after 1 h at 0 °C EGTA was added to 2 mM. The *inset* shows the result of proteolysis performed on fraction 2 using the same procedure as in Fig. 3, *Panel C*. *Panel C*, 1 mM Ca^{2+}, but no micrococcal nuclease, was added, followed after 1 h at 0 °C by the addition of EGTA to 2 mM. *Panel D*, 1 mM Ca^{2+}, 2 mM EGTA, and 1000 units/ml micrococcal nuclease were added in that order at the beginning of the reaction.

In contrast, after extraction with cholate only the components of the glycosyltransferase complex remained in significant quantities (Fig. 5*B*, *lanes 7* and *10*). It is likely, then, that a subset of the membrane proteins remaining associated with ribosomes after extraction with digitonin, presumably including the Sec61 complex, is responsible for the protection of the 70-aa fragment of the nascent chain.

Conversion of the 70-aa Band to the 40-aa Band—The absence of a significant 40-aa band upon proteolysis of the digitonin extract allowed us to demonstrate directly that the 40-aa and 70-aa bands were derived from the same population of nascent chains. To this end we treated the proteolyzed digitonin extract with the ionic detergent cholate, which disrupts the ribosome-translocon junction (data not shown). With continued proteolysis after the addition of cholate, the 70-aa band was converted to the 40-aa band (Fig. 6, *lane 3*).

Fragments of Different Secretory Proteins Are Contained in the 70-aa Band—The protease protection assay monitors the environment of a heterogeneous population of nascent chains translated by pancreatic membrane-bound polysomes. The data shown in Fig. 7 verify that the 70-aa band was indeed generated from more than one species of nascent secretory protein. Labeled RMs were extracted with digitonin as described above and proteolyzed

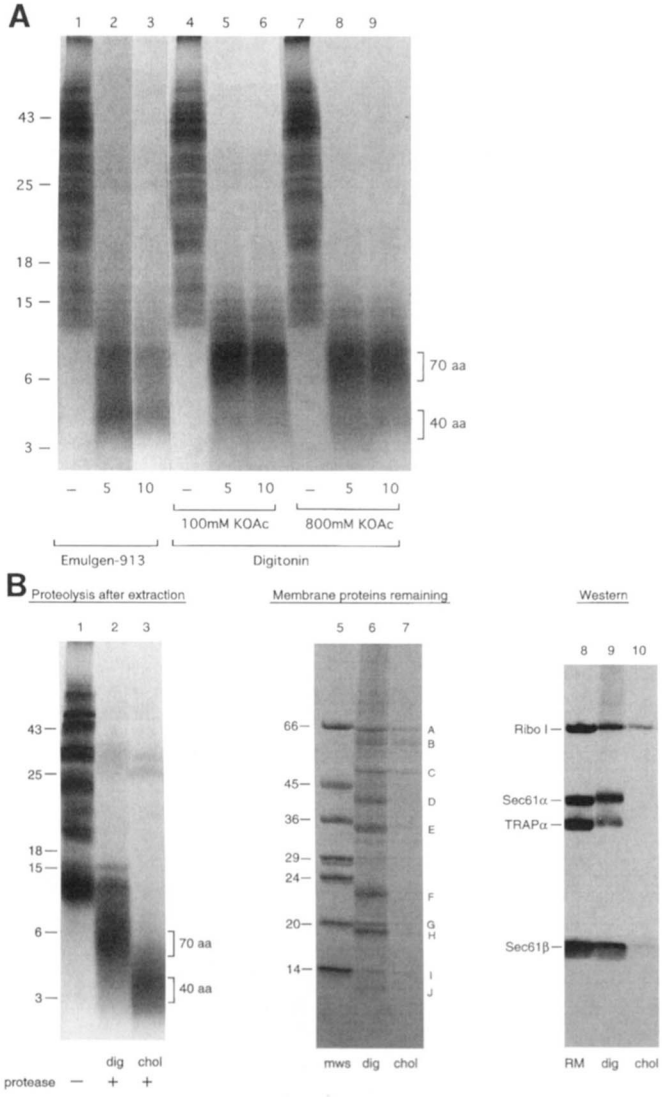

FIG. 5. *Panel A,* proteolysis of labeled nascent chains after extraction of rough microsomes with digitonin. Nascent chains contained in RMs were labeled and the microsomal membranes were subsequently extracted with 2% digitonin at either 100 or 800 mM KOAc. After extraction the mixtures were diluted to 100 mM KOAc and digested with trypsin and chymotrypsin. After either 5 or 10 min of digestion aliquots were removed, precipitated with CTABr, and the precipitates electrophoresed on 27.5% polyacrylamide gels (*lanes 5, 6, 8,* and *9*). For comparison, *lanes 2* and *3* show the 5- and 10-min time points of digestion after extraction with 0.5% Emulgen-913 as in Fig. 1. *Lanes 1, 4,* and *7* show the CTABr-precipitable products with no protease added. Molecular mass standards and the approximate positions of fragments of 40 and 70 amino acids are as in Fig. 1. *Panel B,* integral membrane proteins associated with ribosomes under conditions in which the 70-aa band is protected from proteolysis. RMs with labeled nascent chains were extracted with 2% digitonin at 800 mM KOAc, or with 2% cholate at 300 mM KOAc. After dilution of the extracts to 100 mM KOAc, they were subjected to proteolysis, CTABr precipitation, and gel electrophoresis (*lanes 2* and *3*) as in *A*. Larger aliquots of unlabeled RMs were extracted under the same conditions, and the ribosomes and any associated material were collected from the extracts by centrifugation. The ribosomal pellets were further extracted with 1% CHAPS at 1 M KOAc. The material removed from the ribosomes by CHAPS was subjected to Triton X-114 phase partitioning (Bordier, 1981) to enrich for integral membrane proteins. The detergent phases were trichloroacetic acid-precipitated, the precipitates electrophoresed on 10–15% SDS-polyacrylamide gels, and the gels either stained with Coomassie Blue (*lanes 6* and *7*) or transferred onto nitrocellulose for Western blotting (*lanes 9* and *10*). Molecular mass standards are shown in *lane 5*, with their sizes in kilodaltons indicated. The *letters* to the right of *lane 7* identify prominent species either on the basis of Western blotting (*lanes 8–10*), or on the basis of coincidence of molecular mass with other subunits of the glycosyltransferase, Sec 61, or TRAP complexes. The probable identities of these species are: *A*, ribophorin I; *B*, ribophorin II; *C*, ribophorin-associated protein; *D*, Sec61 α; *E*, TRAP α; *F*, TRAP β; *G*, TRAP γ; *H*, TRAP δ; *I*, Sec61 β; *J*, Sec61 γ. The Sec61β portion of *lanes 8–10* is from a different exposure of the blot than that used for the other three proteins.

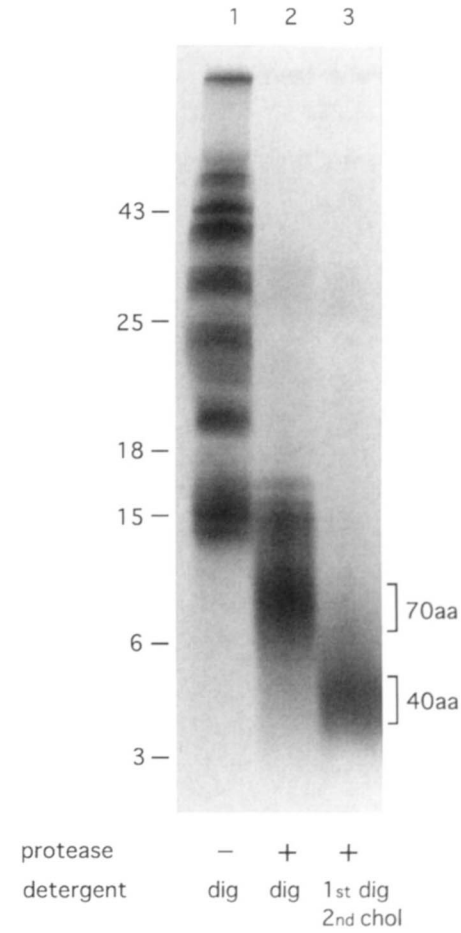

FIG. 6. **Conversion of the 70-aa band to the 40-aa band.** Nascent chains contained in RMs were labeled and the membranes then extracted with 2% digitonin at 300 mM KOAc and proteolyzed as in Fig. 5*A*. This generated an extract containing the population of carboxyl-terminal nascent chain fragments shown in *lane 2*. The proteolyzed extract was then brought to 1.5% cholate at 300 mM KOAc. Continued proteolysis produced the population of fragments shown in *lane 3*. CTABr precipitation, electrophoresis, molecular mass standards, and the positions of the 40-aa and 70-aa bands are as in previous figures.

to produce the 70-aa band. This collection of fragments was then immunoprecipitated with antibodies specific to three different pancreatic secretory proteins, amylase, chymotrypsinogen 2, and proelastase. Each of the three secretory proteins tested produced a distribution of fragments in the 70-amino acid range. These distributions are slightly different, indicating that they are not the result of nonspecific precipitation. Thus, these three nascent secretory proteins, and most likely all secretory proteins, give rise to similar fragments upon protease digestion and hence are likely to pass through the membrane in the same or similar environments.

DISCUSSION

We have used proteolysis to probe the disposition of nascent chains during their translocation across the membrane of the endoplasmic reticulum. We chose pancreatic RMs containing labeled nascent chains as the experimental system because it allowed us to monitor the behavior of a population of many

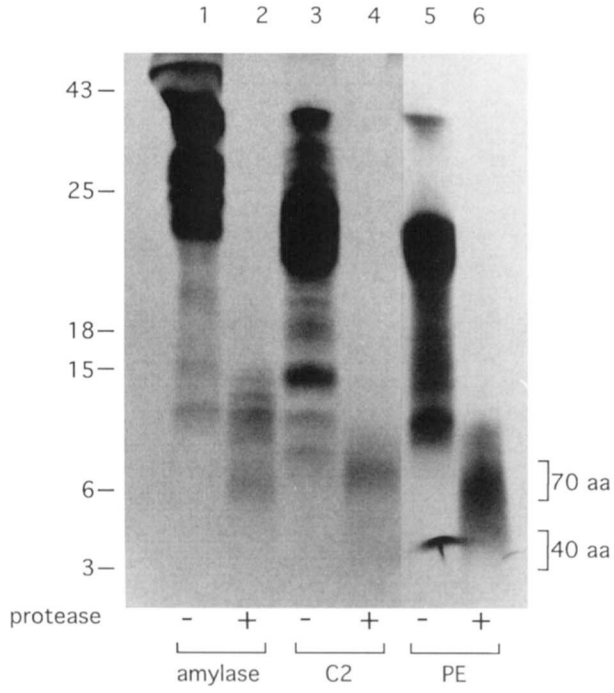

FIG. 7. **Immunoprecipitation of individual secretory proteins after proteolysis of digitonin-extracted membranes.** Nascent chains contained in RMs were labeled, and the RMs subsequently extracted with 2% digitonin at 100 mM KOAc. The extract was then proteolyzed with trypsin and chymotrypsin, and the tRNA-associated proteolytic fragments of nascent chains precipitated with CTABr. The procedure was exactly as for *lane 5* of Fig. 5*A*. The CTABr precipitate was resuspended and immunoprecipitated with an antiserum against one of three secretory proteins: amylase, chymotrypsinogen 2 (*C2*), or proelastase (*PE*). The immunoprecipitates were electrophoresed and processed as in all previous figures (*lanes 2, 4,* and *6*). *Lanes 1, 3,* and *5* show the results of immunoprecipitation after having omitted the proteolysis step. Molecular mass standards and the positions of the 40-aa and 70-aa bands are as in previous figures. The conditions required for this experiment were suboptimal for recognition by these sera of their antigens. The antisera were therefore not present in excess.

different nascent chain species at many different stages of translocation. Thus, the results of these studies provide a general picture of the environment of translocating nascent chains during the steady state.

Our interpretation of the results is presented schematically in Fig. 8. As demonstrated originally by Malkin and Rich (1967), and later by Blobel and Sabatini (1970), and as confirmed here in Fig. 2*C*, the ribosome protects about 40 amino acids of the nascent chain from proteolysis. This protection is responsible for the 40-aa band that we observe (Fig. 8*A*). During steady state translocation of a secretory protein the nascent chain, on exiting from the large ribosomal subunit, enters directly into a transmembrane structure, the translocon, capable of protecting it for an additional approximately 30 amino acids. The combined protection by ribosome and translocon gives rise to the 70-aa band observed. In all cases, because a large number of different species of secretory proteins are synthesized at any one time, proteolysis produces heterogeneous populations of fragments, centered around 40 and 70 amino acids. Thus, despite the use of a mixture of trypsin and chymotrypsin, which should cut nascent chains frequently at many different positions, the two bands are broad.

With intact RMs proteases cannot gain access to the lumenal side of the membrane, so that all proteolysis occurs between the ribosome and the translocon. Consequently, only the 40-amino acid fragment protected by the ribosome is produced (data not shown). In the presence of detergent, proteases have access to the lumenal side of the translocon, generating the 70-amino acid band. The translocon is likely composed of some or all of the membrane proteins found associated with ribosomes under conditions in which protection was observed. The removal of those proteins correlated with loss of protection. Among them is Sec61, one of the few integral membrane proteins isolated with digitonin-solubilized polysomes (Görlich *et al.*, 1992a). The junction between the ribosome and the translocon is either tight enough to offer some, but not complete, protection from proteolysis, or, as it is composed of protein itself, can be degraded to allow eventual access of proteases to the nascent chain. We favor the latter explanation, because the results of Crowley and colleagues (1993, 1994) point to a tight seal between the ribosome and translocon, impermeable to species as small as iodide ions. Such a seal would likely provide an effective barrier to protect the nascent chain from limited proteolysis. A previous study concluded that translocating nascent chains longer than about 100 amino acids were succeptible to proteolysis between the ribosome and the membrane, probably because proteolysis was extensive enough to degrade the proteins forming the seal (Connolly *et al.*, 1989).

At low concentrations of detergent (*e.g.* after Sarkosyl permeabilization in Fig. 2*A*) the membrane is perforated, but not extracted (represented by the holes in the membrane in Fig. 8*B*), limiting access of proteases to the lumenal side of the membrane. As a result the protease concentration in the lumen is less than that on the exterior of the vesicle, making the cut between the ribosome and the membrane more frequent than that at the lumenal face. Thus, the 70-amino acid fragment is produced, but the products are dominated by the 40-amino acid fragment. At detergent concentrations high enough to completely extract the membrane (*e.g.* after Emulgen-913 extraction in Fig. 1), the protease concentrations available to cut at each of the two sites are the same (Fig. 8*C*). Cuts on the lumenal side of the membrane therefore increase in frequency relative to those between the ribosome and the translocon, increasing the ratio of the 70-amino acid fragment to the 40-amino acid fragment. After extraction with digitonin only the 70-amino acid band is observed (Fig. 5). This probably reflects the less disruptive manner in which digitonin interacts with membrane proteins, resulting in greater preservation of the ribosome-membrane junction (Hartmann *et al.*, 1993; Görlich and Rapoport, 1993).

The length of the nascent chain protected by the translocon is insufficient to cross the thickness of a lipid bilayer more than once. Thus, we conclude, as shown in Fig. 8, that, on emerging from the ribosome during steady state translocation, the path of a nascent secretory protein traverses the thickness of the membrane only once and without substantial folding. We can draw no conclusions from these experiments about the path of the nascent chain after it becomes accessible to the ER lumen. These conclusions are consistent with those of Mothes and colleagues (1994), who did not observe cross-links between the nascent chain and Sec61α when the cross-linking group was positioned more than about 70 amino acids from the carboxyl-terminal end. Cross-links to other membrane species were detected beyond that length, but they were not over extended regions of the nascent chain, and hence are unlikely to be directed toward components of the translocation channel. We note, however, that the pattern of carboxyl-terminal products includes several less prominent bands larger than 70 amino acids (*e.g.* Fig. 1, *lanes 5* and *6*). This raises the possibility that the nascent chain may make additional contacts beyond 70 amino acids, possibly with ER lumenal chaperones. These bands are most pronounced with amylase (Fig. 7, *lane 2*).

Previous measurements of the length of the nascent chain

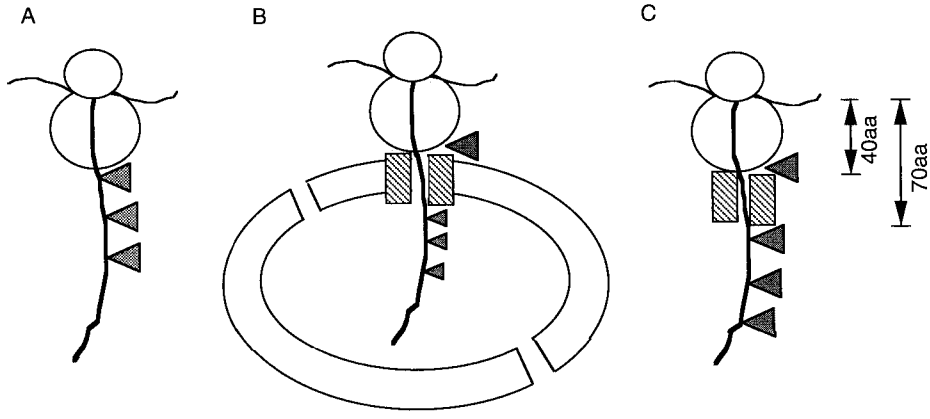

FIG. 8. **Model describing the proteolysis of nascent chains.** *Shaded triangles* pointing to the left indicate proteolytic attack at a point on the nascent chain, with the size of a triangle proportional to the relative accessibility of the nascent chain at that point. *Panel A*, on free ribosomes only the large subunit of the ribosome itself protects the nascent chain from proteolysis. The 40-aa band is generated. This result is shown in Fig. 2*C*. *Panel B*, in barely permeabilized RMs the protease concentration in the lumen of a vesicle is much lower than that on its exterior. Proteases digest the ribosome-membrane junction to gain access to the nascent chain, but a few nascent chains escape proteolysis at this point and are cut only within the lumen. The population of carboxyl-terminal nascent chain fragments is dominated by 40-amino acid fragments, but a few 70-amino acid fragments are present. This result is shown in Fig. 2*A*. *Panel C*, in completely solubilized RMs all of the nascent chain beyond the translocation apparatus is freely accessible to proteolysis. In a detergent that weakens the ribosome-membrane junction, proteolysis can also occur between the ribosome and the membrane, but it is no more frequent there than at the point at which the nascent chain exits from the membrane. The 40- and 70-amino acid carboxyl-terminal fragments are generated in equal amounts. This result is shown in Fig. 1, *lanes 5* and *6*. In a detergent that maintains the ribosome-membrane junction, cutting of the nascent chain at that point is not possible. Only the 70-amino acid carboxyl-terminal fragment is generated. This result is shown in Fig. 5*A*.

required to span the membrane have yielded different figures. Glabe and colleagues (1980) determined that a glycosylation site must be at least 30 amino acids from the point at which the nascent chain exits from the ribosome in order to be modified. Because glycosylation occurs on the lumenal face of the ER membrane the glycosylation site would have to have crossed the membrane completely. This makes their figure a measurement of the lower limit of the number of amino acids between the point at which the nascent chain emerges from the ribosome and that at which it enters the lumen of the ER. This length is in good agreement with our estimate of 30 amino acids as the length of nascent chain within the translocon. In contrast, other measurements have estimated the number of amino acids that must separate the peptidyltransferase site from a cysteine residue before that residue can participate in a disulfide bond. Bergman and Kuehl (1979) concluded that a cysteine positioned 100 amino acids from the amino terminus of a nascent chain of 16,700 daltons could participate in a disulfide bond. Using a figure of 110 daltons as the average molecular mass of an amino acid residue, we calculate that the nascent chain would be composed of about 150 amino acids. That would leave only about 50 amino acids between the peptidyltransferase site and the cysteine residue. This is less than the lower end of the distribution of membrane-protected fragments that we observe. Since the cysteine in question bonded with another cysteine positioned 65 amino acids away, considerable folding of the nascent polypeptide would have been required to form the bond. It seems unlikely that such a large domain could fit within the translocon. It seems more likely that the electrophoretic and chromatographic methods used to estimate the sizes of the nascent chains containing disulfide bonds resulted in low estimates.

Our estimate of 70 amino acids as the total length of the nascent chain surrounded by the complex of the ribosome and the translocon together is in precise agreement with the number of amino acids between the peptidyltransferase site and the distal end of the region of continuous proximity between the nascent chain and Sec61α (Mothes *et al.*, 1994). Because proteases have access to the nascent chain at that position, it must be exposed on the lumenal face of the membrane, thereby demonstrating that Sec61α contacts the nascent chain continuously from one side of the membrane to the other, consistent with the idea that Sec61α is the protein that forms or lines the inside of the translocation channel.

Our results support the concept that translocation occurs through a pore formed across the ER membrane and that the translocon is in intimate contact with the ribosome. It is particularly intriguing to speculate that such intimate contact could allow communications between the two that could serve as regulatory mechanisms for translocation. Several suggestions of such communication have already been reported. Thus, the finding that a nascent single spanning integral membrane protein remains in contact with components of the channel until the very end of translation suggests that the presence of a ribosome is required for the integrity of the channel (Thrift *et al.*, 1991). Also consistent with this idea are the results of Simon and Blobel (1991), who observed disappearance of puromycin-dependent aqueous channels at salt concentrations known to remove ribosomes from the membrane. Given these tantalizing suggestions it will be extremely interesting to uncover the regulatory mechanisms that govern the formation or opening, maintenance, and closure of the translocation pore.

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