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Tryptic dissection and reconstitution of translocation activity for nascent presecretory proteins across microsomal membranes

(prolactin mRNA/translation in reticulocyte lysate/signal and ribosome receptor domain/transport domain/signal peptidase)

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ABSTRACT **The ability of microsomal membranes to translocate nascent presecretory proteins across their lipid bilayer into the intravesicular space was investigated by using trypsin as a proteolytic probe. We found that under defined conditions trypsin is able to dissect the translocation activity of microsomal membranes into components that can be separated into two fractions, one soluble and the other membrane bound. The trypsinized membrane fraction has lost its translocation activity. Addition of the trypsin-generated soluble fraction, however, results in reconstitution of translocation activity. These results are compatible with the notion proposed in the signal hypothesis that the translocation activity of the microsomal membrane resides in transmembrane protein(s). We propose that trypsin effects solubilization from the membrane of cytosol-exposed domain(s) involved in recognition of the signal sequence or ribosome or both, leaving behind membrane-integrated domain(s) that provide the environment for the passage of the nascent chain across the membrane. Signal peptidase activity was unaffected by trypsinization of microsomal vesicles consistent with a localization of the active site of this enzyme on the cisternal side of the vesicles.**

Most of the secretory proteins (1) and several membrane proteins (2–4) so far investigated are synthesized as preproteins containing an amino-terminal extension (signal peptide) when their mRNAs are translated in a cell-free system in the absence of microsomal membranes. The signal peptide portion of the nascent chain has been proposed (5) to contain the necessary information to direct the translating ribosome to form a functional complex with receptor(s) of the rough endoplasmic reticulum membrane and thereby to provide the conditions for a unidirectional translocation of the nascent peptide chain across the membrane into the intracisternal space. Before the nascent chain is completed, the signal peptide is removed by signal peptidase, an endoprotease located on the cisternal side of the membrane (6), except for chicken ovalbumin (7), which is proposed to contain an uncleavable signal sequence (8).

Little is known about the recognition–transport machinery in the membrane or the ribosomal components interacting with it. Two membrane proteins, restricted in their location to rough endoplasmic reticulum, have been proposed (9) to function as ribosome receptors, primarily on the basis of their physical association with membrane-bound ribosomes. Furthermore, it has been reported (10) that microsomal membranes can be largely depleted of their translocation activity when extracted with a solution of relatively high ionic strength (500 mM KCl) and that activity can be regenerated when the salt extract is added back to the depleted membranes.

We present a way to dissect the translocation activity in the microsomal membrane by limited trypsinization into functionally competent components which were separated by centrifugation. Translocation activity of the membrane can be regenerated by recombination of the fractions.

METHODS

Preparation of Stripped Microsomal Membranes. Rough microsomal membranes were prepared from freshly excised dog pancreas (11) as described (12), with the following exceptions: all buffers contained 1 mM dithiothreitol and the rough microsomes were collected by centrifugation for 2.5 hr at 140,000 × g_{av}. All procedures were done at 2–4°C. The resulting pellet, consisting of rough microsomes, was resuspended by manual homogenization in a Potter–Elvehjem homogenizer in solution A (250 mM sucrose/50 mM triethanolamine·HCl, pH 7.5/1 mM dithiothreitol) to a concentration of 50 A_{280} units/ml. [All absorbance determinations of membrane solutions were done in 2% sodium dodecyl sulfate ($NaDodSO_4$).]

Membrane-bound polysomes and peripheral membrane proteins were removed by extracting rough microsomes first with EDTA and then with high salt. A solution of rough microsomes was incubated for 2 min at 2–4°C with 1 vol of solution B (like solution A, except that it was 46 mM in EDTA); 20-ml aliquots were transferred into 45-ml centrifuge tubes underlayered with 15 ml of solution C (like solution A, except that it contained 0.5 M sucrose) and centrifuged for 1 hr at 140,000 × g_{av}. The resulting pellet of EDTA-stripped microsomal membranes was resuspended in solution A to a concentration of 50 A_{280} units/ml (yield after EDTA stripping was ≈30–40% of the A_{280} of the original rough microsomes). Ice-cold 2.0 M KCl was slowly added to a final concentration of 0.5 M. The membranes were centrifuged for 1 hr at 140,000 × g_{av}. Excess KCl was removed from the sedimented membranes by resuspending them in solution A and centrifuging again through a cushion of 15 ml of solution C for 1 hr at 140,000 × g_{av}. The resulting pellet was resuspended in solution A to a concentration of 40 A_{280} units/ml (yield after KCl stripping was ≈60–70% of the A_{280} of the EDTA-stripped microsomes). Aliquots were frozen in liquid N_2 and stored at −80°C. This preparation is called stripped microsomal membranes.

Limited Trypsin Digestion of Stripped Microsomal Membranes. All operations were performed at 1–4°C. Volumes for digestion were 15–500 μl. For a typical trypsin treatment, 78 μl of stripped microsomal membranes was incubated for 30 min on ice with 2 μl of a trypsin solution of the appropriate concentration so that the final concentration of trypsin was 5–600 μg/ml. The trypsin solution was freshly prepared by dilution of a stock solution (10 mg of trypsin per ml in 10 mM triethanolamine·HCl, pH 7.5) kept in small aliquots at −80°C.

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

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Trypsin digestion was terminated by addition of 10 μl of Trasylol. Aliquots of this mixture were assayed directly in the cell-free protein-synthesizing system (see Fig. 1) or fractionated from aliquots by high-speed centrifugation into a membrane and a supernatant fraction.

To prepare the membrane fraction, we layered a 40-μl aliquot of the digestion mixture over a discontinuous sucrose gradient consisting of 50 μl of 0.5 M sucrose over 25 μl of 2.0 M sucrose in 175-μl nitrocellulose tubes; both sucrose solutions also contained 10 mM triethanolamine·HCl, pH 7.5/1 mM dithiothreitol/1% Trasylol. Centrifugation for 5 min at 30 lb./inch^2 (1 lb./inch^2 = 6.9 kPa), generating $\approx$100,000 × g_{av}, was in a Beckman Airfuge kept in a cold room (4–8°C). The membranes banded neatly at the 2.0 M/0.5 M sucrose interface and, by a Hamilton syringe, could be quantitatively recovered (after removal of the 2.0 M sucrose layer). The original volume of 40 μl was restored by addition of 20 μl of 10 mM triethanolamine·HCl, pH 7.5/1 mM dithiothreitol. Membranes prepared this way will be abbreviated Mb(x), in which x indicates the concentration of trypsin in μg/ml of digestion mixture.

To prepare the supernatant fraction, we centrifuged the remaining 45-μl aliquot for 10 min at 30 lb./inch^2 in the Airfuge. The supernatant was withdrawn with a Hamilton syringe and recentrifuged for 10 min at 30 lb./inch^2. Supernatants prepared this way will be abbreviated Sup(x), in which x indicates the concentration of trypsin in μg/ml of digestion mixture. One equivalent of Mb(x) or Sup(x) is arbitrarily defined as the amount of material that is derived from the equivalent (0.035 A_{280} unit) of stripped microsomal membranes. One equivalent of either Mb(x) or Sup(x) is present in a volume of 1 μl.

Cell-Free Protein Synthesis. Rabbit reticulocyte lysate was treated with staphylococcal nuclease (12). Translation of bovine anterior pituitary RNA (13) (0.05 A_{260} unit) in a 25-μl assay was as described (12, 14) except that components such as ATP, GTP, phosphocreatine, creatine phosphokinase, 15 μCi (1 Ci = 3.7 × 10^{10} Bq) of [^{35}S]methionine, and 19 unlabeled amino acids were lyophilized as a mixture and directly taken up in lysate; this procedure saved volume for the titration of other components to be done within the limits of a 25-μl incubation volume. Incubation was at 30°C for 60 min.

Posttranslational Assays. After completion of translation, 10-μl aliquots were cooled on ice; $CaCl_2$ was added to a final concentration of 1 mM, followed by addition of 0.1 vol of a solution of trypsin and chymotrypsin (3 mg of each per ml) and incubation in an ice bath for 90 min. The reaction was terminated by addition of 5 μl of Trasylol. Posttranslational processing to determine signal peptidase activity of different membrane fractions was done as described (6).

Polyacrylamide Gel Electrophoresis. Procedures for polyacrylamide slab gel electrophoresis in $NaDodSO_4$ and subsequent autoradiography of dried slab gels were as described (5, 13). To determine the radioactivity in gel regions that were located by autoradiography, we excised bands and rehydrated them for 1 hr in 100 μl of H_2O. The rehydrated slices were then incubated in 1 ml of NCS tissue solubilizer/H_2O, 9:1 (vol/vol) for 6 hr at 50°C. After cooling, 50 μl of glacial acetic acid was added and radioactivity was measured in 10 ml of Aquasol in a liquid scintillation counter.

Materials. [^{35}S]Methionine (580 Ci/mmol) and NCS tissue solubilizer were from Amersham/Searle; trypsin (from bovine pancreas, 33 units/mg) was from Boehringer Mannheim; Trasylol (10,000 units/ml) was from FBA Pharmaceuticals (New York, NY); Aquasol was from New England Nuclear.

RESULTS

To investigate the nature of the translocation activity that allows the nascent chains of secretory proteins to be transferred into the cisternae of the rough endoplasmic reticulum, we sought limited proteolysis of microsomal vesicles as a means to dissect this activity into functional components. The rationale was that the translocation activity might be represented by transmembrane protein(s) (5) that should contain cytosol-exposed domain(s) that are readily accessible to proteolytic enzymes. On the premise that these domains are globular in nature, conditions were sought that would permit their proteolytic severance in a form sufficiently intact so that their readdition to the proteolytically depleted and inactivated membranes would restore translocation activity.

Translocation activity of microsomal membranes can be assayed in cell-free protein-synthesizing systems (12, 15, 16). The activity is tightly coupled to translation—i.e., it is expressed only *during* but not after synthesis of the polypeptide chain; moreover, it is accompanied by cleavage ("processing") of the signal peptide (13, 15, 17). We used a nuclease-treated rabbit reticulocyte lysate system programmed with bovine pituitary RNA (12, 13). In the absence of microsomal membranes there is synthesis of a larger molecule, preprolactin, whereas in the presence of adequate amounts of membranes there is synthesis primarily of "processed" prolactin which is similar, if not identical, to mature prolactin (13). Among the two forms of prolactin molecules that are synthesized in the presence of microsomal membranes, only the "processed" prolactin, but not the unprocessed preprolactin molecules, were segregated within the microsomal vesicles (12, 13). The absence of detectable amounts of segregated preprolactin molecules indicated that few if any of the *in vitro* translocated molecules escape processing by signal peptidase, which is situated on the cisternal side of the microsomal vesicles (6). Because of the location and the efficiency of signal peptidase, we decided to use the readily measurable ratio of processed prolactin to preprolactin as a quantitative indicator for the translocation activity of microsomal membranes.

In order to ensure that cytosol-exposed domain(s) of the translocation activity would be optimally accessible to the proteolytic probe, we subjected dog pancreas rough microsomes to extraction, first by EDTA and subsequently by high salt. These treatments should "strip" the microsomal membranes of their bound polysomes and the bulk of their extraneous and peripheral membrane proteins. The effect of these treatments on the activity of the microsomal membrane to translocate prolactin is shown in Fig. 1. Neither EDTA extraction (lane 4) nor the subsequent KCl extraction (lane 5) abolished the translocation activity of the microsomal membranes, as is evident from their ability to effect synthesis of processed prolactin. Because of comigration of preprolactin (lane 1) with a major product synthesized by mRNA present in native pancreas rough microsomes (lane 2) and, furthermore, because of competition between prolactin mRNA and the pancreatic mRNAs that are present in native rough microsomes, it is impossible to quantitate the translocation activity (by the ratio of processed prolactin to preprolactin) of native rough microsomes (lane 3) before extraction with EDTA or KCl or both. However, because of the striking extraction of pancreatic mRNA by EDTA, and even more so by high salt (compare banding patterns in lanes 2–6), it is possible to determine (data not shown) the translocation activity of the EDTA-extracted microsomes (lane 4) and of the EDTA/salt-extracted microsomes (lane 5). A comparison of the activities in these two membrane fractions (lanes 4 and 5) shows that high salt extraction *per se* does not result in a significant loss of translocation activity. These data then are at variance

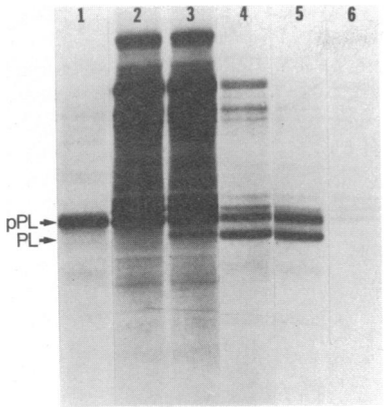

FIG. 1. Effect of EDTA and high-salt treatment on translocation activity of microsomal membranes. Bovine pituitary RNA was translated in the staphylococcal nuclease-treated rabbit reticulocyte lysate in the absence (lane 1) or presence (lanes 3–5) of rough microsomal membranes that were untreated (lane 3), EDTA-stripped (lane 4), or EDTA/KCl-stripped (lane 5). Lanes 2 and 6, translation products of rough microsomal membranes and EDTA/KCl-stripped membranes, respectively, in the absence of exogenous pituitary RNA. Membranes were present at a concentration of 0.15 A_{280} unit (rough microsomes), 0.05 A_{280} unit (EDTA-stripped microsomes), and 0.03 A_{280} unit (EDTA/KCl-stripped microsomes) in 25-μl incubation volume. During membrane preparation, 0.15 A_{280} unit of rough microsomes gave rise to 0.05 A_{280} unit of EDTA-stripped microsomes, which in turn gave rise to 0.03 A_{280} unit of EDTA/KCl-stripped microsomes, so that in fact equivalent amounts of membranes with respect to the original rough microsomes were added in each case. Analysis was by polyacrylamide gel electrophoresis in $NaDodSO_4$ and subsequent autoradiography. pPL, Preprolactin; PL, prolactin.

with those reported by others (10) that extraction of rough microsomes by high salt (500 mM KCl) leads to a complete loss of translocation activity.

With a highly extracted, but translocation-active, microsomal vesicle fraction at hand we proceeded to determine the range of trypsin concentrations within which a dissection of the translocation activity into functionally competent components might be possible. Stripped microsomal membranes were therefore incubated in an ice bath for 30 min in either the absence (control) or presence of various concentrations of trypsin. After termination of proteolysis by addition of a large excess of Trasylol (a noncovalent trypsin inhibitor), aliquots of the incubation mixture were assayed directly in the cell-free system programmed with prolactin mRNA. The results in Fig. 2 show that preincubation of stripped microsomes with increasing concentrations of trypsin resulted in a striking decrease of the ratio of processed prolactin to preprolactin (lanes 3–5), indicating progressive inactivation of the translocation activity. Appropriate controls demonstrated that trypsin is completely inhibited by the amount of Trasylol used and that neither Trasylol nor the trypsin–Trasylol complex interfered with protein synthesis or translocation or both (data not shown).

If a gradual inactivation of the translocation activity by trypsin is at all indicative of functionally competent components being generated as intermediates in the digestion process, it is clear that these will undergo rapid inactivation at high concentrations of trypsin (Fig. 2, lane 5). However, it is conceivable that the generated fragments differ in their susceptibility to further degradation and ultimate inactivation. Thus, a cytosol-exposed domain could be anticipated to be more trypsin sensitive than a membrane-integrated domain, the latter being more resistant largely by virtue of its inaccessibility to trypsin. Based, therefore, on the premise that there might be differential sensitivity of these two domains to trypsin, we prepared a

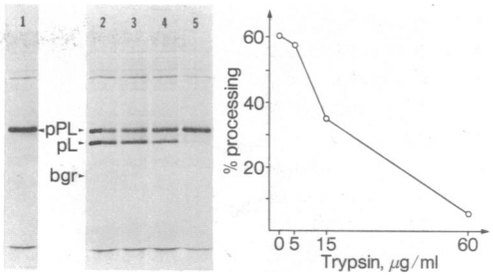

FIG. 2. Progressive inactivation of translocation activity of stripped microsomal membranes by increasing concentrations of trypsin. Bovine pituitary RNA was translated in the cell-free system in the absence (lane 1) or presence (lanes 2–5) of stripped microsomal membranes that had been preincubated in the absence (lane 2) or presence of trypsin: 5 μg/ml (lane 3); 15 μg/ml (lane 4); and 60 μg/ml (lane 5). Preincubated membranes (not subfractionated) were present at a concentration of 0.035 A_{280} unit/25 μl of incubation volume. Analysis and designations are as for Fig. 1. For quantitation, individual pPL and PL bands were sliced from the dried and autoradiographed gels and radioactivity was determined. A background value was obtained by determining the radioactivity in an approximately equal-sized slice from an indicated area (bgr) of each lane; this was subtracted from the radioactivity in the corresponding pPL or PL bands. For example, the amount of radioactivity in lane 4 was: pPL, 8584 cpm; PL, 6089 cpm; and bgr, 3121 cpm. (*Right*) Percent processing is defined as (cpm PL × 100)/(cpm pPL + cpm PL).

membrane fraction—referred to as Mb(60)—that was obtained after resedimentation of stripped microsomal vesicles incubated at a *high* trypsin concentration (60 μg/ml). Assayed by itself, this Mb(60) fraction would be expected to be translocation inactive. It might be able, however, to retain the capacity to interact with its severed cytosol-exposed domain which might be present in a supernatant fraction that was generated from stripped microsomal membranes after incubation at *low* trypsin concentrations (such as 5 or 15 μg/ml).

These expectations were borne out by the data in Fig. 3. One equivalent of an Mb(60) fraction was translocation inactive when assayed by itself (lane 1), but activity could be partially restored upon addition of one equivalent of supernatant fraction that was prepared at 5 μg (lane 3) or 15 μg (lane 4) of trypsin per ml. Furthermore, consistent with the notion that the cytosol-exposed domain undergoes rapid degradation, a Sup(5)

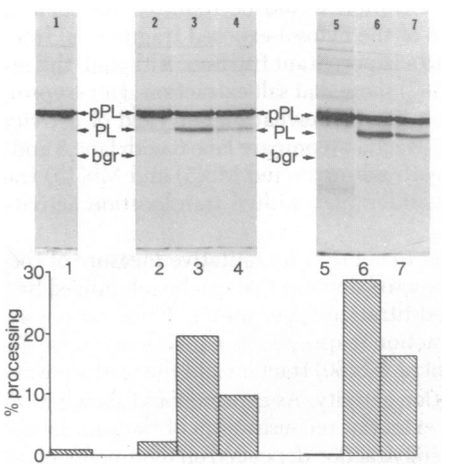

FIG. 3. Reconstitution of translocation activity. One equivalent of Mb(60) was present in 25 μl of the cell-free protein-synthesizing system either alone (lane 1) or together with one equivalent of supernatant: Sup(0), lane 2; Sup(5), lane 3; Sup(15), lane 4. Lanes 5–7 are as lanes 2–4, respectively, except that the digestion mixture was adjusted to 500 mM KCl before the supernatant was prepared. Percent processing (*Lower*) was determined as in Fig. 2.

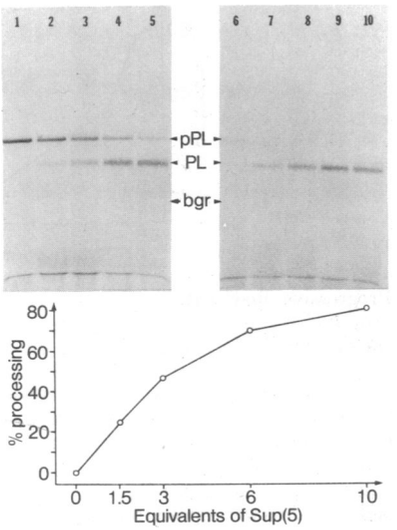

FIG. 4. Progressive reconstitution of translocation activity by titration of one equivalent of Mb(60) with increasing equivalents of Sup(5), and resistance of translocated prolactin chains to proteolysis. One equivalent of Mb(60) was present in 25 μl of the cell-free protein-synthesizing system together with: 0, 1.5, 3, 6, or 10 equivalents of Sup(5) (lanes 1–5, respectively). Aliquots corresponding exactly to those shown in lanes 1–5 were subjected to posttranslational proteolysis with trypsin and chymotrypsin (lanes 6–10, respectively). Percent processing (*Lower*) was determined as in Fig. 2. Exactly the same results as shown in lane 1 were obtained when 10 equivalents of Sup(0) were assayed in the absence of Mb(60); this eliminated the possibility that Sup(5) contained a small amount of translocation-active vesicles that may not have sedimented.

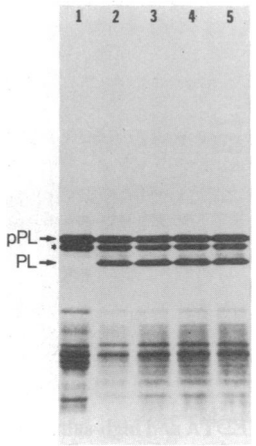

FIG. 5. Trypsin digestion of stripped microsomal membranes does not inactivate signal peptidase. Membrane fractions (see *Methods*) prepared after incubation of stripped microsomal membranes in the absence or presence of increasing concentrations of trypsin, were solubilized with sodium deoxycholate and assayed for signal peptidase activity, by using [^{35}S]Met-labeled preprolactin (prepared by *in vitro* translation of pituitary RNA in the wheat germ cell-free system) as substrate as described (6). The products were analyzed by polyacrylamide gel electrophoresis in $NaDodSO_4$ followed by radioautography of the dried gel. Lane 1, preprolactin incubated without solubilized membrane; lanes 2, 3, 4, and 5, posttranslational processing of preprolactin by sodium deoxycholate-solubilized Mb(0), Mb(5), Mb(15), and Mb(60), respectively. Asterisk (*), bovine pregrowth hormone. Although pregrowth hormone is also cleaved in the posttranslational signal peptidase assay (6), the processed growth hormone band is faint and not clearly visible in this figure.

fraction was more active than an equivalent Sup(15) fraction. A control (lane 2) shows that the addition to an Mb(60) fraction of a Sup(0) fraction [prepared exactly like Sup(5) or Sup(15), except that preincubation of stripped microsomal membranes was in the absence of trypsin] does not result in reconstitution of translocation activity. This control rules out the trivial possibility that the activity present in Sup(5) or Sup(15) is due to a trypsin-independent extraction.

The possibility existed that at low trypsin concentration much of the translocation activity was actually cleaved, but that high salt concentration would be required for solubilization and severance of the cytosol-exposed fragment(s) from the membrane into a supernatant fraction. Although the results in Fig. 3 (lanes 5–7) show that salt extraction after trypsin incubation does result in a somewhat higher yield of activity in the supernatant fraction (compare lane 6 with lane 3 and lane 7 with lane 4), both salt-extracted Mb(5) and Mb(15) fractions contained considerable residual translocation activity (data not shown).

In order to obtain a quantitative measure of the amount of cytosol-exposed domain that can be solubilized by trypsin, we performed titration experiments. Thus, various amounts of a Sup(5) fraction (expressed as equivalents) were added to one equivalent of Mb(60) fraction and the mixture was assayed for translocation activity. As expected and shown in Fig. 4 (lanes 1–5), the extent of reconstitution of the translocation activity of the Mb(60) fraction depended on the amount of added Sup(5) fraction. Approximately five equivalents of Sup(5) fraction reconstituted all of the translocation activity that was originally present in untreated (i.e., nontrypsinized) stripped microsomal membranes [notice 60% processing by one equivalent of untreated vesicles (Fig. 2, *right*) compared to 60% processing by addition of about five equivalents of Sup(5) to one equivalent of Mb(60) (Fig. 4, *lower*)]. These quantitative data indicate that ≈20% of the cytosol-exposed domain was solubilized in the form of reconstitution-competent fragment(s) at 5 μg of trypsin per ml. Furthermore, our observation that the reconstituted translocation activity could be increased over that present in untreated vesicles [compare 80% processing after reconstitution by 10 equivalents of Sup(5) (Fig. 4, *lower*) with 60% processing in original, untreated vesicles (Fig. 2, *right*)] indicated that nontrypsinized microsomal vesicles had already lost some of their cytosol-exposed domain (most likely by endogenous proteolysis) but retained their membrane-integrated domain in a reconstitution-competent form. Endogenous proteolysis, particularly during cell fractionation for the preparation of rough microsomes, and the subsequent loss of cytosol-exposed domain could therefore account for the variability in the translocation activity which we have encountered among various batches of membrane preparations. However, other, still undefined, variables may well affect the translocation activity of microsomal membranes when assayed *in vitro*.

To verify the validity of using the ratio of processed prolactin to preprolactin as a quantitative measure for the translocation activity of microsomal membranes, even when reconstituted from components, we subjected aliquots of each of the various assays shown in lanes 1–5 of Fig. 4 to posttranslational proteolysis. All preprolactin molecules were degraded (Fig. 4, lanes 6–10), consistent with the notion that they were not translocated into the intravesicular space. In contrast, most if not all processed prolactin molecules were protected from proteolysis, consistent with the notion that they had been translocated.

The results shown in Figs. 3 and 4 suggested that the active site of signal peptidase had apparently not been affected by proteolysis used for preparation of the Mb(60) fraction. This conclusion was corroborated by determining signal peptidase activity in a translocation-independent assay (6). For this assay, stripped microsomal membranes that were preincubated in the

absence or presence of various trypsin concentrations were treated subsequently with sodium deoxycholate to solubilize signal peptidase. The solubilized membranes were assayed with preprolactin as a substrate (6). The results (Fig. 5) demonstrate that the activity of signal peptidase was not affected by trypsinization of microsomal vesicles, adding further evidence to that already provided (6) that the enzyme—or at least its active site—is located on the cisternal side of the vesicle.

DISCUSSION

Our results are most readily discussed in view of the notion [proposed in the signal hypothesis (5)] that the translocation activity of the microsomal membrane is represented by transmembrane protein(s) that possess at least two domains: a cytosol-exposed domain, which may function as a recognition site for the signal sequence or the ribosome or both, and a membrane-integrated domain, which may provide the hydrophilic environment for the actual passage of the nascent chain across the lipid bilayer. By using trypsin as a dissecting tool, we have demonstrated that the translocation activity of stripped microsomal membrane vesicles can indeed be cleaved and subsequently fractionated by high-speed centrifugation into two fractions: a supernatant fraction in part composed of trypsin-solubilized fragments, which could be derived largely from the cytosol-exposed portions of integral membrane proteins (most peripheral membrane proteins were presumably removed by EDTA treatment and a high salt wash before trypsinization of the vesicles), and a pellet fraction, consisting of the trypsinized membrane vesicles. Under certain digestion conditions (30-min incubation at 0°C with 60 μg of trypsin per ml), these trypsinized vesicles [Mb(60)] have lost all of their translocation activity. Most strikingly, however, translocation activity of Mb(60) could be restored by addition of a high-speed supernatant fraction, Sup(5), which was generated by a 30-min incubation of stripped microsomal membranes at 0°C with 5 μg of trypsin per ml. In view of the presumed transmembrane disposition of the translocation activity, the Mb(60) vesicles would have lost activity because of severance of the cytosol-exposed recognition domain, but would have retained the membrane-integrated transport domain which can be restored to function in cotranslational translocation when supplemented with solubilized recognition domain. Thus, the tryptic cleavage of these two domains must have proceeded in such a way that both of them retain sufficient information for mutual interaction. The capacity of the putative transport domain to act as a functional acceptor for the recognition domain is impressively resistant to trypsin digestion; membrane vesicles that were trypsinized as described above, but with 600 μg of trypsin per ml, still retained 70% of their acceptor activity (data not shown). This relative trypsin resistance was important since it permitted us to prepare trypsinized membranes that were by themselves free of any "background" translocation activity but were able to function as acceptors for the putative recognition domain.

In contrast to the relative trypsin resistance of the transport domain, the putative recognition domain is rapidly inactivated by trypsin. Therefore, its preparation as a functionally competent entity (e.g., able to interact with the transport domain and thereby to restore translocation activity) is preferably done at low trypsin concentrations, albeit at the cost of a reduced yield. From the data in Fig. 4, we estimate that ≈20% of the recognition domain can be dissected from the membrane in a functionally competent form. Because of the relative abundance of recognition domain in the microsomal membrane, this low-yield, tryptic product should nevertheless provide a good starting material for purification.

Our proposal that the translocation activity of microsomal membranes is represented by integral transmembrane protein(s) is in apparent conflict with evidence (10) that the translocation activity, or at least part of it, resides in membrane-associated protein(s) that can be extracted with high salt concentrations and by this criterion, therefore, would constitute peripheral rather than integral membrane protein(s). This conflict, however, can be readily resolved if one assumes that the reported (10) salt extractability of translocation activity is a result of proteolysis—e.g., due to the action of endogenous proteases during cell fractionation. Proteolysis by these endogenous proteases could be less extensive than our intentional one by trypsin and, therefore, high salt extraction may be required to dislodge the cleaved recognition domain from the membrane. Definitive evidence for or against this interpretation, however, has to await purification of the salt-extracted and the trypsin-extracted activities and biochemical comparison with each other and the "holoprotein(s)" from which the trypsin factor(s) and presumably also the salt factor(s) were derived.

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