Secretion of a type II integral membrane protein induced by mutation of the transmembrane segment

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Signal peptide/membrane anchor (SA) domains of type II membrane proteins initiate the translocation of downstream polypeptides across the endoplasmic reticulum (ER) membrane. In contrast with signal peptides, however, SA domains are not cleaved by signal peptidase and thus anchor the protein in the membrane. In the present study we have introduced mutations in the SA domain of neprilysin (neutral endopeptidase-24.11; NEP) to identify structural elements that would favour the processing of SA domains by signal peptidase. Mutants of full-length and truncated (without cytoplasmic domain) protein were constructed by substitution of the sequences SQNS, QQTT or YPGY for VTMI starting at position 15 of the NEP SA domain. In addition, a Pro residue was substituted for Thr at position 16 of the SA domain. The rationale for the use of these sequences was decided from our previous observation that substitution in the NEP SA domain of the sequence SQNS, which is polar and has α-helix-breaking potential, could promote SA domain processing under certain conditions (Roy, Chatellard, Lemay, Crine and Boileau (1993) J. Biol. Chem. **268**, 2699–2704; Yang, Chatellard, Lazure, Crine and Boileau (1994) Arch. Biochem. Biophys. **315**, 382–386). The QQTT sequence is polar but, according to secondary structure predictions, is compatible with the α-helix structure of the NEP SA domain. The YPGY sequence and single Pro residue are less polar and have α-helix-breaking potential. The predicted effects of these mutations on the structure of the NEP SA domain were confirmed by CD analysis of 42-residue peptides encompassing the hydrophobic segment and flanking regions. Wild-type and mutated proteins were expressed in COS-1 cells and their fate (membrane-bound or secreted) was determined by immunoblotting and by endoglycosidase digestions. Our biochemical and structural data indicate that: (1) the cytosolic domain of NEP restricts the conformation of the SA domain because mutants not secreted in their full-length form are secreted in their truncated form; (2) α-helix-breaking residues are not a prerequisite for cleavage; (3) the presence, in close proximity to a putative signal peptidase cleavage site, of a polar sequence that maintains the α-helical structure of the SA domain is sufficient to promote cleavage. Furthermore pulse–chase studies suggest that cleavage is performed in the ER by signal peptidase and indicate that cleavage is not a limiting step in the biosynthesis of the soluble form of the protein.

INTRODUCTION

The first steps in the transport of secretory and transmembrane proteins towards the cell surface are their targeting to and translocation across the endoplasmic reticulum (ER) membrane. Both of these events are co-translational and are promoted by the presence of an N-terminal signal peptide (reviewed in [1,2]). The signal peptide of secretory and type I membrane proteins is co-translationally cleaved during translocation by a specific signal peptidase located on the luminal side of the ER [3,4]. In type II membrane proteins the uncleaved signal peptide [signal peptide/membrane anchor (SA) domain] is also responsible for anchoring the proteins in the membrane.

Typically, signal peptides consist of three domains or regions: a positively charged N-terminal n-region, a central h-region characterized by a stretch of 7–15 hydrophobic residues critical for efficient translocation of the protein, and a C-terminal c-region containing the signal peptidase cleavage site (reviewed in [5,6]). Residues in the c-region located at positions -3 and -1 with respect to the cleaved peptide bond are particularly important for specifying the cleavage site. Statistical analyses have shown that small apolar side chain residues are preferred at these positions [7–9]. SA domains of type II membrane proteins differ substantially from cleaved signal peptides. First, their n-region, which forms the cytoplasmic domain of the protein, is usually longer. Secondly, the hydrophobic segment is at least 20 apolar residues in length to allow it to span the lipid bilayer as an α-helix. Finally, they are not recognized by signal peptidase.

Studies *in vitro* have shown that some SA domains of type II membrane proteins do in fact contain cryptic signal peptidase cleavage sites. Indeed, the SA domain of both the invariant chain (Iγ) of class II histocompatibility antigens and asialoglycoprotein receptor H1 were processed after deletion of the positively charged cytoplasmic domains [10,11], suggesting that the presence of the cytosolic segment somehow restricts access to the cleavage site. In contrast, other type II membrane proteins are not secreted on deletion of the cytosolic domain. For example, deletion of the 27-residue cytosolic domain of neutral endopeptidase-24.11 (EC 3.4.24.11; NEP) did not promote the secretion of the protein when expressed in COS-1 cells [12] despite the presence of a putative signal peptidase cleavage site identified with the algorithm of von Heijne [8]. One explanation

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; endo F, endoglycosidase F; endo H, endoglycosidase H; ER, endoplasmic reticulum; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside; NEP, neutral endopeptidase-24.11 (EC 3.4.24.11); octyl glucoside, n-octyl- β-D-glucopyranoside; SA, signal peptide/membrane anchor.

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for this discrepancy might be the nature of the residues in the SA domains which, in some proteins, may favour access to the cleavage site. This possibility is supported by the observation that the NEP SA domain was processed by signal peptidase when the hydrophilic sequence SQNS was substituted 14 residues from the beginning of the SA domain and the cytoplasmic domain was deleted [12,13]. The mechanism by which this sequence promotes cleavage of the SA domain is, however, unknown. Predictions of secondary structure [14] have suggested that it can induce formation of a β-turn structure. Because potential α-helix-breaking residues were shown to enhance cleavage by signal peptidase [15–17] we speculated that this structural feature could play an important role in SA domain cleavage [12,13].

To test this hypothesis we have created new mutants of full-length and truncated (without a cytosolic domain) protein by substitution of the sequences QQTT or YPGY starting at position 15 of the SA domain of NEP. According to Chou and Fasman [14], the polar QQTT sequence is compatible with the α-helix structure of the SA domain, whereas the less polar YPGY sequence shows a high propensity for the formation of a β-turn structure, as does the SQNS sequence. In addition, a Pro residue, which is known to break α-helix stuctures, was substituted at position 16 of the SA domain. Results obtained from the expression of NEP and NEP mutants in COS-1 cells, and from CD analysis of peptides corresponding to the SA domains of NEP and NEP mutants, do not support the hypothesis that α-helix-breaking residues promote cleavage by signal peptidase. On the contrary, our biochemical and structural data suggest that the substitution of a polar sequence that maintains the α-helical structure of the hydrophobic segment and is in close proximity to a putative signal peptidase cleavage site is sufficient to promote the cleavage of the NEP SA domain. Furthermore because all truncated mutants were secreted except the one with the Pro mutation, we suggest that the cytosolic domain of NEP restricts the conformation that the SA domain might adopt in the membrane and also access to the signal peptidase cleavage site.

MATERIALS AND METHODS

Manipulation of DNA and construction of plasmids

DNA manipulations were performed in accordance with standard procedures [18,19]. The construction of plasmids pSVNEP, pSVNEP Δcyto, pSVNEP(SQNS) and pSVNEP Δcyto(SQNS), coding for NEP, NEP Δcyto, NEP(SQNS) and NEP Δcyto(SQNS) respectively, have been described previously [12,13]. To create new mutants, DNA fragments of interest were subcloned in M13mp19. In the first series of mutants the sequence Val^{42}-Thr-Met-Ile in the SA domain of NEP and NEP Δcyto was changed to Gln^{42}-Gln-Thr-Thr or Tyr^{42}-Pro-Gly-Tyr by site-directed mutagenesis. The mutated fragments were then ligated back into the simian virus 40-based expression vectors, thus creating vectors pSVNEP(QQTT), pSVNEP(YPGY), pSVNEP Δcyto(QQTT) and pSVNEP Δcyto(YPGY) coding for NEP(QQTT), NEP(YPGY), NEP Δcyto(QQTT) and NEP Δcyto(YPGY) proteins respectively. In the second series of mutants, Thr^{43} in the SA domain of NEP and NEP Δcyto was changed to Pro^{43} to produce NEP(P) and NEP Δcyto(P) proteins. The mutations were verified by sequencing the mutated DNA regions [20]. A summary of the mutations is presented in Figure 1. The pGEX-2T vectors were constructed by digesting pGEX-2T (Pharmacia) with *Bam*HI and *Eco*RI and ligating into it PCR-amplified fragments from pSVNEP, pSVNEP(SQNS), pSVNEP(QQTT), pSVNEP(YPGY) and pSVNEP(P) flanked by *Bam*HI and *Eco*RI restriction sites. pGEX-2T NEP, pGEX-2T NEP(SQNS), pGEX-2T NEP(QQTT), pGEX-2T NEP(YPGY) and pGEX-2T NEP(P) are fusion proteins of glutathione S-transferase (GST) with residues 15–54 of full-length NEP and NEP-related mutants.

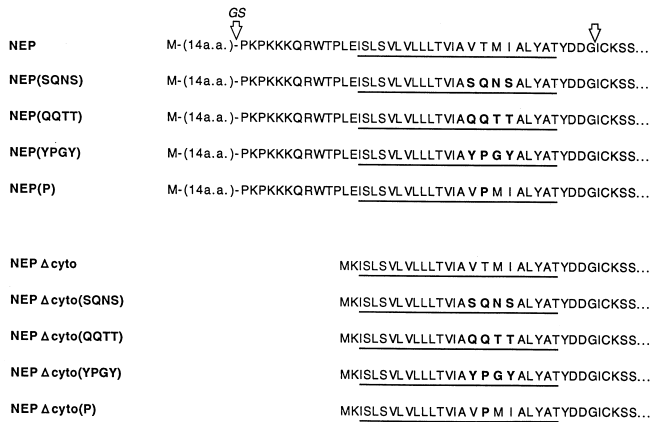

Figure 1 N-terminal sequences of NEP and NEP mutants

The transmembrane domain is underlined and the substituted amino acids are shown in bold. The two arrows define the amino acid sequence of the related peptides used for CD measurements. The amino acids shown above the left arrow are present at the N-terminus of each peptide and are derived from the GST portion of the fusion protein after its cleavage by thrombin. Abbreviation: a.a., amino acids.

Transfection of COS-1 cells and immunoblotting of proteins

COS-1 cells [21] were transfected as previously described [12]. The serum-containing medium was exchanged 24 h after transfection for synthetic medium (modified from the procedure of Murakami [22]) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5 μg/ml insulin, 17 μg/ml transferrin, 20 μM ethanolamine, 100 μg/ml soybean trypsin inhibitor and 10 μg/ml aprotinin. This medium was recovered after 16 h of incubation and concentrated by centrifugation with a Centricon-10 (Amicon). Transfected cells were harvested and lysed with 1% n-octyl β-D-glucopyranoside (octyl glucoside) in Tris-buffered saline as described previously [12]. The solubilized extracts and concentrated culture media were analysed by SDS/PAGE [7.5% (v/v) gel] [23]; the proteins were transferred to nitrocellulose filters [24]. Wild-type and mutated NEP were detected by immunoblotting with an NEP-specific monoclonal antibody (18B5) as described elsewhere [25] and the immunocomplexes were detected with a Vectastain ABC kit (Burlingame, CA, U.S.A.). Endoglycosidase digestions before immunoblotting were performed as described previously [26].

Pulse–chase studies

At 48 h after transfection, COS-1 cells were washed with DMEM lacking methionine and cysteine and preincubated for 45 min at 37 °C in the same medium. The cells were then labelled for 15 min at 37 °C by the addition of [^{35}S]methionine and [^{35}S]cysteine (100 μCi/ml) (Amersham; specific radioactivity 1192 Ci/mmol). After the pulse, the cells were rinsed in DMEM containing an excess of non-radioactive methionine and cysteine (0.15 mg/ml each; Sigma) and were chased for various periods. The chase media were then collected and concentrated, then the harvested cells were lysed with 1% octyl glucoside in Tris-buffered saline. In some metabolic labelling experiments, transfected COS-1 cells were labelled for 20 min in the presence of

Brefeldin A at a concentration of 5 μg/ml and immediately harvested as described above. In both types of experiment, NEP-related proteins present in cell extracts or culture media were immunoprecipitated with a specific polyclonal antibody as described elsewhere [27]. The immunoprecipitated proteins were subjected to SDS/PAGE, either a 4–20 % gel gradient or 10 % gel [23], and detected by fluorography.

N-terminal sequence determination

Transfected COS-1 cells were labelled with [^{3}H]tyrosine as described previously [26] and the labelled secreted proteins were recovered by immunoprecipitation [27]. Proteins were then transferred to ProBlot membranes (Applied Biosystems) for sequencing in a gas-phase sequenator (Applied Biosystems; Model 470A).

Expression and isolation of GST fusion proteins

A single colony of DH-5 (Gibco-BRL) transformed with the plasmid of interest was grown overnight in 10 ml of 2YT medium containing 50 μg/ml ampicillin. The 10 ml overnight culture was then used to inoculate 500 ml of 2YT containing 50 μg/ml ampicillin, which was then incubated at 37 °C with constant agitation until D_{595} reached 0.4. Isopropyl β-D-thiogalactoside (IPTG) was then added to a final concentration of 0.5 mM and the incubation was continued for a period of 4 h to induce overexpression of the fusion protein (Figure 2A, compare lanes 1 and 2). Bacteria were pelleted by centrifugation (10 min, 5000 ***g***, 4 °C) and pellets were resuspended, at 25 ml per g of bacterial pellet, in ice-cold STE [10 mM Tris (pH 8.0)/150 mM NaCl/1 mM EDTA] containing 100 μg/ml lysozyme. The suspension was incubated for 10 min on ice before adding dithiothreitol to a final concentration of 5 mM. Lysis was achieved by sonication on ice (four periods of 30 s, power level 5; Sonifier Cell Disruptor; Heat Systems Co.) and the lysate was centrifuged (10000 ***g***, 20 min, 4 °C). At this point most of the NEP–GST fusion protein was found in the post-sonication pellet. We thus decided to use this insolubility as the first purification step because a significant amount of unrelated proteins were solubilized (Figure 2A, lane 3). The post-sonication pellet was then resuspended, at 12.5 ml per g of initial bacterial pellet, in STE containing 0.2 % *N*-laurylsarcosine and sonicated on ice (two periods of 30 s). The suspension was then clarified by centrifugation (10000 ***g***, 20 min, 4 °C) and the fusion protein was recovered in the *N*-laurylsarcosine supernatant (Figure 2A, lane 4). The protein concentration of the supernatant was determined by the Bradford assay and then incubated overnight at 4 °C with constant agitation in the presence of 0.2 NIH unit of human plasma thrombin (Sigma) per mg of fusion protein to cleave the peptide from the GST protein. It should be noted that the cleavage of the fusion protein by thrombin left two residues (Gly-Ser) of the GST protein at the N-terminus of the peptides (see Figure 1). Figure 2A (lane 2) shows that the fusion protein is induced as a doublet of 32 and 29 kDa. The reason for this is not clear and does not seem to be due to the NEP peptide moiety. Indeed, the primary sequence of the four NEP mutant peptides was confirmed by amino acid composition analysis (results not shown) and their homogeneity by SDS/PAGE (Figure 2C).

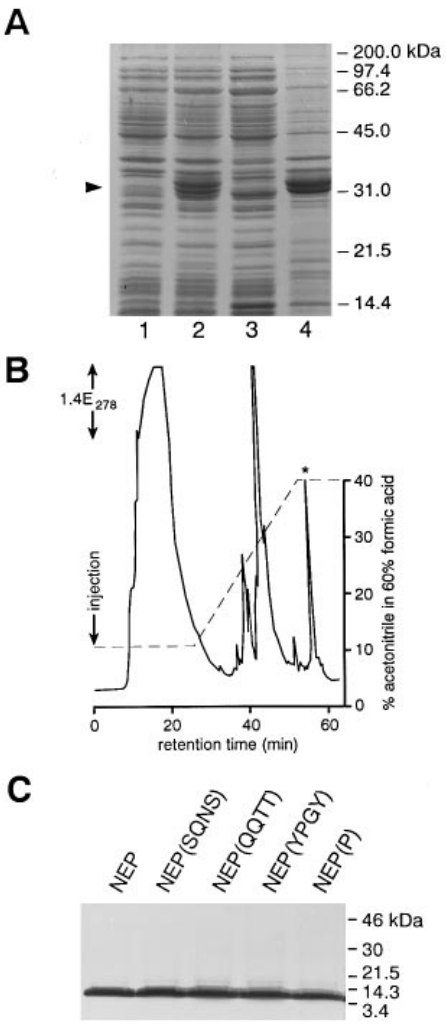

Figure 2 Peptide production and purification

(**A**) Bacteria transformed with the appropriate pGEX-2T vectors were induced by IPTG to overexpress the related fusion protein with GST. The bacterial lysate was centrifuged, and the pellet was resuspended in 0.2% *N*-laurylsarcosine then clarified by centrifugation. During overexpression and partial purification of the fusion protein, aliquots of the different steps were taken and analysed by SDS/PAGE (12.5% gel) and Coomassie staining. Lanes 1 and 2, non-induced and induced total lysate respectively; lane 3, first supernatant; lane 4, clarified supernatant (S2). The positions of molecular mass standards are shown at the right. (**B**) Peptide purification was performed with reverse-phase HPLC. Peptide samples (S2) adjusted to 10% (v/v) acetonitrile and 60% (v/v) formic acid were loaded at 2 ml/min on a C_{18} μbondapak column (8 mm × 100 mm). After a 25 min wash, peptide products were resolved with a 10–40% gradient of acetonitrile in 60% formic acid as indicated by the broken line. (**C**) Homogeneity of the purified peptides was monitored by SDS/PAGE on a 4–20% gradient gel. The fusion GST protein induced by IPTG is marked with a solid arrowhead. The peak corresponding to the purified peptide is indicated by a star. The positions of molecular mass standards are shown at the right.

Peptide purification

NEP peptide purification was performed by reverse-phase HPLC (modified from the procedure of Heukeshoven and Dernick [28]). Samples, containing 12 mg of proteins adjusted to 10 % (v/v) acetonitrile and 60 % (v/v) formic acid, were loaded at 2 ml/min on a C_{18} μbondapak column (8 mm × 100 mm, 10 μm particle size, 12.5 nm pore size; Waters) previously equilibrated with 10 % acetonitrile and 60 % formic acid. After a 25 min wash with 10 % acetonitrile and 60 % formic acid, peptide products were resolved with a 30 min linear gradient of 10–40 % acetonitrile in 60 % formic acid. The eluted peptides were detected by their absorbance at 278 nm and the peak fractions were collected (Figure 2B, see star). Solvents were evaporated and the peptides solubilized in trifluoroethanol. This last procedure was repeated

twice and samples were precipitated by the addition of water and freeze-dried. We used the same purification method for NEP(SQNS), NEP(QQTT), NEP(YPGY) and NEP(P) peptides. However, the retention time of each mutant peptide was slightly different from that of NEP and seemed to vary with the hydrophobic nature of the mutant peptide. The homogeneity of the purified peptides was monitored by SDS/PAGE on a 4–20 % gradient gel [23] (Figure 2C) and their primary structures were confirmed by sequencing and/or amino acid composition (results not shown).

CD measurements

CD spectra were performed at room temperature (25 °C) with a JASCO model J-710 spectropolarimeter. Cylindrical fused quartz cells of 0.05 cm path length were used. Samples were prepared by dissolving weighed quantities of the peptide in 1 % (w/v) octyl glucoside. The peptide concentration was 60 μM. All spectra were baseline-corrected and smoothed. The secondary structure of the peptides was determined by the Lincomb method as described by Perczel [29], the method of Chang [30] as accessed by the Antheprot package [31], and the method of Yang [32] supplied by JASCO.

RESULTS

The SQNS sequence, which promoted the cleavage of the NEP SA domain in some mutant proteins, is highly polar and according to Chou and Fasman [14] should promote the formation of a β-turn structure. To gain further insight into the structural determinants most important in promoting cleavage of the NEP SA domain we designed mutants where V^{42}TMI (the wild-type sequence) was replaced by the highly polar α-helix-compatible sequence Q^{42}QTT or the less polar but β-turn-promoting sequence Y^{42}PGY (according to Chou and Fasman [14]). These mutations were done in both full-length and truncated forms of NEP. As the presence of a Pro residue midway into the SA domain of sucrase–isomaltase was shown to promote protein secretion [17] *in vitro*, we also performed the single mutation $T^{43} \rightarrow P^{43}$. A representation of the mutants studied in this work is shown in Figure 1.

Expression of full-length NEP mutants in COS-1 cells

COS-1 cells were transfected with plasmids encoding the wild-type and mutated NEPs. Proteins from the culture media and the cell extracts were resolved by SDS/PAGE and detected by immunoblotting. NEP and NEP(P) were detected in the cell extracts as homogeneous bands, whereas no bands were observed for these proteins in the culture media (Figure 3A, lanes 3 and 4, and 11 and 12 respectively). This observation suggests that the α-helix-breaking proline residue cannot promote the secretion of NEP as it does in sucrase–isomaltase [17].

NEP(SQNS) was also found mostly in the cell extracts (Figure 3A, lanes 5 and 6) but the apparent molecular mass of the major polypeptide band was higher than that of NEP (Figure 3A, compare lanes 5 and 3). Because this difference in migration is not due to differences in glycosylation (see below) it can be best explained by the replacement of a highly hydrophobic sequence (V^{42}TMI) by a hydrophilic sequence (S^{42}QNS), which might change the amount of detergent bound to the polypeptide chain [33–35]. A second polypeptide of lower molecular mass could also be observed in the cell extracts of NEP(SQNS) as well as a faint band in the culture medium (Figure 3A, lanes 5 and 6). Densitometric evaluation of these bands showed that they represented no more than 5 % of the total NEP(SQNS) expression

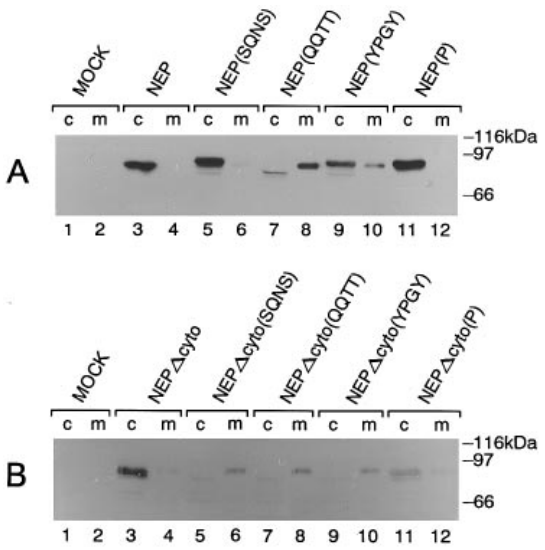

Figure 3 Immunoblotting of NEP and NEP mutant proteins

Equivalent quantities of proteins from cell extracts or culture media were separated by SDS/PAGE (7.5% gel), transferred to nitrocellulose membranes and revealed with an NEP-specific monoclonal antibody. (**A**) full-length proteins; (**B**) truncated proteins. Abbreviations: c, cellular extracts; m, culture media. The positions of molecular mass standards are shown at the right.

level. These results are in agreement with those previously obtained with this mutant [13]. In contrast, most of the NEP(QQTT) protein was detected in the culture medium, whereas only a faint band of lower molecular mass was observed in the cell extracts (Figure 3A, lanes 7 and 8). With NEP(YPGY) very little protein was found in the culture medium and most of the protein was present in cell extracts (Figure 3A, lanes 9 and 10).

Endoglycosidase digestion of the immunoreactive polypeptides found in the cell extracts was undertaken to distinguish between proteins that had reached the cell surface and those delayed in the ER. As expected, NEP was mostly resistant to digestion with endoglycosidase H (EC 3.2.1.96; endo H) but sensitive to treatment with endoglycosidase F (EC 3.2.1.96; endo F) (Figure 4A, lanes 4–6). These observations suggest that the protein had acquired complex sugars by travelling through the Golgi apparatus. Similar results were obtained for NEP(P) (Figure 4A, lanes 16–18) and for the major species of NEP(SQNS) and NEP(YPGY) (Figure 4A, lanes 7–9 and 13–15 respectively). It was not possible to evaluate clearly the effect of endoglycosidase treatment on the minor species of NEP(SQNS) and NEP(YPGY) because these bands were too weak. In comparison, the intracellular form of NEP(QQTT) was sensitive to endo H digestion as well as to endo F digestion (Figure 4A, lanes 10–12), suggesting that it represents NEP(QQTT) trapped in the ER as was shown previously for other mutants [12].

Similar studies were performed on proteins from the culture media of COS-1 cells expressing NEP(SQNS), NEP(QQTT) and NEP(YPGY). The results indicate that all the secreted forms had acquired complex sugars because they were endo H-resistant (Figure 4B, lanes 4–12).

Expression of truncated NEP in COS-1 cells

Analysis of cell extracts and culture media from transfected COS-1 cells showed that NEP Δcyto and NEP Δcyto(P) were

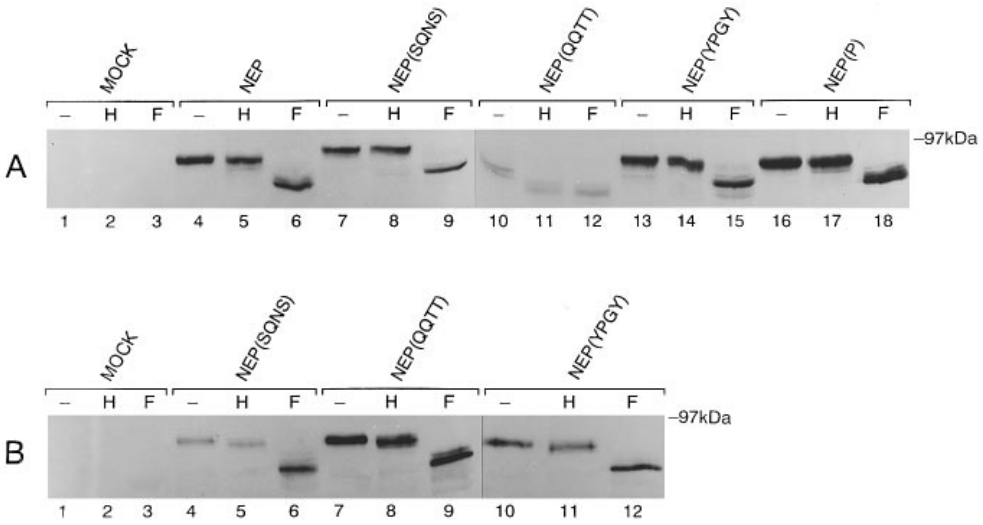

Figure 4 Endoglycosidase treatment of NEP and full-length NEP mutant proteins

Equivalent quantities of proteins from cell extracts (**A**) or culture media (**B**) were treated with endo H (H) or endo F (F), or left untreated (—). The position of a molecular mass standard is shown at the right.

found mostly in the cell extracts, whereas minor amounts were detected in the culture media (Figure 3B, lanes 3 and 4, and 11 and 12 respectively). As was shown previously, deletion of the cytoplasmic tail of NEP is not sufficient to promote efficient cleavage of the SA domain [12], and the presence of Pro does not significantly increase the processing of the SA domain.

For NEP Δcyto(SQNS), NEP Δcyto(QQTT) and NEP Δcyto(YPGY) the situation is somewhat different because proteins were detected mostly in the culture media (Figure 3B, lanes 6, 8 and 10 respectively). These results indicate that the presence of a hydrophilic and/or a β-turn-promoting sequence inside the SA domain of NEP Δcyto can promote secretion of the truncated protein. In addition to the secreted forms, intracellular forms of NEP Δcyto(SQNS), NEP Δcyto(QQTT) and NEP Δcyto(YPGY) were also observed (Figure 3B, lanes 5, 7 and 9 respectively). These intracellular forms had lower molecular masses and were sensitive to endo H digestion (results not shown). These bands most probably represent molecules trapped in the ER.

Although mutations in the full-length series did not significantly influence the expression levels of the mutants, we observed a different situation when the cytoplasmic tail was deleted. Indeed, the expression levels of most truncated mutants were very low (Figure 3B) compared with the full-length series (Figure 3A), suggesting that the deletion of the cytoplasmic tail interfered with the biosynthesis of these proteins. Translation *in vitro* of mRNA species encoding NEP and NEP mutants lacking the cytosolic domain in the presence of microsomes indicated that these mutant proteins were mostly inserted in the microsome membrane as type III membrane protein [36]. In a cellular environment this would surely result in their rapid degradation.

Biosynthesis of NEP and NEP(QQTT)

Substitution of Q^{42}QTT for V^{42}TMI without previous deletion of the cytoplasmic tail resulted in secretion of the protein. To compare the biosynthesis of the secreted protein with that of the membrane-bound protein, COS-1 cells expressing NEP or

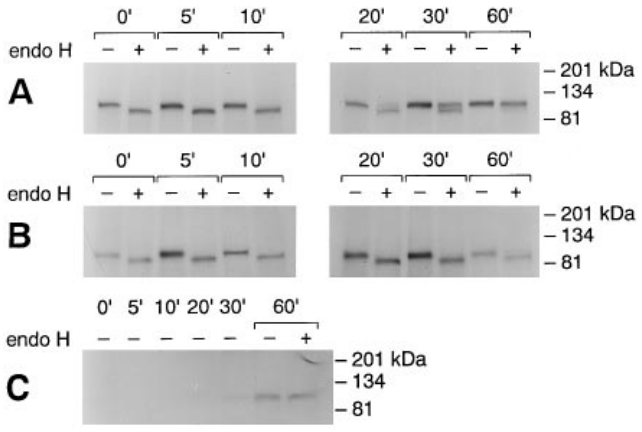

Figure 5 Biosynthesis of NEP and NEP(QQTT) in COS-1 cells

COS-1 cells expressing either NEP or NEP(QQTT) were labelled with Tran^{35}S-label for 15 min. After various periods, cells were solubilized or culture media were concentrated and NEP or NEP(QQTT) was immunoprecipitated. Half of the immunoprecipitated product was subjected to endo H digestion (+). Proteins were resolved by SDS/PAGE on a 4–20% gradient and then revealed by fluorography. The exposure time on Fuji X-ray films was 3 days for (**A**) and (**B**), and 6 days for (**C**). (**A**) NEP cell extracts; (**B**) NEP(QQTT) cell extracts; (**C**) NEP(QQTT) culture media. The positions of molecular mass standards are shown at the right. Abbreviation: ′, min.

NEP(QQTT) were labelled with Tran^{35}S-label followed by various chase times (Figure 5). NEP was initially synthesized as an endo H-sensitive form that started to acquire endo H resistance after approx. 20 min of chase (Figure 5A). After 60 min, all NEP was endo H-resistant, suggesting effective carbohydrate processing and therefore efficient transport out of the ER to the mid-Golgi apparatus, where these reactions take place. The soluble protein NEP(QQTT) was also synthesized as an endo H-sensitive form but no endo H-resistant form could be observed in cell extracts even after 60 min of chase (Figure 5B). However, after 30 min we detected an endo H-resistant soluble form in the

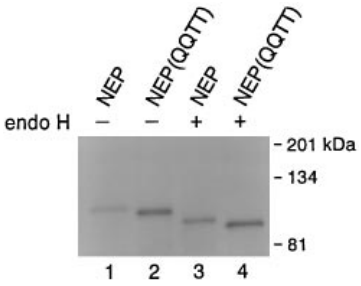

Figure 6 Metabolic labelling of COS-1 cells in the presence of Brefeldin A

COS-1 cells expressing either NEP or NEP(QQTT) were labelled with Tran^{35}S-label for 20 min in the presence of Brefeldin A. Cells were then solubilized and NEP or NEP(QQTT) was immunoprecipitated. Half of the immunoprecipitated product was subjected to endo H digestion (+). Proteins were resolved by SDS/PAGE (10% gel) and revealed by fluorography. The exposure time was 7 days on Fuji X-ray film. The positions of molecular mass standards are shown at the right.

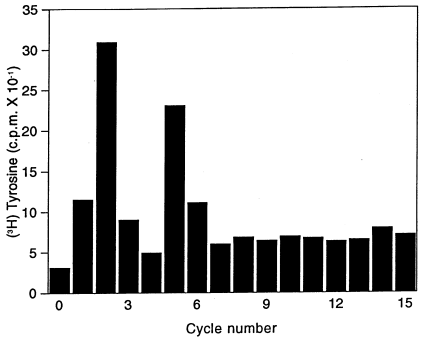

Figure 7 Partial N-terminal sequence of NEP(QQTT)

COS-1 cells expressing NEP(QQTT) were grown in the presence of [^{3}H]tyrosine. The ^{3}H-labelled NEP(QQTT) was recovered by immunoprecipitation and subjected to 15 cycles of automated Edman degradation.

culture medium (Figure 5C), suggesting that once the intracellular form had reached the mid-Golgi compartment, rapid transport of the protein occurred.

It has been suggested that a few type II membrane proteins such as sialyltransferase, dopamine β-mono-oxygenase, and tumour necrosis factor were not released from the membrane by the signal peptidase [37] but rather by other proteases in post-ER compartments [38]. To verify that NEP(QQTT) was solubilized in the ER by signal peptidase, COS-1 cells expressing this NEP mutant were labelled with Tran^{35}S-label. Brefeldin A was added to the culture medium to increase the amount of ER-associated proteins because it is known to rapidly block the exocytic transport of proteins from the ER to the Golgi apparatus [39]. Under these conditions, only endo H-sensitive forms of NEP and NEP(QQTT) accumulated in the cell extracts, suggesting their presence in the ER (Figure 6, compare lane 1 with lane 3, and lane 2 with lane 4). A difference of 2 kDa can be observed between the processed and unprocessed proteins rather then the 5.5 kDa expected from the loss of the NEP cytoplasmic and transmembrane domains. One explanation for this discrepancy might again be the migration of the soluble NEP(QQTT), which is underestimated compared with NEP by the SDS/PAGE technique. Indeed, the cleavage of the cytoplasmic and the highly hydrophobic transmembrane domains of NEP(QQTT) might change the amount of detergent bound to the polypeptide chain [33–35]. Because the molecular mass of the ER-accumulated form of NEP(QQTT) digested with endo H is similar to that of the endo F-treated soluble form (Figure 6, lane 4, and Figure 4B, lane 9; also results not shown) we conclude that no further processing of NEP(QQTT) occurred in post-ER compartments. These results confirm that solubilization of NEP(QQTT) occurred in the ER presumably by signal peptidase.

N-terminal sequencing of NEP(QQTT)

To confirm cleavage and to determine the precise site of cleavage, COS-1 cells expressing NEP(QQTT) were labelled with [^{3}H]tyrosine. The labelled NEP-related proteins secreted into the culture medium were collected by immunoprecipitation and submitted to 15 cycles of Edman degradation. Peaks of [^{3}H]tyrosine were observed at positions 2 and 5 (Figure 7). The molecular mass determined for the soluble form of NEP(QQTT) and the positions of tyrosine residues in the primary structure are consistent with cleavage of NEP(QQTT) on the CO_2H side of Ala^{46} in the SA domain [40] (Figure 1).

Analysis of the secondary structure of NEP and mutant NEP transmembrane domains

Because we observed striking differences in the processing of NEP and its mutants, we decided to investigate the structural changes induced by the mutations in the transmembrane segment. Peptides corresponding to residues 15–54 of NEP and related NEP mutants (SQNS, QQTT, YPGY and P; Figure 1) were produced in *Escherichia coli* as GST fusion proteins. We chose this strategy to obtain significant amounts of peptides (5–10 mg), which might have been difficult to produce by the usual synthetic method because of the peptide length and hydrophobicity. It can be argued that the conformations in solution of the peptides corresponding to NEP wild-type and mutant transmembrane domains might differ greatly from their conformation within the context of the intact protein. At least two observations suggest that this is not so. First, we have shown that the ectodomain of NEP can acquire a conformation compatible with activity in the absence of the transmembrane domain [26], thus ruling out the existence of significant interaction between these domains during protein folding. Secondly, we have shown that NEP transmembrane domain translated in a reticulocyte system *in vitro* in the presence of microsomes can anchor the protein in the membrane even in the absence of the full NEP ectodomain and with or without the cytosolic tail [36], suggesting that the NEP transmembrane domain can achieve a function-competent conformation *in vitro*.

The secondary structure of the purified peptides was analysed by CD measurements in the interfacial micellar environment of 1% octyl glucoside (Figure 8). Octyl glucoside was selected on the one hand for its capacity to solubilized native NEP in its enzymically active form [41] and on the other hand as a simple interfacial environment mimicking a membrane. The CD spectrum of NEP peptide in octyl glucoside has two negative bands at approx. 208 and 222 nm, and one positive band at approx. 192 nm (Figure 8). This suggests that the peptide has mostly a helical structure [42]. Computer analysis of the CD spectrum by different methods [29,30,32], to estimate the relative contributions of α-helix, β-sheet, coil and turn, indicated that this sequence has

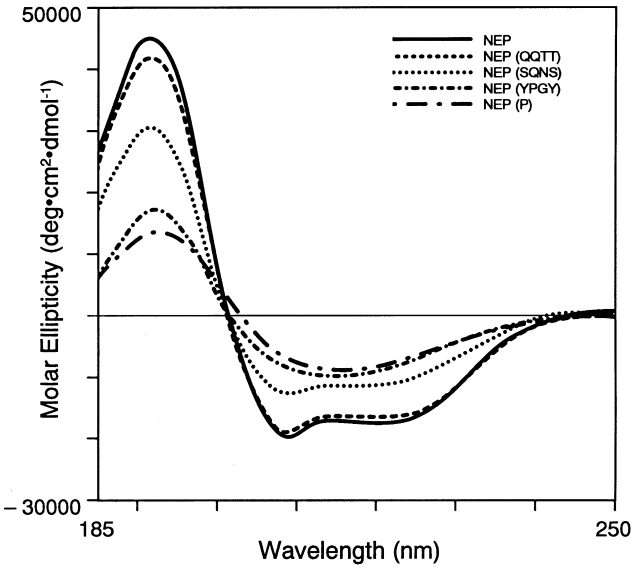

Figure 8 CD spectra of NEP related peptides in 1% octyl glucoside

an α-helical content of 55–60% (depending on the computer software used). This result is in good agreement with the generally accepted idea, which suggests that a transmembrane domain spans the lipid bilayer as an α-helix. However, the α-helical content is low compared with that of a synthetic signal peptide (70%) [43] or of a synthetic transmembrane peptide (90%) [44]. This discrepancy might best be explained by differences in the lengths of the regions flanking the hydrophobic core and by the nature of the residues in these flanking regions. Except for NEP(QQTT) peptide, the CD spectra of the other mutant peptides were different from that of NEP (Figure 8). The intensities of the different bands described earlier for NEP decreased slightly for the NEP(SQNS) mutation and drastically for the NEP(YPGY) and NEP(P) mutations. Computer analysis of these spectra revealed that the α-helix content of these peptides decreased to 45–50%, 30–40% and 40–45% respectively. Similar results were obtained when the peptides were dissolved in 95% (v/v) trifloroethanol (results not shown).

DISCUSSION

The SA domain of type II transmembrane proteins promotes translocation and anchors the protein in the lipid bilayer. This domain, which is thought to form an α-helical structure in the membranous environment [44], is not cleaved by signal peptidase. The experiments presented in this paper were designed to obtain insights into the properties of structural elements that could promote processing of SA domain by signal peptidase. For this study we constructed six mutants of NEP by mutation of the SA domain of full-length and truncated NEP with two different sequences (QQTT and YPGY) and with the single residue Pro. In addition, the previously described NEP mutants having the SQNS substitution in the SA domain were also studied [13]. The rationale for the use of these sequences was derived from the observation that substitution in the NEP SA domain of the sequence SQNS, which is polar and has a high propensity for β-turn formation (α-helix-breaking sequence), could promote SA domain processing under certain conditions [12,13]. The QQTT sequence is polar but according to Chou and Fasman [14] is compatible with the α-helix structure of the NEP SA domain. The YPGY sequence and the single Pro residue are less polar and have α-helix-breaking potential [14]. It is interesting to note that the predicted effects of these mutations on the structure of the NEP SA domain were confirmed by CD analysis of the corresponding peptides. The full-length series of mutants were referred to as NEP(SQNS), NEP(QQTT), NEP(YPGY) and NEP(P), whereas the truncated (without the cytoplasmic domain) series of mutants were referred to as NEP Δcyto(SQNS), NEP Δcyto(QQTT), NEP Δ(YPGY), and NEP Δcyto(P). The mutants as well as the control proteins NEP and NEP Δcyto were expressed *in vivo* by using simian virus 40-based vectors and COS-1 cells.

Analysis of the different mutations undertaken in the SA domain of full-length and truncated NEP shows that the introduction of the α-helix-breaking residue Pro is not enough to promote the secretion of the proteins as was shown for sucrase–isomaltase [17]. In the latter case, the Pro mutation, which was positioned 15 amino acids from the beginning of the SA domain (as in the NEP Pro mutants), induced cleavage at a site located two residues upstream of the mutation. Because neither NEP nor sucrase–isomaltase SA domains contain other helix-breaking residues, we suggest that other structural elements present in the flanking regions of the sucrase–isomaltase hydrophobic core might be important in specifying the signal peptidase cleavage site. This hypothesis is consistent with the observations that deletions and other mutations in the mature domain of a secreted protein [45,46] as well as drastic shortening of the long n-region of some SA domains [10,11] can affect signal sequence cleavage. Furthermore we show that deletion of the cytoplasmic domain of NEP in addition to the introduction of the Pro residue inside the SA domain does not significantly increase processing of the truncated protein. Our results thus indicate that proline itself can be tolerated inside the NEP SA domain as it is in rat dipeptidylpeptidase IV [47].

The situation is somewhat different with the other mutations that we introduced inside the SA domain of NEP. We showed that the QQTT sequence induces the efficient secretion of NEP, whereas the SQNS and YPGY sequences cannot promote efficient cleavage of the full-length proteins (Figure 3A). In contrast, we observed that their respective truncated mutants were all secreted into the culture medium (Figure 3B), indicating that the three sequences promote cleavage of NEP Δcyto SA domain independently of their effect on the SA domain structure (see Figure 8). One explanation for these results might be that, on cytoplasmic domain deletion, the NEP SA domain structure is more relaxed and therefore allows the putative signal peptidase cleavage site to acquire a structure and/or to be placed in a position favourable for recognition by the enzyme.

The secretion of full-length NEP is induced efficiently only by the hydrophilic QQTT sequence. Microsequencing experiments showed that cleavage occurs 1 residue downstream of the sequence. In NEP(QQTT) and other mutants, this site was predicted as a potential signal peptidase cleavage site by the algorithm of von Heijne [8]. That signal peptidase does cleave at this site is supported by metabolic labelling studies in the presence of Brefeldin A, which showed that the ER-accumulated form of NEP(QQTT) was already processed. We also showed that no further processing of NEP(QQTT) occurred in post-ER compartments because the molecular mass of the ER-accumulated form digested with endo H was the same as that of the secreted form digested with endo F. Thus our results indicate that processing of the NEP(QQTT) SA domain is performed by signal peptidase. This cleavage does not seem to be a rate-limiting step in the biosynthesis of soluble NEP because pulse–chase studies showed that it reached the culture medium after 30 min of chase, which

compares well with the 40 min observed for cell-surface delivery of membrane-bound NEP [48]. Furthermore we observed no accumulation of endo H-resistant forms of NEP(QQTT) in cell extracts, suggesting that rapid transport of the protein occurred after processing in the ER.

Structural predictions and CD analysis showed that the sequence QQTT is compatible with the α-helix structure of the NEP SA domain, whereas the sequences SQNS and YPGY decrease the α-helix content of the peptide, possibly by increasing the propensity for β-turn formation [14]. That the QQTT sequence but not the SQNS or YPGY sequence promoted cleavage of the NEP SA domain is rather surprising, considering that the presence of β-turn promoting residues in the vicinity of signal peptidase cleavage sites was shown to enhance cleavage [15–17]. One explanation for our observations might be that the sequences SQNS and YPGY, which have been introduced in close proximity to the putative cleavage site, promote structural changes that preclude recognition of the site by signal peptidase in full-length NEP. This hypothesis is consistent with our previous results showing that moving the SQNS sequence three residues upstream promoted efficient cleavage at the same site in full-length NEP [13]. At present we cannot totally exclude the possibility that the specific structures promoted by the substituted sequences mediate different interactions with the components of the ER translocase, resulting in the different positioning of the SA domain in the proteinaceous channel [49,50]. In conclusion, our structural and biochemical results suggest that substitution of a polar sequence that maintains the α-helical structure of the hydrophobic segment and that is in close proximity to a putative signal peptidase cleavage site is sufficient to promote cleavage of the NEP SA domain.

We thank Dr. Joanne Turnbull of the Concordia University for invaluable help in CD analysis and for stimulating discussions. This work was supported by a grant from the Medical Research Council of Canada to G.B.

REFERENCES

1 Walter, P., Gilmore, R. and Blobel, G. (1984) Cell **38**, 5–8
2 Wickner, W. T. and Lodish, H. F. (1985) Science **230**, 400–407
3 Jackson, R. C. and Blobel, G. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5598–5602
4 Strauss, A. W., Zimmerman, M., Boime, I., Ashe, B., Mumford, R. A. and Alberts, A. W. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4225–4229
5 von Heijne, G. (1990) J. Membr. Biol. **115**, 195–201
6 Bird, P., Gething, M.-J. and Sambrook, J. (1990) J. Biol. Chem. **265**, 8420–8425
7 von Heijne, G. (1983) Eur. J. Biochem. **133**, 17–21
8 von Heijne, G. (1986) Nucleic Acids Res. **14**, 4683–4690
9 Perlman, D. and Halvorson, H. O. (1983) J. Mol. Biol. **167**, 391–409
10 Lipp, J. and Dobberstein, B. (1986) Cell **46**, 1103–1112
11 Schmid, S. R. and Spiess, M. (1988) J. Biol. Chem. **263**, 16886–16891
12 Roy, P., Chatellard, C., Lemay, G., Crine, P. and Boileau, G. (1993) J. Biol. Chem. **268**, 2699–2704
13 Yang, X.-F., Chatellard, C., Lazure, C., Crine, P. and Boileau, G. (1994) Arch. Biochem. Biophys. **315**, 382–386
14 Chou, P. Y. and Fasman, G. D. (1978) Annu. Rev. Biochem. **49**, 251–261
15 Yamamoto, Y., Taniyama, Y. and Kikuchi, M. (1989) Biochemistry **28**, 2728–2732
16 Nothwehr, S. F. and Gordon, J. I. (1989) J. Biol. Chem. **264**, 3979–3987
17 Hegner, M., von Kieckebush-Guck, A., Falchetto, R., James, P., Semenza, G. and Mantei, N. (1992) J. Biol. Chem. **267**, 16928–16933
18 Sambrook, J., Fritsh, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D. and Smith, J. A. (1988) Current Protocols in Molecular Biology, Wiley Interscience, New York
20 Tabor, J. M. and Richardson, C. C. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4767–4771
21 Gluzman, Y. (1981) Cell **23**, 175–182
22 Murakami, H., Masui, H., Sato, G. H., Sueoka, N., Chow, T. P. and Kano-Suoka, T. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 1158–1162
23 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
24 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
25 Dion, N., Lemoual, H., Crine, P. and Boileau, G. (1993) FEBS Lett. **318**, 301–304
26 Lemay, G., Waksman, G., Roques, B. P., Crine, P. and Boileau, G. (1989) J. Biol. Chem. **264**, 15620–15623
27 Corbeil, D., Boileau, G., Lemay, G. and Crine, P. (1992) J. Biol. Chem. **267**, 2798–2801
28 Heukeshoven, J. and Dernick, R. (1985) J. Chromatogr. **326**, 91–101
29 Perczel, A., Park, K. and Fasman, G. D. (1992) Anal. Biochem. **203**, 83–93
30 Chang, C. T., Wu, C.-S. C. and Yang, J. T. (1978) Anal. Biochem. **91**, 13–31
31 Deléage, G., Clerc, S. S., Roux, B. and Gautheron, D. C. (1988) Cabios **4**, 351–356
32 Yang, J. T., Wu, C.-S. C. and Martinez, H. M. (1986) Methods Enzymol. **130**, 208–269
33 Virella, G. and Marques Coelho, I. (1974) Immunochemistry **11**, 157–160
34 de Jong, W. W., Zweers, A. and Cohen, L. H. (1978) Biochem. Biophys. Res. Commun. **82**, 532–539
35 Noël, G., Nikaido, K. and Ferro-Luzzi Ames, G. (1979) Biochemistry **18**, 4159–4165
36 Lemire, I., Roy, P. and Boileau, G. (1994) Biochem. Cell Biol. **72**, 182–187
37 Ehlers, M. R. W. and Riordan, J. F. (1991) Biochemistry **30**, 10065–10074
38 Paulson, J. C., Weinstein, J., Ujita, E. L., Riggs, K. J. and Lai, P.-H. (1987) Biochem. Soc. Trans. **15**, 618–620
39 Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L. and Klausner, R. D. (1991) Cell **67**, 601–616
40 Devault, A., Lazure, C., Nault, C., Le Moual, H., Seidah, N. G., Chrétien, M., Khan, P., Powell, J., Mallet, J. and Beaumont, A., et al. (1987) EMBO J. **6**, 1317–1322
41 Aubry, M., Bertelott, A., Beaumont, A., Roques, B. P. and Crine, P. (1987) Biochem. Cell Biol. **65**, 398–404
42 Fasman, G. D. (1993) Biotechnol. Appl. Biochem. **18**, 111–138
43 McKnight, C. J., Briggs, M. S. and Gierasch, L. M. (1989) J. Biol. Chem. **264**, 17293–17297
44 Davis, J. H., Clare, D. M., Hodges, R. S. and Bloom, M. (1983) Biochemistry **22**, 5298–5305
45 Folz, R. J. and Gordon, J. I. (1986) J. Biol. Chem. **261**, 14752–14759
46 Burgess, T. L., Craik, C. S., Matsuuchi, L. and Kelly, R. B. (1987) J. Cell Biol. **105**, 659–668
47 Hong, W. and Doyle, D. (1990) J. Cell Biol. **111**, 323–328
48 Howell, S., Lanctôt, C., Boileau, G. and Crine, P. (1994) Biochem. J. **299**, 171–176
49 Nilsson, I., Whitley, P. and von Heijne, G. (1994) J. Cell Biol. **126**, 1127–1132
50 Martoglio, B., Hofmann, M. W., Brunner, J. and Dobberstein, B. (1995) Cell **81**, 207–214

Received 27 July 1996/17 October 1996; accepted 17 October 1996