Biochimica et Biophysica Acta, 1152 (1993) 119–127

BBAMEM 76094

The initial association of a truncated form of the *Neurospora* plasma membrane H^+-ATPase and of the precursor of yeast invertase with microsomes are distinct processes

Randolph Addison *

Department of Biochemistry, The University of Tennessee, Health Science Center, 858 Madison Avenue, Suite G01, Memphis, TN 38163 (USA)

(Received 9 February 1993)
(Revised manuscript received 3 June 1993)

Key words: ATPase, H^+-; Microsome; Translocation; Integration; Invertase; (Yeast); (*N. crassa*)

Translocation and integration activities were assessed in *Neurospora* microsomes (nRM) after modification either by a sulfhydryl alkylating reagent or by a proteinase. A *Neurospora* in vitro system was programmed with RNA transcripts that encode the amino-terminal 194 amino-acid residues of the *Neurospora* plasma membrane H^+-ATPase (pma^+_{194}) or the 262 amino-acid residues of the precursor of yeast invertase ($preinv_{262}$). The processing of $preinv_{262}$ was blocked in *N*-phenylmaleimide- and in trypsin-pretreated nRM. In contrast, the binding of $preinv_{262}$ to microsomes was unaffected in the chemically alkylated nRM, but was affected in the trypsin-pretreated nRM. In the chemically alkylated vesicles, the integration of the pma^+_{194} was not affected, but was partially blocked in the trypsin-pretreated vesicles. These data imply that trypsin-sensitive components are required for these activities in nRM, and that binding, translocation and integration can be differentiated by their sensitivity to chemical alkylation of sulfhydryl groups in nRM. Evaluated also were the effects of temperature on translocation and integration activities in the nRM. These were maximal at 20°C, whereas the binding of $preinv_{262}$ was maximal at 0°C. Taken together, these data demonstrate that the processing of $preinv_{262}$ by nRM can be resolved into two steps: binding of the precursor protein to nRM and subsequent translocation into the lumen of the vesicles. Whereas, the integration of the pma^+_{194} into nRM could not be resolved into separable steps. Taken together, these results are interpreted to imply that the initial association of truncated forms of the pma^+ and the precursor of invertase to the surface of the nRM are distinct processes.

Introduction

It is now fairly clear that reconstituted systems provide valuable insights into the secretory pathway. These in vitro systems are reconstituted from subcellular fractions: a high-speed supernatant fraction, that serves to translate the RNA transcripts as well as serving as a source of soluble secretory components, and a membrane fraction, that acts as the in vivo site for the interaction of the precursor proteins with the organelle. Sections of the secretory pathway have been resolved and reconstituted: the translocation of proteins across the lipid bilayer of the endoplasmic reticulum (RER) [8]; the transport of vesicles from the RER to the *cis* face of the Golgi complex [24]; the intercompartmental vesicular transport of the Golgi complex [23]; the budding of secretory vesicles from and their fusion with competent membrane [7,13,25]. These in vitro systems are essential, not only as starting material for purifying components of the secretory pathway, but they also serve as a functional assay for these components. Although other methods may lead to the identification of these components, the exact role of these proteins in the secretory pathway and an insight into their molecular mechanisms will be gained only when these components are studied in a chemically well-defined functional assay.

An ideal system for studying the secretory pathway is one that is amenable to genetic and to biochemical analyses. Toward this goal, I have developed an in vitro system that reconstitutes the processing of proteins by the RER [3]. This in vitro system was developed using subcellular fractions from the filamentous fungus *Neu-*

* Corresponding author. Fax: +1 (901) 5287360.

Abbreviations: nRM, microsomes from *Neurospora*; pma^+_{194}, the amino-terminal 194 amino-acid residues of the *Neurospora* plasma membrane H^+-ATPase; $preinv_{262}$, the amino-terminal 262 amino-acid residues of the precursor of the yeast invertase; cRM, microsomes from canine pancreas; RER, rough endoplasmic reticulum; SRP, signal recognition particle; NPhM, *N*-phenylmaleimide; NEM, *N*-ethylmaleimide; IMP, integral membrane protein.

rospora crassa. A similar system was developed with subcellular fractions from yeast [15,22,29]. As previously reported, I directly demonstrated that the hydrolysis of a ribonucleotide is required for translocating truncated forms of the precursor of invertase into microsomes. Whereas, the binding of the precursor protein to the vesicles is ribonucleotide independent [4]. Subsequently, similar results, using the precursor of OmpA, were obtained with the in vitro system from yeast [26]. I have demonstrated also that the integration of truncated forms of the *Neurospora* plasma membrane H^+-ATPase (pma^+) into microsomes requires a GTPase [6]. These early studies suggested that the *Neurospora* pma^+ and the precursor of yeast invertase apparently interact with the RER by distinct processes.

In this communication, I have assessed the effects of the chemical alkylation of sulfhydryl groups in and of the proteolytic digestion of microsomes on binding, on translocation, and on integration. Chemically alkylated microsomes were competent with respect to binding and integration, but not to translocation. Binding, translocation, and integration were differentiated by their sensitivity to proteolytic digestion. Taken together, these results imply that the initial interactions of the pma^+ and the precursor of invertase with the RER are distinct processes.

Materials and Methods

Materials

The sources of most of the materials have been previously described [3,5]. Trypsin (TPCK-treated) and soybean trypsin inhibitor were obtained from Worthington. Puromycin was obtained from Boehringer-Mannheim and *N*-phenylmaleimide from Aldrich. The other reagents were of the highest commercially available grade.

Plasmids

The plasmid pSPM8 [1] contains the cDNA that encode the *Neurospora* pma^+. This was digested with the restriction endonuclease EcoRV (New England Biolabs) which cleaved the cDNA within codon 195. The plasmid pAB208 [3] contains the cDNA that encode the precursor of the yeast invertase [27]. This was digested with the restriction endonuclease *Bam*HI (New England Biolabs) to yield a cDNA cleaved within codon 263. The RNA transcripts were prepared as described [3].

Neurospora translation

The *Neurospora* translation extract and the microsomes (nRM) were prepared as previously described [3]. Post-translational processing of $preinv_{262}$ by and the integration of the pma^+_{194} into nRM were conducted as described [4]. All of the results depicted in this report were obtained by adding the nRM post-translationally; results obtained co-translationally are identical to those given here. Adding nRM co-translationally inhibits translation by 30–75%. Accordingly, all experiments were conducted post-translationally, to obtain a better signal to noise ratio. Unless stated otherwise, the translation reactions were conducted for 45 min at 16°C. Afterwards, to release the nascent polypeptide chain from the ribosome-peptidyl-tRNA complex, puromycin was added to a concentration of 200 μM. The mixtures were incubated for an additional 5 min at 16°C. Each mixture was layered over a 200-μl cushion and centrifuged at $436\,000 \times g_{max}$ for 4 min at 4°C in a Beckman TLA-100.1 fixed angle rotor [3,6]. The applied volume and the upper half of the sucrose cushion were removed; the proteins were precipitated by TCA as described [3]. The remaining portion of the cushion was discarded. The pelleted microsomes, or microsome remnants, and the TCA-precipitated proteins were prepared for and resolved on SDS-7.5–15% polyacrylamide gradient gels [3]; the products were detected by autoradiography.

Alkylating sulfhydryl groups in microsomes

DTT was removed from stored microsomes by layering 50 μl of the nRM over a sucrose step gradient: the top layer (600 μl) contained 0.5 M sucrose, 40 mM K^+-Hepes (pH 7.5); the bottom layer (100 μl) contained 2 M sucrose, 40 mM K^+-Hepes (pH 7.5). The resultant gradient was centrifuged in a Beckman TLS-55 swinging bucket rotor at $173\,502 \times g_{max}$ for 6 min at 4°C. The nRM were collected from the 0.5 M/2 M sucrose interface and were adjusted to 50 μl, using 0.25 M sucrose, 40 mM K^+-Hepes (pH 7.5). The nRM (10 A_{280} units/ml) were treated with *N*-phenylmaleimide (NPhM) in the presence of 0.25 M sucrose, 40 mM K^+-Hepes (pH 7.5), 50 mM KOAc for 15 min at 20°C, in a final reaction volume of 50-μl. The alkylation reaction was terminated by adding an equimolar amount of β-mercaptoethanol and then incubating the mixture for 5 min at 20°C. Afterwards, the sample was placed on ice. The control was obtained by adding premixed NPhM and β-mercaptoethanol to nRM and incubating the mixture as mentioned.

Digesting microsomes with trypsin

The nRM (10 A_{280} units/ml) were digested by trypsin (TPCK treated) in the presence of 0.25 M sucrose, 50 mM KOAc, 1 mM $CaCl_2$, 40 mM K^+-Hepes (pH 7.5), and 1 mM DTT for 20 min at 0°C, in a final reaction volume of 50-μl. To stop the reaction, a 3-fold excess of soybean trypsin inhibitor and 10 mM of phenylmethylsulfonyl fluoride were added. Control was obtained by adding to the nRM premixed trypsin and

inhibitors. The control sample was incubated as mentioned. All samples were used directly in the assay.

Determination of the percent processing

An estimate of the relative quantity of the forms that were either integrated, bound or glycosylated was performed by densitometric analysis of the autoradiograms by a Bio Image Radioanalytic Imaging System (MilliGen/Biosearch, Bio Image, Ann Arbor, MI, USA). The percent processing of $preinv_{262}$ was determined by dividing the amount of glycosylated products by the sum of the glycosylated products and the bound form in the pellet. The percent of the product that was bound was determined by dividing the amount of the bound form in the pellet by the sum of the glycosylated forms and the bound form in the pellet. There are two unglycosylated bands in the pellet fraction. The slower migrating band is the bound unprocessed $preinv_{262}$ [4]. Because the identity of the faster migrating band in the pellet is uncertain, it was not used in any of the calculations. Yeast $preinv_{262}$ has six methionyl residues, but the processed form has five [27]. The calculation corrected for the lost of one methionyl residue in the processed $preinv_{262}$ when the signal sequence is cleaved by using the ratio 6:5.

Results

Treatment of microsomes with NPhM blocks translocation

The sulfhydryl alkylating reagent *N*-ethylmaleimide (NEM) has proven useful for identifying proteins in canine pancreatic microsome vesicles (cRM) whose functions are required for processing secretory proteins and for demonstrating that various classes of secretory proteins share common components in their initial interactions with the RER [17,21]: One component, the SRP receptor (SRP-docking protein) [11,20], is required for displacing SRP from the nascent peptidyl-tRNA-ribosome complex [12]. Another component, that functions subsequent to the SRP-SRP receptor interaction, is required for translocating preprolactin into the vesicular lumen of the cRM [21]. The activities of these proteins are impaired by NEM [18,21]. Is the use of NEM a useful strategy that is applicable to other systems? To determine this, NEM was assessed for its

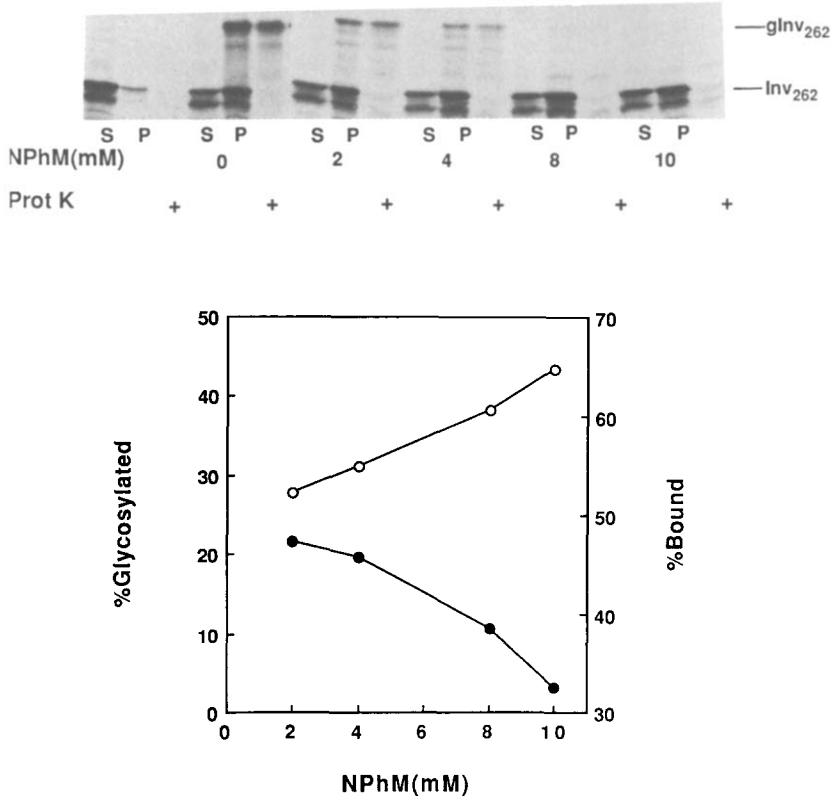

Fig. 1. Glycosylation of $preinv_{262}$ by NPhM-alkylated nRM. The RNA transcripts for $preinv_{262}$ were translated in the *Neurospora* in vitro system essentially as described [3]. The nRM were treated with NPhM as outlined in Materials and Methods. Where noted, the nRM were added post-translationally to a concentration of 3 A_{280} units/ml. The first three lanes represent a translation mixture where no nRM were added. The next three lanes represent an incubation with control nRM. Proteinase digestions were performed with 100 μg/ml of proteinase K for 30 min at 0°C. Samples were fractionated into membrane (P) and supernatant (S) fractions by centrifugation through sucrose cushions containing 0.5 M KOAc. The $preinv_{262}$ (Inv_{262}) and the glycosylated $preinv_{262}$ ($glnv_{262}$) were resolved using SDS-7.5–15% PAGE and detected by autoradiography. Quantitation of the relative amount of bound (○) and the glycosylated (●) forms was performed on a BioImage System.

affects on the translocation and integration activities of microsome vesicles from *Neurospora*. The nRM were treated with 15 mM of NEM for 30 min at 25°C; The processing of the precursor of the yeast invertase was only marginally affected by the NEM pretreated nRM (data not shown). This suggests that either there are no sulfhydryl groups in *Neurospora* microsomes that are involved in these activities or the sulfhydryl groups that are presumed to function in these processes are not accessible to the alkylating activity of NEM, i.e., they may exist in a more hydrophobic milieu. To test this, *N*-phenylmaleimide, a more hydrophobic sulfhydryl-directed alkylating reagent, was evaluated for its ability to block the formation of glycosylated $preinv_{262}$.

The RNA transcripts that encode for $preinv_{262}$ have no stop codon. Therefore, puromycin, an aminoacyl-tRNA analog, was added, after the addition of nRM, to the translation mixture to release the ribosome-bound precursor. As previously demonstrated, the ribosome-$preinv_{262}$ complex bounds to the nRM and puromycin causes the release of the $preinv_{262}$ which is then translocated into and processed by nRM [4]. When NPhM-treated nRM were added to the translation mixture post-translationally, the glycosylation of $preinv_{262}$ was blocked, but binding was not impaired (Fig. 1). The bound form of $preinv_{262}$ is the unglycosylated $preinv_{262}$ that co-sedimented with the microsomes. Clearly, translocation, a step subsequent to binding [4], is blocked in the NPhM-treated nRM. The amount of pelleted $preinv_{262}$ increases, however, suggesting that the association of $preinv_{262}$ with nRM is not impaired in these treated microsomes. It could be that the form that was bound initially has been transferred to another component(s) whose function has been compromised by the activity of NPhM. Therefore, the bound form(s) would accumulate at a point subsequent to the initial binding step. Nevertheless, in the quantitation of the data, the unglycosylated $preinv_{262}$ that is found in the pellet is treated as the bound form, regardless of the exact component(s) to which it may be associated. The identity of the faster migrating band in the membrane fraction is unclear. This could be the signal-sequence-cleaved product of preinvertase or the nonspecific association of the cytosolic form of invertase with the microsomes. In the absence of nRM, less than 1% of the products were found in the pellet (Fig. 1), suggesting that the products that co-sedimented with the nRM were associated, specifically or nonspecifically, with the membranes (see below).

The glycosylation of secretory proteins at asparagine residues of appropriate receptor sites serves as an excellent reporter of translocation, for the glycosylating enzymes are localized to the lumen of the vesicles [16]. The inhibition of the formation of glycosylated $preinv_{262}$ by NPhM-treated nRM indicates either that NPhM chemically alkylated a lumenal protein(s) that is required, directly or indirectly, for the glycosylation of $preinv_{262}$ or it modified a protein(s) in the nRM whose function is required for translocating $preinv_{262}$ into the lumen of the nRM. To distinguish between these possibilities, a proteinase was added to the reaction mixture. If the precursor is translocated and unglycosylated, it will not be digested by the proteinase. The unglycosylated $preinv_{262}$ was digested by proteinase K (Fig. 1), demonstrating that the unprocessed $preinv_{262}$ was not translocated into the nRM and that NPhM alkylated a protein(s) in the nRM whose function is apparently required for translocating $preinv_{262}$ into nRM.

Are the NPhM-pretreated nRM integration competent? To determine this, the integration of the pma^{+}_{194} into the chemically-alkylated nRM was assessed. The manipulation of the cDNA clones of various IMPs has demonstrated that transmembrane spanning domains of IMPs serve as signals to initiate and to stop translocation [9,19]. Using truncated forms of the pma^{+}, it was demonstrated that topogenic sequences for these events exist within the first 200 amino-acid residues of the pma^{+} [5,6]. Therefore, the cDNA for the pma^{+} was treated with an endonuclease to cleave the cDNA

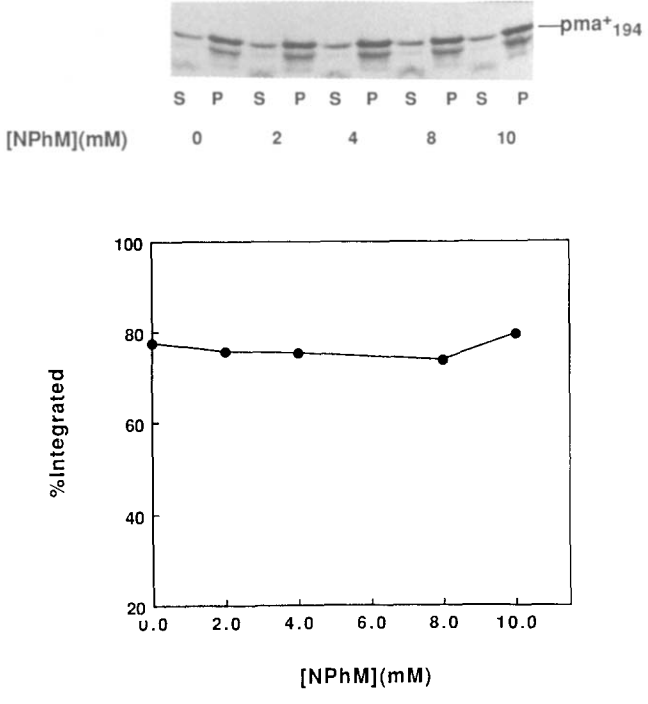

Fig. 2. Integration of the pma^{+}_{194} into NPhM-pretreated nRM. Translation of the RNA transcripts that encode for the pma^{+}_{194} and the incubation of the products with NPhM pretreated nRM were as described in the legend to Fig. 1. The first two lanes represent a translation mixture that was incubated with control nRM. Samples were extracted with Na_2CO_3 (pH 11.5) and were fractionated into membrane (P) and supernatant (S) fractions by centrifugation through sucrose cushions containing 0.1 M Na_2CO_3 (pH 11.5). The products were resolved by SDS-PAGE using a 7.5%–15% polyacrylamide gradient gel and detected by autoradiography. Quantitation was preformed on a BioImage System.

within codon 195. The resultant RNA transcripts encode the amino-terminal 194 amino-acid residues that contains the first two putative transmembrane-spanning domains of the pma^+ [2,14]. These RNA transcripts contain no stop codon. Accordingly, puromycin was added to the translation mixture after incubating the mixture with nRM. The truncated forms of the pma^+ are completely released from the ribosome by the addition of puromycin [5]. When the NPhM-treated nRM were added to the translated product, the pma^+_{194} was integrated into the chemically-alkylated nRM, as judged by the resistance of the membrane-associated pma^+_{194} to extraction by Na_2CO_3 (pH 11.5) (Fig. 2). Extracting microsomal vesicles by Na_2CO_3 (pH 11.5) converts them into open sheets, this allows for the extraction of peripheral proteins from the exposed faces of the microsome sheets [10]. This technique has served as a criterion to demonstrate that in vitro synthesized proteins are integrated into microsomes. Furthermore, after extracting the microsomes with 0.5 M KOAc, 4 M urea, or with 25 mM EDTA, the pma^+_{194} remained associated with the pelleted NPhM-treated microsomes (data not shown). Under these conditions, the IMPs are recovered with the microsomes (P) fraction; the soluble and peripheral proteins are recovered in the supernatant (S) fraction. The effectiveness of the differential centrifugation step is demonstrated by the observation that in the absence of microsomes the pma^+_{194} is found exclusively in the supernatant fraction (see Fig. 6).

Trypsin treatment of microsomes inhibits translocation and integration

To gain additional insight into these activities, binding, translocation and integration were assessed in trypsin-pretreated nRM. The processing of $preinv_{262}$ was blocked in trypsin-pretreated nRM (Fig. 3). When nRM were treated with trypsin at a concentration of 0.5 μg/ml, the glycosylation of $preinv_{262}$ by these was 95% inhibited, but the binding of the preprotein was only marginally affected. Most of the bound form was sensitive to proteinase K digestion (Fig. 3): There is a portion of the bound product that was not digested by proteinase K (Fig. 3). This form could not be digested by proteinase K, even in the presence of Triton X-100 (data not shown). With increasing concentrations of trypsin, the binding of $preinv_{262}$ to the treated vesicles decreased (Fig. 3); This is in contrast to what was observed with the NPhM-treated nRM, there the amount of $preinv_{262}$ that was bound to the treated microsomes was inverse to the formation of glycosylated products (Fig. 1). This implies that binding of

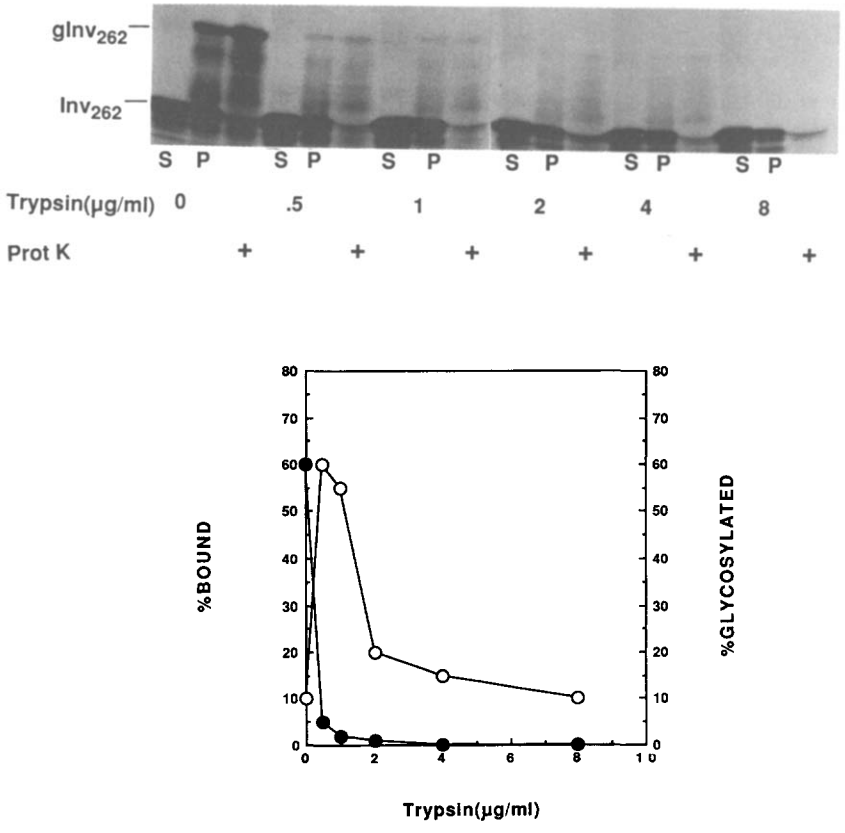

Fig. 3. Glycosylation of $preinv_{262}$ by trypsin-pretreated nRM. The experimental details and the quantification of relative amount of the bound (○) and the glycosylated (●) forms were as described in the legend to Fig. 1. The nRM were treated with trypsin as outlined in Materials and Methods. The first three lanes represent a translation mixture that was incubated with control nRM.

preinv$_{262}$ to nRM depends on trypsin-sensitive component(s) of the microsomes. Furthermore, these data imply that processing of preinv$_{262}$ by nRM can be resolved into two steps: The binding of preinv$_{262}$ to the surface of the nRM and the subsequent translocation of the preprotein into the nRM. These steps are differentiated by their sensitivity to trypsin digestion and to the chemical alkylation of sulfhydryl groups in nRM.

When the trypsin-treated nRM were assessed for their integration competence, the integration of the pma$^{+}_{194}$ into the treated microsomes was diminished (Fig. 4). The concentration of trypsin that was required to reduce integration by 70% was at least 8-times that required to block by 95% the processing of preinv$_{262}$ (see Fig. 3). There is a level of integration activity that was resistant to trypsin digestion, even after treating the nRM with higher concentrations of the proteinase (Fig. 4). The latter, after the treatment with the proteinase, were still not competent for the binding of preinv$_{262}$ (data not shown), implying that the microsomes were not extensively damaged by trypsin so that nonspecific associations with these begin to occur. It is not apparent if this trypsin-resistant integration activity represents an alternate pathway for integrating pma$^{+}_{194}$ into the microsomes. Clearly, additional experiments will be required to gain insight into this intriguing possibility.

Temperature dependence of translocation and integration

The initial association of preinv$_{262}$ to nRM was not blocked by the alkylation of sulfhydryl groups in nRM nor by the treatment of nRM with a low concentration of trypsin. The subsequent glycosylation of preinv$_{262}$ by the pretreated nRM was blocked, however, as judged by the diminution of the glycosylated forms. This suggests that the association of the preprotein with nRM and its subsequent translocation across the membrane can be resolved. To further substantiate this, another approach was taken. These reactions were studied at temperatures between 0°C to 30°C. The translocation of preinv$_{262}$ into nRM, as judged by the glycosylation of preinv$_{262}$ by nRM, displays maximal activity at 20°C and minimal at 0°C. Whereas, binding to nRM was maximal at 0°C (Fig. 5). The integration of the pma$^{+}_{194}$ into nRM was evaluated also at various temperatures. The integration of the pma$^{+}_{194}$ into nRM was maximal at 20°C (Fig. 6), similar to what was observed for the processing of preinv$_{262}$. In contrast to translocation, however, approx. 50% of the pma$^{+}_{194}$ was integrated into nRM at 0°C.

Discussion

In this report, three criteria were used to gain insight into the initial interaction of precursor proteins with the surface of the RER of *Neurospora*. These were (1) chemical alkylation of sulfhydryl groups of the nRM by NPhM; (2) proteolytic digestion of nRM by trypsin and (3) modification of the milieu temperature. The processing of preinv$_{262}$ by nRM, as assessed by the addition of high-mannose oligosaccharide chains to the protein, was blocked in NPhM- and in trypsin-treated nRM. In contrast, the binding of preinv$_{262}$ to nRM was unimpaired in NPhM treated nRM and was only marginally affected in nRM that were treated with low concentrations of trypsin. The unglycosylated preinv$_{262}$ that co-sedimented with the NPhM-treated nRM was digested by proteinase K, precluding the possibility that the protein was translocated and unprocessed. The effects, therefore, of NPhM chemically-alkylating sulfhydryl groups in nRM indicate that this reagent affected a protein(s) whose function is essential for translocating preinv$_{262}$ into the nRM, but not for the binding of the precursor to the surface of the nRM. As previously demonstrated, the binding of the precursor of invertase to nRM is a ribonucleotide-independent process: the bound form, without being released from the microsomes, could be 'chased' into the lumen of the microsomes by the hydrolysis of a ribonucleotide [4]. This implies that the bound form is an intermediate of the translocation process.

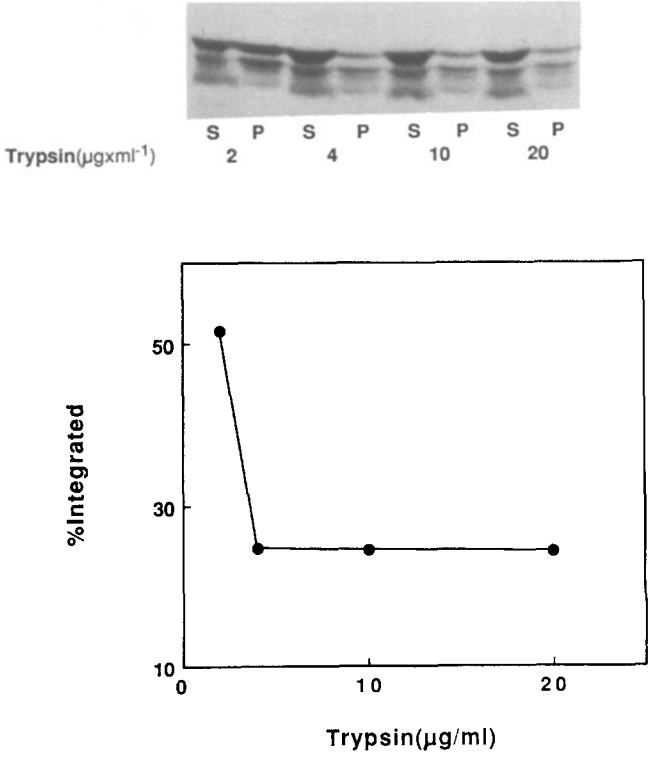

Fig. 4. Integration of the pma$^{+}_{194}$ into trypsin-pretreated nRM. The translation of the RNA transcripts for pma$^{+}_{194}$ and the integration of the product into trypsin-pretreated nRM were as described in the legend to Fig. 3. Methods for the fractionation of the products, their detection and quantitation were as described in the legend to Fig. 2.

It is clear, therefore, that the processing of $preinv_{262}$ by nRM can be resolved into two events: binding of the precursor protein to the surface of the nRM and subsequent translocation into the lumen of the microsomes. This conclusion is further substantiated by the observed differences in the temperature dependence of the two events: the formation of the glycosylated $preinv_{262}$ is maximal at 20°C, minimal at 0°C; whereas, the association of $preinv_{262}$ to the surface of the nRM was maximal at 0°C.

The alkylation of sulfhydryl groups in nRM had no effect on the integration of the pma^{+}_{194}. The digestion of nRM by trypsin, however, did affect the integration of the pma^{+}_{194}, but this inhibition of integration was less sensitive to trypsin digestion than the processing of $preinv_{262}$, because higher concentrations of trypsin were required to inhibit integration activity than to block the glycosylation of $preinv_{262}$. The effects of the trypsin digestion of nRM imply that trypsin-sensitive protein component(s) is involved in the integration process. In the absence of a ribonucleotide, truncated forms of the pma^{+} do not associate with nRM, with or without the nascent polypeptide chain being attached to ribosomes [6]. Taken together, this suggests that the association of the pma^{+}_{194} with the nRM is not the promiscuous insertion of the product into microsomes but is a specific process, requiring a GTPase and, presumably, other nRM proteins. In contrast, therefore, to the processing of $preinv_{262}$ by nRM, only integration, one event, could be demonstrated for the interaction of the pma^{+}_{194} with the nRM.

These data suggest that the initial association of the pma^{+}_{194}, an IMP, and of $preinv_{262}$, a secreted protein, with nRM are distinct processes. These observations differ from observation obtained from in vitro systems reconstituted with cRM. There the evidence indicates that different classes of preproteins apparently share identical components in their initial interaction with the cRM [28]. For example, the initial step in the processing of precursor proteins, the binding of the nascent secretory proteins to the surface of the cRM, is blocked by NEM [11,18,21]. These distinctions could reflect evolutionary differences in the two in vitro systems. In support of this notion, the translocation of

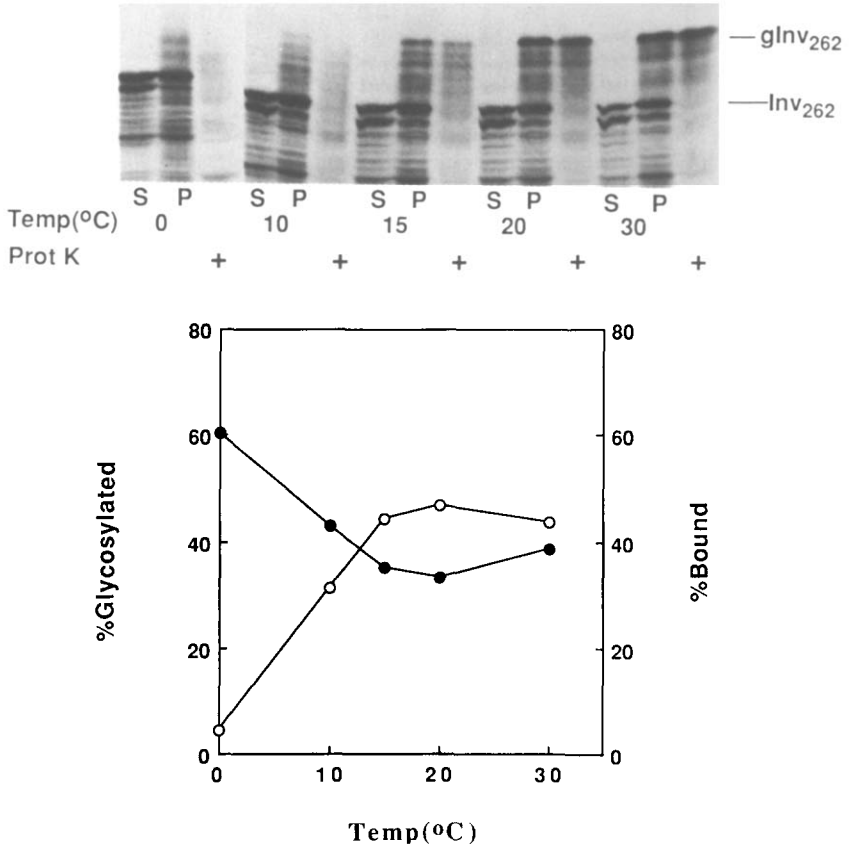

Fig. 5. The effects of temperature on the glycosylation of $preinv_{262}$ by nRM. The RNA transcripts for $preinv_{262}$ were translated as described [3]. The translated products were incubated at the indicated temperatures for 5 min before adding the nRM, which were stored on ice, to a concentration of 3 A_{280} units/ml. The resultant mixtures were incubated for 20 min at the indicated temperature. Afterwards, the mixtures were either fractionated or treated with proteinase K, as outlined in the legend to Fig. 1. The detection and the quantification of the relative amount of bound (Inv_{262}) (●) and the processed ($glnv_{262}$) (○) forms were as outlined in the legend to Fig. 1.

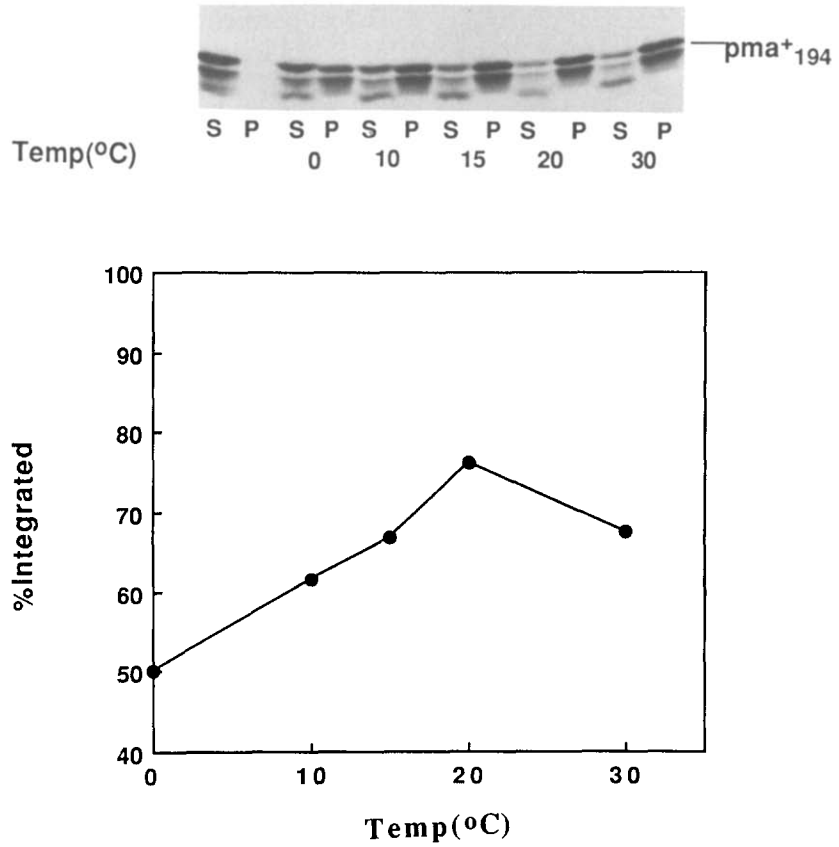

Fig. 6. The effects of temperature on the integration of the pma^{+}_{194} into nRM. The translation of the RNA transcripts for the pma^{+}_{194} and the incubation of the products with the nRM at the various temperatures were as described in the legend to Fig. 2. The fractionation of the mixtures into membrane (P) and supernatant (S) fractions, and the quantification of the products were as outlined in the legend to Fig. 2. The first two lanes represent a translation mixture that was incubated at 0°C without nRM.

the precursor of OmpA into yeast microsomes can be resolved into separatable steps: binding and translocation [26]: binding is a ribonucleotide-independent event that is unaffected by the chemical alkylation of sulfhydryl groups by NEM. Presumably, in these fungi, a putative signal sequence receptor on the surface of the RER recognizes the signal sequence of precursor proteins in a ribonucleotide-independent event. This is analogous to the import of precursor proteins into mitochondria: The preproteins are bound to specific targeting sequence receptors on the surface of the outer membrane of mitochondria and are subsequently translocated across the outer membrane via a general insertion protein, in an energy dependent manner. Although this comparison is conjectural, this concept would imply that the putative signal-sequence receptors of these fungi may represent the evolutionary precursor of the more complex receptor found in canine pancreas and/or they represent components of a functionally redundant targeting pathway to the RER.

The data also indicate that binding, translocation and integration can be resolved, for each displays markedly differing effects to chemical alkylation, to trypsin digestion and to variations in the milieu temperature, suggesting that the essential components for each process are distinct. Binding is independent of translocation, suggesting separate components for each event. It is conceivable that both activities could be combined in a single protein. Integration is insensitive to NPhM alkylation of sulfhydryl groups in nRM, implying that integration and translocation are catalyzed by different machineries. It is conceivable that both activities are combined in a multisubunit proteinaceous effector that is capable of decoding topogenic sequences and performing subsequent reactions for IMPs and for the precursor of secreted proteins. Clearly, to resolve these issues will require the resolution of translocation and integration activities and the reconstitution of these activities in a well-defined chemical system. In the laboratory, current experiments are directed toward this goal.

References

1 Aaronson, L.R., Hager, K.M., Davenport, J.W., Mandala, S.M., Chang, A., Speicher, D.W. and Slayman, C.W. (1988) J. Biol. Chem. 263, 14552–14558.
2 Addison, R. (1986) J. Biol. Chem. 261, 14896–14901.
3 Addison, R. (1987) J. Biol. Chem. 262, 17031–17037.

4 Addison, R. (1988) J. Biol. Chem. 263, 14281–14287.
5 Addison, R. (1990) Biochim. Biophys. Acta 1030, 127–133.
6 Addison, R. (1991) Biochim. Biophys. Acta 1065, 130–134.
7 Balch, W.E., Glick, B.S. and Rothman, J.E. (1984) Cell 39, 525–536.
8 Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 852–862.
9 Friedlander, M. and Blobel, G. (1985) Nature 318, 338–343.
10 Fujiki, Y., Hubbard, A.L., Fowler, S. and Lazarow, P.B. (1982) J. Cell Biol. 93, 97–102.
11 Gilmore, R., Blobel, G. and Walter, P. (1982) J. Cell Biol. 96, 463–469.
12 Gilmore, R. and Blobel, G. (1983) Cell 35, 677–685.
13 Gorvel, J.-P., Chavrier, P., Zerial, M. and Gruenberg, J. (1991) Cell 64, 915–925.
14 Hager, K.M., Mandala, S.M., Davenport, J.W., Speicher, D.W., Benz, E.J., Jr. and Slayman, C.W. (1986) Proc. Natl. Acad. Sci. USA 83, 7693–7697.
15 Hansen, W., Garcia, P.D. and Walter, P. (1986) Cell 45, 397–406.
16 Hirschberg, C.B. and Snider, M.D. (1987) Annu. Rev. Biochem. 56, 63–87.
17 Hortsch, M., Avossa, D. and Meyer, D.I. (1986) J. Cell Biol. 103, 241–253.
18 Jackson, R.C., Walter, P. and Blobel, G. (1980) Nature 286, 174–176.
19 Lipp, J., Flint, N., Haeuptle, M.-T. and Dobberstein, B. (1989) J. Cell Biol. 109, 2013–2022.
20 Meyer, D.I. and Dobberstein, B. (1980) J. Cell Biol. 87, 503–508.
21 Nicchitta, C.V. and Blobel, G. (1989) J. Cell Biol. 108, 787–795.
22 Rothblatt, J.A. and Meyer, D.I. (1986) Cell 44, 619–628.
23 Rothman, J.E. and Orci, L. (1990) FASEB J. 4, 1460–1468.
24 Ruohola, H., Kastan-Kabcenell, A. and Ferro-Novick, S. (1988) J. Cell Biol. 107, 1465–1476.
25 Salamero, J., Sztul, E.S. and Howell, K.E. (1990) Proc. Natl. Acad. Sci. USA 87, 7717–7721.
26 Sanz, P. and Meyer, D.I. (1989) J. Cell Biol. 108, 2101–2106.
27 Taussig, R. and Carlson, M. (1983) Nucleic Acid Res. 11, 1943–1954.
28 Walter, P. and Lingappa, V.R. (1986) Annu. Rev. Cell Biol. 2, 499–516.
29 Waters, M.G. and Blobel, G. (1986) J. Cell Biol. 102, 1543–1550.