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). 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; Wiedemann 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 protein (TRAM) protein and the translocating-associated protein (TRAP) complex) can also be cross-linked to the nascent chain (Görlich et al., 1992b). The TRAP 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 luminal 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...
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 luminal 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 Sec61a to translocating nascent chains suggests the possibility that it may be a component of the channel wall. Sec61a 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 peptide transferase 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 the carboxyl-terminal 40 amino acids of a nascent chain, the length and position within the nascent chain of the contact region is consistent with it spanning the plane of the membrane. It is not clear from this data, however, whether Sec61a 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 Sec61a and the TRAM protein (Crowley et al., 1993; Mothes et al., 1994). Similarly, because the contact region between Sec61a and the nascent chain begins just behind 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 Sec61a 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**—[35S]Methionine (>1000 Ci/mmol) was from Amersham. The detergent Emulgen-913 was the kind gift of Dr. Yoshih Imai (University of Osaka, Japan), and is commercially available from the KAO Atlas Co. Ltd. of Tokyo, Japan. Emulgen-913 is a linear polyoxyethyleneamphiphilic 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 amyrase, 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 (43,000 Da), α-chymotrypsinogen (25,7 KDa), β-lactoglobulin (18,4 KDa), lysozyme (14.9 KDa), bovine trypsin inhibitor (6.9 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 the stock solution of this buffer solution (1 M) was prepared from these stock solutions was not reconditioned 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 -20°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 (1978; 1975). In brief, the reaction mixture contained final concentrations of 10 μM 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 RNA, and 100 units/ml human placental ribonuclease inhibitor. Final salt conditions were 20 mM TEA, 100 mM KOAc, 3 mM magnesium acetate, 20 mM β-glycerolphosphate, and 2 mM ATP. The reaction also included was [35S]methionine, usually at 1 μCi/ml. 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 poly peptide chains was about 15 min under these conditions.

**Quantitation of Nascent Chains**—The endogenous methionine concentration in a standard labeling reaction was measured by including a trace amount of [35S]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 [35S]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. At this time, we estimated that each nascent chain was elongated by approximately 70 or fewer amino acids because at the combined label in the 40- and 70-amino acid bands (see “Results”) is the same as the total label incorporated in an unproteolysed reaction (data not shown). At all subsequent times it was less. Methionine constituted 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 80 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 composed 20% of the engaged translocation machinery (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 (1 μl) was added while the reaction mixture was being vortexed. After 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), and the pellet washed with 1 ml of 0.3 M sodium acetate (pH 5.4). The precipitates were washed 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 membrane after Sarkosyl permeabilization and proteolysis, a sample (12 µl) was loaded on top of a 50-µl cushion of 250 µm 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 Product—A labeling reaction (70 µl at 30% (v/v) reticulocyte lysate was incubated for 3 min under the same conditions described above for labeling reactions with RNAs. 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) was added to the remaining reaction mixture. The supernatant remaining after centrifugation was mixed 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 RNAs was treated identically except that the final KOAc concentration was 300 mM and a 3.33% (w/v) solution of cholate. After a 30-min incubation, each mixture was centrifuged for 52 min at 75,000 rpm (~200,000 × g) 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 mM KOAc, 2 mM Mg(OAc)2, 1 mM DTT, and 1% (w/v) CHAPS. After resuspension (300 µl) was added to 500 µl of a buffer containing 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 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, 5 mM Mg(OAc)2, and 1% digitonin by the addition of 50 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 35 µl was then removed and mixed with a mixing solution (6 µl; 0.5% (w/v) digitonin in 0.15 M NaCl 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 RNAs—A labeling reaction (490 µl) was diluted to 300 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 NaAc, 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% (w/v) Triton X-100, and 0.1% SDS. This mixture was split into three, 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
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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 encased 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 chicken liver. These membranes 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 [35S]methionine, tRNA, ATP, GTP, and an energy regenerating system. Under such conditions, we observed the incorporation of [35S]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 vitro prior to the disruption of the cells. The incorporation of [35S]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 detergent, the proteins were completely digested (data not shown). Additionally, when a labeling reaction was subjected to an equivalent flotation, 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 50%, 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 luminal 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 carboxy-terminal ends were at the peptide transferase site of the ribosome.

The results of a representative experiment are shown in Fig. 1. At each detergent concentration a large fraction of carboxy-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-aa 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 carboxy-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 carboxy-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-aa 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 luminal 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-
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Permeabilization with 0.016% Sarkosyl. After protease treatment for different times, CTABr-precipitable peptides were analyzed on SDS-polyacrylamide gels (Fig. 2A). 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 RMRS but not free ribosomes, the 70-aa band was recovered exclusively in the pellet fraction (Fig. 2B, lane 3). In contrast, most of the 40-aa band (presumed to be protected by the ribosome) was recovered in the supernatant (Fig. 2B, 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 were less than 15 kilodaltons, the approximate molecular mass of the hemoglobin chains (Fig. 2C, 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. 2C, lane 2). This result supports the identification of the 40-aa band observed after proteolysis of the permeabilized 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 luminal 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 RMRS 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. 3B). The distribution of nascent chains within the gradient was similar to that of reticulocyte polysomes (Fig. 3D). In the absence of detergent, the nascent chains were recovered in the bottom fraction of the gradient (Fig. 3A). 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. 3C). Note that proteolysis of fraction 2 (containing the monosome peak; see Fig. 3D) 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 in a marked loss of the 70-aa band.
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. 4C), or when the micrococcal nuclease was added after the addition of the calcium chelator EGTA (Fig. 4D), indicating that the observed shift resulted from nuclease 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. 4B, fraction 2) were still protected from protease digestion to yield the 70-aa band (Fig. 4B, 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 Digestion 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. 5A, lanes 5, 6, 8, and 9), identical to that seen under the solubilization conditions using Emulgen-913 at low KOAc concentrations (Fig. 5A, 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. 5B, 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. 5B, 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. 5B, 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).
In contrast, after extraction with cholate only the components of the glycosyltransferase complex remained in significant quantities (Fig. 5B, 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...
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**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 I, 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% chol 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 mM 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 81, 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, Sec61β; J, Sec61γ. 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. 5A. 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% chol 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.

**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
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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. 5A. 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. 2C, 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. 8A). 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 luminal 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 luminal 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 (Gorlich 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 susceptible 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. 2A) the membrane is perforated, but not extracted (represented by the holes in the membrane in Fig. 8B), limiting access of proteases to the luminal 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 luminal 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. 8C). Cuts on the luminal 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; Gorlich 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 luminal chaperones. These bands are most pronounced with amylase (Fig. 7, lane 2).

Previous measurements of the length of the nascent chain
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 luminal face of the ER membrane the glycosylation site would have to cross the membrane completely. This makes their 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 peptide transferase 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 peptide transferase 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 peptide transferase 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 luminal 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 purmerycin-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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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 40S band is generated. This result is shown in Fig. 2C. 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 40S amino acid fragments, but a few 70S amino acid fragments are present. This result is shown in Fig. 2A. 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, 40S 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 70S 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 70S amino acid carboxyl-terminal fragment is generated. This result is shown in Fig. 5A.
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