Nascent Secretory Chain Binding and Translocation Are Distinct Processes: Differentiation by Chemical Alkylation

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Abstract. We have investigated the effects of chemical alkylation of microsomal membranes on nascent chain binding and translocation. Assays were conducted using either full-length or truncated preprolactin transcripts in combination with a reconstituted membrane system consisting of proteolyzed rough microsomes and the cytoplasmic domain of the signal recognition particle receptor. Treatment of rough microsomes with *N*-ethylmaleimide was observed to inhibit preprolactin processing at a site other than the signal recognition particle or the signal recognition particle receptor. As formation of a translocation competent junction between the ribosome/nascent chain complex and the membrane has recently been demonstrated to require GTP (Connolly, T., and R. Gilmore. *J. Cell Biol.* 1986. 103:2253–2261), the effects of membrane alkylation on this parameter were assessed. *N*-ethylmaleimide treatment did not inhibit nascent chain targeting or GTP-dependent signal sequence insertion. Translocation of the targeted and inserted nascent chain was, however, blocked. These data indicate (*a*) that the process of nascent chain translocation is distinct from targeting and signal sequence insertion, and (*b*) translocation of the peptide chain across the membrane is mediated by an *N*-ethylmaleimide–sensitive membrane protein component(s). To further substantiate the observation that nascent chain targeting and signal sequence insertion can be distinguished from translocation, the temperature dependencies of the two phenomena were compared. Signal sequence insertion occurred at low temperatures (4°C) and was maximal between 10 and 15°C. Translocation was only observed at higher temperatures and was maximal between 25 and 30°C.

COMMITMENT of an appropriate protein precursor to the secretory pathway is mediated through specific recognition and compartmentalization events. Recognition of nascent secretory chains occurs most commonly through the interaction of the signal recognition particle (SRP),[1] an 11-S ribonucleoprotein, with the amino-terminal signal sequence of the nascent chain (26–28). Signal sequences, although lacking in a conserved primary sequence, do have in common distinct physical characteristics, with the most prominent being a central hydrophobic core of at least six amino acid residues (23). Furthermore, they have been demonstrated to contain the information necessary for the initiation of processing (18). Subsequent to the recognition event, the ribosome/SRP/nascent chain complex is targeted to the RER, a process which is dependent upon an integral RER membrane protein termed the SRP receptor, or docking protein (9, 10, 17, 22). Transfer of the peptide chain into the lumen of the RER then serves as the primary step in a series of cellular processing events leading, ultimately, to secretion of the protein into the extracellular environment.

The process of nascent chain transfer across the membrane has been hypothesized to occur by a protein-independent pathway, with the free energy for translocation being provided either through the formation of an energetically favorable secondary protein structure within the lipid bilayer (7) or through the free energy differences arising from direct transfer of the individual amino acid residues into the bilayer (24). Interaction of the completed polypeptide chain with the lipid bilayer, leading to spontaneous insertion of the precursor into the membrane, has also been postulated as a potential mechanism for protein translocation (29). An earlier, alternative hypothesis predicts that nascent chain transfer occurs through a proteinaceous pore (4, 8, 19, 21). In the latter postulate, peptide transfer is mediated through an aqueous environment, thus freeing the translocation event from the thermodynamic barriers presented by the movement of charged amino acid side chains through the lipid bilayer.

To date, the molecular mechanism of protein transfer across the RER membrane remains undefined, although recent evidence indicates that ribonucleotides as well as protein components of the RER membrane are required. Thus, in studies of the ribonucleotide requirement for the binding of elongation-arrested polysomes to the RER membrane, Connolly and Gilmore (6) observed that GTP, or nonhydrolyzable GTP analogues, were necessary for the formation of

1. *Abbreviations used in this paper*: KOAc, potassium acetate; NEM, *N*-ethylmaleimide; PL, prolactin; pPL, preprolactin; RM_{ek}, EDTA and KOAc washed rough microsomes; RM_{ekt}, EDTA and KOAc washed rough microsomes, proteolyzed with 5 μg/mg trypsin; SRP, signal recognition particle.

a translocation-competent ribosome/membrane junction, as assayed by signal peptide cleavage and protease protection after puromycin termination of the nascent chain. In the absence of GTP, nascent preprolactin chains remained accessible to added protease and were not cleaved by signal peptidase (6). Evidence for the involvement of membrane proteins other than the SRP receptor in the regulation of protein translocation is somewhat indirect. It was recently reported that the stable association of nascent, elongation-arrested preprolactin chains with the endoplasmic reticulum membrane could be disrupted in the presence of 4 M urea, an observation consistent with the maintenance of the signal sequence/membrane junction by protein–protein interaction (8). A candidate signal sequence receptor has, in fact, been identified through cross-linking studies (30). In an alternative approach, Hortsch et al. (13) noted that the ability of RER membranes to process IgG κ light chain precursor was blocked by pretreatment of the membrane fraction with *N*-ethylmaleimide (NEM). The function and identity of this activity(s) remains, however, to be determined.

In this study we have assessed the functional consequences of chemical alkylation of RER membranes on nascent chain binding, signal sequence insertion, and nascent chain translocation. As assessed by sensitivity to protease digestion, chemically modified membranes, reconstituted with the cytoplasmic fragment of the SRP receptor, were competent with respect to nascent chain targeting and signal sequence insertion. Under these conditions, however, translocation of the nascent chain into the lumen of the RER was blocked. These results indicate that nascent chain targeting and signal sequence insertion are distinct from chain translocation and implicate the activity of a protein component of the RER membrane in the process of protein translocation.

Materials and Methods

Materials

[^{35}S]Methionine (1,000 Ci/mmol) was from New England Nuclear (Boston, MA). Nuclease-treated rabbit reticulocyte lysate and RNasin, a placental RNase inhibitor, were obtained from Promega Biotec (Madison, WI). Trypsin, elastase, puromycin dihydrochloride, ATP, and GTP were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). T7 RNA polymerase was obtained from United States Biochemical Corp. (Cleveland, OH).

Cell-free Protein Synthesis

Cell-free translations were conducted in a 20-μl reaction volume containing 8 μl of nuclease-treated rabbit reticulocyte lysate, 40 μCi [^{35}S]methionine, 2 U RNasin, and 1 equivalent of rough microsomes, as defined in reference 28. All translations were adjusted to 140 mM KOAc, 2.5 mM $Mg(OAc)_2$, 2 mM DTT. mRNA, coding for bovine preprolactin, was present at a concentration of 300 ng per reaction and was prepared by transcription of the plasmid pGEMBP1 (kindly provided by Dr. Reid Gilmore, University of Massachusetts Medical Center, Worcester, MA) linearized with either Eco RI, to yield a full-length transcript, or with Pvu II, to yield a transcript truncated within codon 87. Unless otherwise stated, translation reactions were conducted for 45 min at 30°C. After incubation, reactions including the truncated transcript were fractionated by treatment with saturated ammonium sulfate, to a final concentration of 66%. Ammonium sulfate precipitates were washed with 5% trichloroacetic acid before analysis by SDS-PAGE. All samples were prepared for SDS-PAGE as described previously (27). Salt exchange of translation reactions was accomplished by chromatography on Sephacryl S-200 columns equilibrated in 140 mM KOAc, 50 mM TEA, pH 7.5, 2.5 mM $Mg(OAc)_2$, 2 mM DTT, as described by Connolly and Gilmore (6).

Transcription reactions were conducted in a final volume of 250 μl containing 30 μg of linearized plasmid DNA, 50 mM Tris/Cl, pH 7.5, 10 mM NaCl, 10 mM DTT, 5 mM $MgCl_2$, 2 mM spermidine, 0.5 mM ATP, GTP, CTP, UTP, 1 U/μl RNasin, and T7 RNA polymerase (200 U). mRNA was purified by phenol/chloroform extraction and ethanol precipitation.

Preparation of Microsomes and SRP Receptor Fragment

Canine pancreas rough microsomes were prepared as described in reference 25 and washed with 0.5 M KOAc and 25 mM EDTA. KOAc/EDTA-washed membranes (RM_{ek}) were subjected to limited proteolysis after resuspension in 0.25 M sucrose, 50 mM TEA, pH 7.5, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM DTT (buffer A) to a concentration of 1 equivalent/μl (25). Trypsin (0.5 mg/ml in buffer A) was added to a final concentration of 5 μg/ml and the microscome suspension was maintained on ice for 60 min. After proteolysis, EGTA and PMSF were added to final concentrations of 20 and 5 mM, respectively. Trypsinized microsomes (RM_{ekt}) were collected by centrifugation through a cushion of 0.5 M sucrose, 50 mM TEA, pH 7.5 for 60 min at 100,000 rpm in the SW-27 rotor (Beckman Instruments Inc., Palo Alto, CA) and resuspended in 0.25 M sucrose, 50 mM TEA, pH 7.5 to a concentration of 1 equivalent/μl. Under these conditions the cytoplasmic domain of the SRP receptor is removed and the microsome fraction is, therefore, translocation incompetent.

The 52-kD cytoplasmic fragment of the SRP receptor was prepared as follows. RM_{ek}, at a concentration of 1 equivalent/μl in buffer A supplemented with 10 U/ml Trasylol, were digested with elastase, at a final concentration of 1 μg/ml, for 60 min on ice. After addition of PMSF to a concentration of 1 mM, microsomes were collected by centrifugation at 4°C for 45 min at 100,000 rpm in the SW-28 rotor. The proteolytic digestion was repeated and the supernatants from the two treatments were combined and applied to a 1.0-ml column of CM-Sephadex equilibrated in 150 mM KOAc, 50 mM Tris/Hepes pH 7.4, 2 mM DTT at a flow rate of 0.5 ml/min. All chromatography steps were performed at 4°C. The column was washed at a flow rate of 0.5 ml/min with five column volumes of equilibration buffer and five column volumes of 250 mM KOAc, 50 mM Tris/Hepes, pH 7.4, 2 mM DTT. The 52-kD SRP receptor fragment was eluted with a step gradient to 425 mM KOAc, 50 mM Tris/Hepes, pH 7.4, 2 mM DTT at a flow rate of 0.2 ml/min. Low molecular mass (<30 kD) protein contaminants were removed by ultrafiltration in a Centricon PM-30 column (Amicon Corp., Danvers, NH).

Unless otherwise noted, RM_{ekt}, at a concentration of 1 equivalent/μl, were alkylated for 30 min at 25°C in the presence of 3 mM NEM. All NEM stocks were prepared fresh in DMSO. The final DMSO concentration was 2%. Control membranes were treated with an equivalent volume of DMSO. Alkylation reactions were quenched by addition of DTT to a final concentration of 50 mM and collected by centrifugation in an airfuge at 100,000 g for 5 min (Beckman Instruments Inc., Palo Alto, CA). Alkylated membranes were resuspended in 0.25 M sucrose, 50 mM TEA, pH 7.5, 2 mM DTT. Control membranes were mock treated and processed similarly.

Analytical Methods

Samples were subjected to SDS-PAGE on either 15% gels (full-length translation product) or 12–20% gradient gels (truncated translation product). Quantitation was performed by direct analysis of the dried gels using an AMBIS Radioanalytic Imaging System (Automated Microbiology Systems, Inc., San Diego, CA) or by densitometric analysis of the autoradiograms with a Pharmacia UltroScan XL laser densitometer (Pharmacia, Piscataway, NJ). Values were corrected for the differences in [^{35}S]methionine content between processed and unprocessed forms of the translation products.

Results

NEM Treatment of Rough Microsomes Inhibits Preprolactin Processing

Chemical alkylating agents have proven useful in determining whether the process of protein translocation is mediated by protein components (13–15). In a recent study, Hortsch et al. (13) noted that treatment of canine pancreas microsomes with high (40 mM) concentrations of NEM inhibited the processing of IgG κ light chain precursor and that NEM was act-

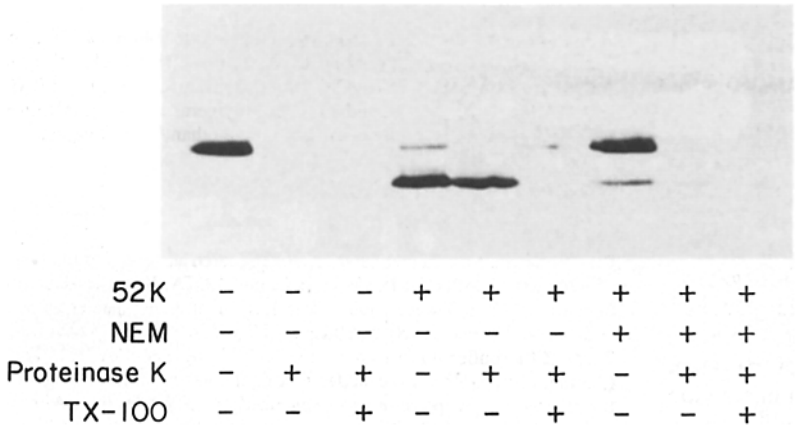

Figure 1. The effects of NEM treatment on preprolactin processing. Preprolactin mRNA was translated in a rabbit reticulocyte lysate system for 60 min in the presence of 1 eq of RM_{ekt}. Where noted, the 52-kD cytoplasmic fragment of the SRP receptor was present at a concentration of 0.75 μg/ml. NEM-treated RM_{ekt} were obtained by incubation of RM_{ekt} with 3 mM NEM for 30 min at 25°C. Protease digestions were performed in the presence of 100 μg/ml proteinase K for 60 min at 0°C. Triton X-100 was present at a final concentration of 1%. pPL (*upper band*) and PL (*lower band*) were resolved by SDS-PAGE on 15% gels and detected by autoradiography. Quantitation was performed by direct radioanalytic scanning.

ing at a site other than SRP or the SRP receptor. We have observed a similar response with respect to preprolactin processing. As shown in Fig. 1, preprolactin (pPL) synthesized in the presence of trypsinized RM_{ek} (RM_{ekt}) was not efficiently translocated and remained sensitive to digestion by added proteinase K in the presence and absence of detergent. Reconstitution of RM_{ekt} with the purified cytoplasmic fragment of the SRP receptor restored targeting and subsequent processing functions (Fig. 1). Under the described conditions, translocation, as assayed by the resistance of the processed form to protease digestion, was very efficient (>90%). pPL processing by NEM-treated RM_{ekt}, reconstituted with the 52-kD SRP receptor fragment, was reduced to ~5% of control (Fig. 1). In the presence of NEM-treated membranes, the unprocessed precursor remained sensitive to protease digestion, precluding the possibility that the precursor was in a translocated, but uncleaved form (Fig. 1). The possibility that the observed effects of NEM arise through an inhibition of signal peptidase can also be excluded as this enzyme has previously been demonstrated to be insensitive to sulphydryl directed alkylating agents (13, 14). In separate experiments, the 52-kD fragment, bound to NEM-treated membranes, retained full activity, that is, the capacity to release elongation arrest in an SRP-supplemented wheat germ translation system (data not shown).

Characterization of the NEM-dependent Inactivation of RM_{ekt}

To further investigate the functional consequences of NEM treatment of RM_{ekt} on secretory protein processing, and to aid in the future identification of the site(s) of NEM action, the inactivation reaction was investigated with respect to the dose dependence, time course, and pH and temperature sensitivity. As shown in Fig. 2 *A*, the inactivation of pPL processing was dependent on the NEM concentration, with half-maximal inhibition being observed at 1.5 mM NEM and maximal inhibition at 3.0 mM NEM. The inactivation reaction was relatively slow, requiring incubation times of ~15 min for half-maximal and 30 min for maximal inhibition (Fig. 2 *B*). It is uncertain whether the relatively slow time course of inactivation is due to low reactivity of the relevant sulphydryl group(s) or if the site(s) of action is in a poorly accessible environment, i.e., within the lipid bilayer. With more hydrophobic *N*-alkylmaleimides, such as *N*-phenylmaleimide, maximal inactivation was observed at a concentration of 1 mM and was more rapid, with maximal inhibition being observed after a 15-min incubation. These data indicate that the sulphydryl group(s) of interest are likely to be within a hydrophobic environment (data not shown).

As *N*-alkylmaleimides preferentially react with the $R\text{-}S^-$, or thiolate, form of the −SH group, this class of chemical reactions is pH sensitive (11). In the experiment depicted in Fig. 2 *C*, RM_{ekt} were treated with either vehicle (control) or NEM at differing pH values, reisolated, and translocation competence assayed at pH 7.4. At pH 6.5 microsomes were relatively resistant to NEM treatment. The sensitivity of RM_{ekt} to inactivation was markedly enhanced at more alkaline pH values and was maximal at pH 7.5. Exposure of RM_{ekt} to an ambient pH of 8.5 resulted in an irreversible loss of translocation competence. Microsomes exposed to pH 8.5 were also observed to be inhibitory to protein synthesis (Fig. 2 *C*; compare total pPL + PL at pH 7.5 vs. pH 8.5).

The NEM-dependent inactivation of secretory protein processing was sensitive to the reaction temperature (Fig. 2 *D*). Half-maximal inhibition was observed at 23°C; maximal inhibition at 37°C. At reaction temperatures below 15°C, RM_{ekt} were relatively insensitive to NEM (data not shown). It would be expected that the NEM-dependent modification of reactive sulphydryl groups would occur readily at temperatures below 15°C. The observed loss of NEM sensitivity at lower temperatures is, therefore, likely to reflect a temperature-dependent decrease in the accessibility of the relevant site(s).

Alkylation Blocks Translocation but not Ribosome/Nascent Chain Targeting or Signal Sequence Binding

Recent experimental evidence indicates that targeting of the ribosome/nascent chain complex promotes the interaction of the signal sequence with an integral membrane protein component(s) of the RER membrane (8, 30). After targeting and signal sequence insertion, the nascent chain is resistant to both extraction and protease digestion. This binding interaction has recently been shown to require GTP and has been proposed to represent the formation of a translocation-competent junction between the RER membrane and the ribosome/nascent chain complex (6). Through use of an 86–amino acid truncated preprolactin precursor (pPL^t), we have

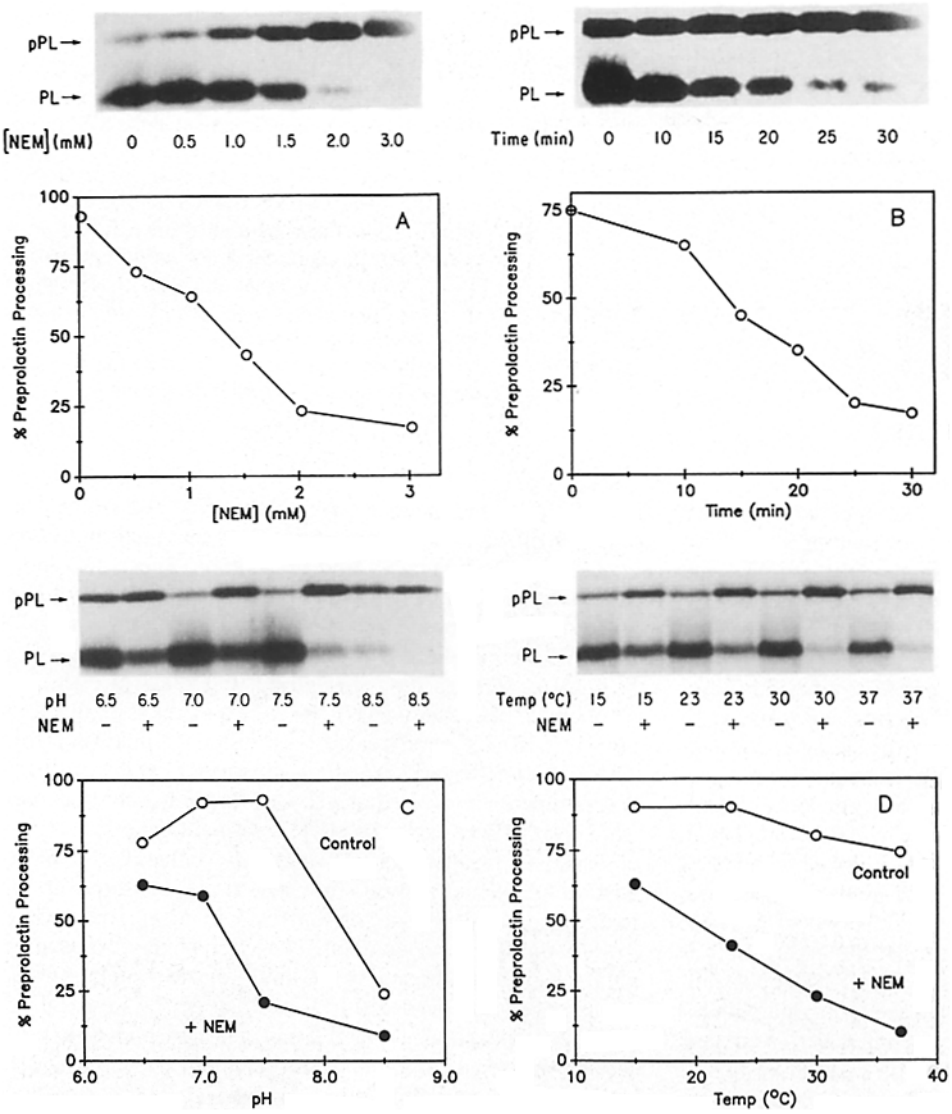

Figure 2. Characterization of the NEM-dependent inhibition of preprolactin processing. Preprolactin mRNA was translated in a rabbit reticulocyte lysate translation system for 60 min in the presence of RM_{ekt} (1 eq) reconstituted with the 52-kD cytoplasmic fragment of the SRP receptor (0.75 μg/ml). (*A*) RM_{ekt} were treated with various concentrations of NEM for 30 min at 25°C, quenched by addition of DTT to 50 mM, collected by centrifugation, and resuspended in 0.25 M sucrose, 50 mM triethanolamine, pH 7.5, 2 mM DTT. (*B*) RM_{ekt} were treated with 3 mM NEM for various time periods and processed as described in the legend to *A*. (*C*) RM_{ekt} were treated in the presence or absence of 3 mM NEM for 30 min at 25°C in 0.20 M sucrose supplemented with 80 mM Pipes (pH 6.5, pH 7.0) or 80 mM Hepes (pH 7.5, 8.5), quenched by addition of DTT to 50 mM, and processed as above. (*D*) RM_{ekt} were treated with 3 mM NEM for 30 min at the described temperatures and processed as described above. Samples were analyzed by SDS-PAGE on 15% gels. Bands were quantitated as described in the legend to Fig. 1.

investigated the effects of alkylation of RM_{ekt} on nascent chain targeting and signal sequence binding. As depicted in Fig. 3 *A*, the ability of GTP to promote the formation of such a ribosome/nascent chain membrane junction is not blocked by alkylation of the membrane fraction. Thus, when a desalted polysome/pPL^t fraction is incubated with both control and alkylated membranes, reconstituted with the 52-kD SRP receptor fragment, GTP was found to promote an increase in protease resistance of the nascent chain. In the absence of GTP, nascent pPL^t chains are readily digested by added protease, with the resulting appearance of a faint limit digestion product. This protease-derived fragment presumably represents the portion of the nascent chain present in the ribosome. We have also observed that nascent pPL^t chains, bound to either control or NEM-treated RM_{ekt}, are resistant to extraction with EDTA, further evidence that alkylation does not block nascent chain targeting or signal sequence binding (data not shown). Although alkylation of RM_{ekt} does not inhibit the initial targeting reaction, subsequent translocation, as assayed after puromycin termination and signal sequence cleavage, is blocked (Fig. 3 *B*). Alkylation of RM_{ekt} appears, therefore, to inhibit the activity of the membrane component(s) responsible for transmembrane peptide chain transfer. These data indicate that the phenomenon of GTP-dependent nascent chain binding is likely to be distinct from peptide chain transfer. In the experiment depicted in Fig. 3 *B*, the ribosome/pPL^t membrane complex was formed cotranslationally and translocation, after puromycin termination, assayed at 30°C. Under these conditions partial pPL^t processing was observed in the absence of puromycin. Puromycin-independent processing of pPL^t was not observed when reactions were performed at 25°C (data not shown) (6). At present it is uncertain whether the partial processing observed at 30°C reflects the activity of the translocation apparatus or whether there is partial, puromycin-independent, chain termination at more elevated temperatures.

Temperature Dependence of Nascent Chain Binding and Translocation

On the basis of the results presented in Fig. 3 it appears that the molecular events associated with the GTP dependent for-

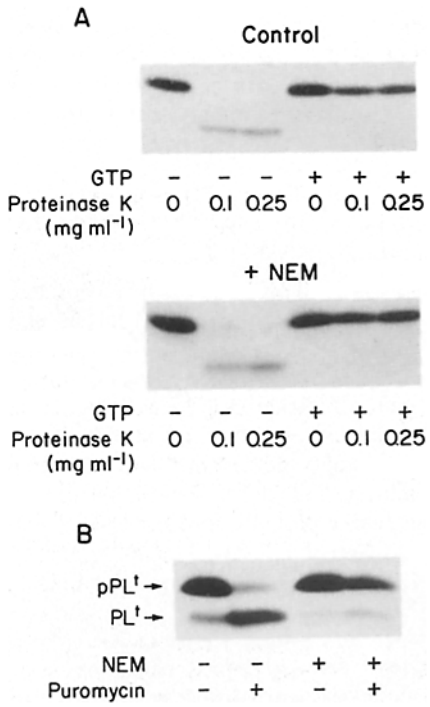

Figure 3. The effects of NEM treatment of RM_{ekt} on GTP dependent nascent chain binding and puromycin dependent translocation. (*A*) mRNA, coding for an 86–amino acid, truncated preprolactin translation product (pPL^t) was translated for 10 min in a rabbit reticulocyte lysate system in a final volume of 100 μl. Translation reactions were adjusted to 0.5 mM emetine, chilled on ice, and salt exchanged on 1.0 ml Sephacryl S-200 columns equilibrated in 140 mM KOAc, 50 mM triethanolamine, 2.5 mM $Mg(OAc)_2$, 1 mM DTT. The void volume was collected, adjusted to 0.5 mM emetine, and incubated for 10 min at 25°C in the presence of RM_{ekt} supplemented with 0.75 μg/ml of the 52-kD fragment of the SRP receptor. RM_{ekt} were alkylated for 30 min at 25°C with 3 mM NEM. (*B*) mRNA, coding for an 86–amino acid truncated preprolactin precursor was translated for 15 min at 30°C in a rabbit reticulocyte lysate system in the presence of control or NEM-treated RM_{ekt} supplemented with 0.75 μg/ml of the 52-kD fragment of the SRP receptor. Assays were supplemented with emetine to a final concentration of 0.5 mM. Puromycin (250 μM), or water, was added and incubations continued for 10 min. Where indicated, protease digestions were performed for 60 min at 0°C. Protease digestions were quenched by addition of PMSF to a final concentration of 2.5 mM. Samples were analyzed by SDS-PAGE on 12–20% gradient gels and subsequent autoradiography.

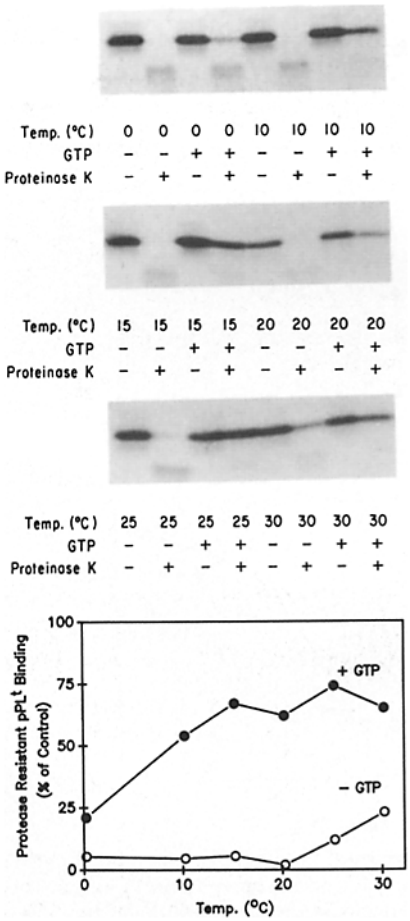

Figure 4. The temperature dependence of protease-resistant pPL^t binding to rough microsomes. mRNA, coding for an 86–amino acid truncated preprolactin precursor was translated in a rabbit reticulocyte lysate system for 10 min at 25°C. Translation reactions were supplemented with emetine to a final concentration of 0.5 mM and salt exchanged as described in the legend to Fig. 3. Aliquots of the polysome fraction, supplemented with 0.5 mm emetine, were incubated with nuclease-treated rough microsomes in the presence or absence of 0.5 mM GTP for 10 min at the described temperatures. Incubations were then chilled on ice and subjected to protease digestion as described in the legend to Fig. 3. Samples were analyzed by SDS-PAGE on 12–20% gradient gels and subsequent autoradiography. Incorporation of [^{35}S]methionine was quantitated by laser densitometry of the autoradiograms.

mation of a translocation competent junction between the ribosome/nascent chain complex and the membrane are distinct from the process of nascent chain translocation. In an effort to better distinguish the two phenomena, we have determined the temperature requirements for each event. The temperature dependence for GTP-dependent nascent chain binding is shown in Fig. 4. In the presence of GTP, protease-resistant binding of the truncated (86 amino acid) translation product is apparent at 0°C. The relative fraction of pPL^t bound in the presence of GTP increases with increasing temperature and plateaus at ~15°C. In the absence of GTP, and at low temperatures in the presence of GTP, addition of proteinase K leads to the formation of a limit digestion product which, as noted previously, presumably represents that portion of the nascent chain protected by the ribosome. No GTP-independent binding of pPL^t was observed at temperatures below 25°C. At 30°C, however, a clear increase in protease-resistant pPL^t binding was observed in the absence of added GTP. At present, we cannot eliminate the possibility that the observed GTP-independent tight binding is a reflection of residual, tightly bound GTP that is not removed during the desalting procedure. It is also possible that the binding reaction can occur in the absence of GTP and that GTP acts to enhance the rate of binding, a response that would be accentuated at a reduced temperature.

The temperature dependence of pPL^t translocation is depicted in Fig. 5. In this series of assays pPL^t was translated in the presence of rough microsomes for 10 min at 25°C. After addition of the protein synthesis inhibitor emetine, the

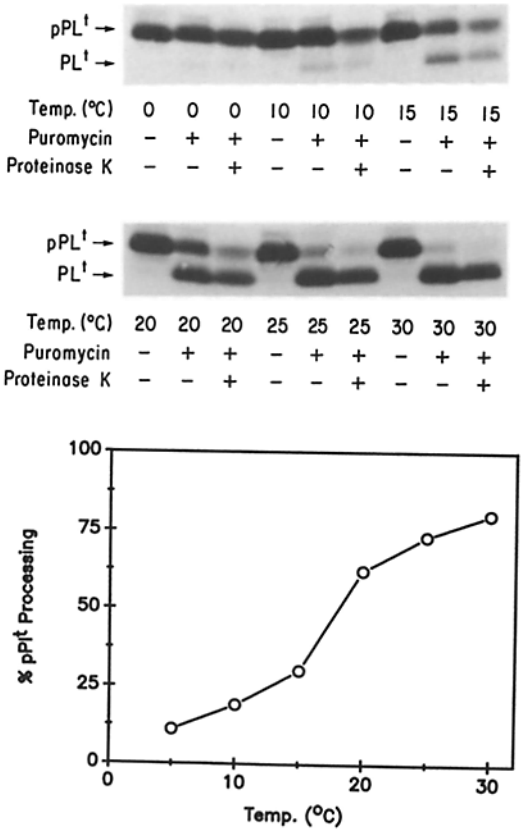

Figure 5. The temperature dependence of pPL' translocation. mRNA coding for an 86-amino acid truncated preprolactin was translated for 15 min in a rabbit reticulocyte lysate system in the presence of nuclease-treated rough microsomes. After translation, incubations were supplemented with emetine, to a final concentration of 0.5 mM, and chilled on ice. Aliquots were then incubated for 2 min at the described temperatures followed by addition of either water or puromycin, to a final concentration of 250 μM. Incubations were continued for an additional 10 min at the described temperatures, chilled on ice, and subjected to proteolysis as described in the legend to Fig. 3. Samples were analyzed by SDS-PAGE on 12-20% gels. Incorporation of [^{35}S]methionine was quantitated by analysis of the dried gels as described in Fig. 1.

translation reaction was chilled on ice. Aliquots of the unfractionated lysate were equilibrated at the defined temperatures and subsequently treated with puromycin. Very little translocation, as assessed by protease protection of the processed form, was observed at temperatures below 15°C. Translocation was markedly stimulated at temperatures above 15°C and was maximal at between 25 and 30°C. As can be discerned from comparison of the sensitivity of the unprocessed form to protease digestion after puromycin termination, the rate-limiting step in the translocation event did not appear to be the puromycin-dependent release of the peptide from the ribosome (Fig. 5). Thus, at lower temperatures (15°C), it can be seen that after puromycin termination a significant fraction of the unprocessed form was sensitive to protease digestion but not translocated. Comparison of the data depicted in Fig. 5 with that of Fig. 4 clearly indicates that the process of GTP-dependent nascent chain binding is maximal at a far lower temperature (15°C) than that of chain translocation (25-30°C). By virtue of the observed differences in both sensitivity to inhibition by chemical alkylation and temperature optima, it is indeed probable that nascent chain binding and translocation are separate phenomena subject to regulation by distinct components of the RER membrane.

Discussion

In the present study we report that translocation of nascent secretory proteins across the RER membrane is mediated by an NEM-sensitive membrane component. Analysis of the effects of NEM treatment of RM_{ekt} on the initial ribonucleotide-dependent insertion of the signal sequence and on nascent chain translocation indicate that NEM is acting at a step subsequent to signal sequence binding but before signal sequence cleavage. These data indicate that a protein component of the RER membrane mediates, at least in part, translocation of nascent secretory proteins across the RER membrane. These findings are of relevance to the controversy concerning the molecular mechanism of protein translocation (3, 7, 21, 24, 29).

The initiation of secretory protein translocation is known to involve at least two, defined events; ribosome/nascent chain targeting and signal sequence insertion. It has recently been demonstrated that signal sequence insertion, and formation of a translocation-competent junction between the ribosome/nascent chain complex and the membrane, displays an obligatory requirement for GTP (6, 12, 31). We have observed that treatment of microscomes with NEM does not inhibit GTP-dependent signal sequence insertion. In terms of the capacity to provide the initial targeting and insertion functions, therefore, NEM-treated membranes are indistinguishable from mock-treated membranes. Alkylated membranes are, however, incapable of translocating nascent secretory chains. On the basis of these data we conclude that peptide chain transfer across the membrane occurs by a process that is distinct from the initial GTP-dependent insertion event. This conclusion is further substantiated by observations of the markedly differing temperature dependencies of the two processes; GTP-dependent signal sequence insertion being maximal at 15°C and translocation being maximal at 25-30°C. Perhaps most significantly, the inhibition of protein translocation, by NEM, indicates that the translocation event is mediated by a protein component(s) of the RER membrane.

The conclusions drawn from these studies are derived primarily from analysis of the behavior of an 86-amino acid truncated preprolactin precursor. Synthesis of this precursor in the presence of either mock- or NEM-treated membranes resulted in the formation of a protease-protected form of the nascent chain. Resistance to protease digestion could reflect at least two phenomena, insertion into the membrane or simple inaccessibility of the substrate to protease, perhaps through interaction with membrane-bound protein components. At present, we cannot unequivocally discern whether either, or both, phenomena are responsible for the observed protease resistance of pPL' synthesized in the presence of membranes.

Of the 86-amino acid residues present in this translation product ~40 residues, at the carboxy terminus, reside in a protease-protected domain of the ribosome (2, 5, 16). The remaining 46 amino acid portion of the chain is composed

of a 30-amino acid signal sequence and a 16-amino acid portion of the mature protein (20). If the observed protease resistance represents protection by the membrane then both mock- and NEM-treated microsomes must be capable of inserting a fairly substantial (46 amino acid) polypeptide into the membrane. Regardless of the conformation assumed by this portion of the chain, be it extended, alpha helix, or 3.10 helix, it would be of more than sufficient length to traverse the bilayer, a distance requiring a minimum nascent chain length of ~20 amino acids (1). If both mock- and NEM-treated membranes are capable of inserting a 46-amino acid segment of the chain into the membrane how then can the NEM-dependent inhibition of translocation be explained? On the basis of the described data, we suggest that recognition and insertion of the signal sequence occurs by a process associated with, but independent of, the translocation event. If, for example, the signal sequence of the 86-amino acid preprolactin precursor is recognized and bound by a separate, NEM-insensitive, component of the translocation apparatus, protease resistance would be conferred by protection of the remaining 16-amino acid portion of the chain, perhaps through steric phenomena or by the lack of an appropriate site for the enzyme. Translocation, as it requires a distinct, NEM-sensitive component, could therefore be inhibited without affecting targeting and signal sequence insertion. The recently described signal sequence receptor (30), or, perhaps, the beta subunit of the SRP receptor would be candidate proteins for the NEM-insensitive signal sequence recognition component (22). This hypothesis, although conjectural, is supported by the observations that signal sequence insertion and nascent chain translocation can be distinguished by both temperature dependence and sensitivity to alkylation.

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