The nature of topogenic sequences determines the transport competence of topological mutants of neutral endopeptidase-24.11

Xiao-Feng YANG*, Philippe CRINE and Guy BOILEAU

Département de biochimie, Faculté de médecine, Université de Montréal, Montréal, Québec, Canada H3C 3J7

Type II integral membrane proteins are anchored by a signal-peptide/membrane-anchor domain (SA domain) located near their N-terminus, whereas type I membrane proteins are anchored by stop-transfer sequences usually located near the C-terminus. In this study we have attempted to transform neutral endopeptidase-24.11 (EC 3.4.24.11; NEP), a type II membrane protein, into a type I membrane protein. Three type I mutant proteins were constructed by fusion of topogenic sequences to the C-terminus of SecNEP, a soluble form of NEP. The first two type I mutants, SecNEP-TMC and SecNEP-TMIC, were constructed by fusing in frame the cytosolic and SA domains of NEP to the C-terminus of SecNEP. These two fusion proteins differ only in the orientation of the cytosolic tail. The third type I

mutant, SecNEP-ACE, was constructed by fusing in frame the stop-transfer and cytosolic domains of angiotensin I-converting enzyme (EC 3.4.15.1; ACE) to the C-terminus of SecNEP. Our results suggest that: (1) the NEP ectodomain can be anchored with a type I topology in the endoplasmic reticulum (ER) membrane by both NEP and ACE topogenic sequences; (2) SecNEP-TMC and SecNEP-TMIC were transport-incompetent and needed proteolytic cleavage in the C-terminal region to leave the ER, whereas SecNEP-ACE was transported out of the ER as a type I membrane protein. Therefore we concluded that the nature of topogenic sequences determines the transport-competence of topological mutants of neutral endopeptidase-24.11.

INTRODUCTION

Translocation of type I membrane proteins into the endoplasmic reticulum (ER) is initiated by a cleavable signal peptide located at the N-terminus, similar to that found in secretory proteins. A hydrophobic stop-transfer sequence in the C-terminal domain of the protein prevents further translocation and anchors the protein into the lipid bilayer. Mature type I proteins thus have a large N-terminal ectodomain. In type II membrane proteins an uncleaved signal-peptide/membrane-anchor domain (SA domain), located near the N-terminus, is responsible for anchoring the protein in the membrane. Type II membrane proteins thus have a large C-terminal ectodomain [1,2].

The roles of the cytosolic and transmembrane domains of integral membrane proteins have been studied extensively by gene fusion technology [3–5]. This work showed that these protein domains contained information for the translocation, oligomerization and intracellular transport of the membrane proteins. The same approach has also been used to study the topogenic function of these different sequences (signal peptide, SA and stop-transfer domains). It has been suggested that there may be no fundamental difference between these topogenic elements besides their relative position within a given protein [6–10]. However, another study showed that signal and stop-transfer sequences are not interchangeable elements [11]. Furthermore, it is clear that some hydrophobic stretches are entirely translocated across, rather than inserted into, membranes [2]. Thus it appears that stop-transfer activity of hydrophobic sequences cannot simply be predicted on the basis of their position and hydrophobicity [12,13].

Previous studies on the exchange of SA and stop-transfer domains mainly used systems *in vitro*, so that correct folding and intracellular transport-competence of passenger domains were

not considered. Furthermore, this approach has been limited to the exchange of domains between proteins of the same type and, to our knowledge, there exist no reports of the transformation *in vivo* of a type II integral membrane protein into a type I membrane protein. In the present study we have attempted to transform neutral endopeptidase-24.11 (EC 3.4.24.11; neprilysin; NEP), a type II membrane protein, into a type I integral membrane protein *in vivo*. Rabbit NEP is a 749-residue protein consisting of a short N-terminal cytosolic domain (27 residues), a single membrane-spanning segment (23 residues; SA domain) and a C-terminal ectodomain that comprises most of the protein mass [14]. SecNEP, a soluble form of NEP, was engineered by substituting a cleavable signal peptide for the N-terminal cytosolic and SA domains of NEP [15]. In this paper, three mutant proteins were constructed. The first two mutants, SecNEP-TMC and SecNEP-TMIC, were constructed by fusing in frame the SA and cytosolic domains of NEP to the C-terminus of SecNEP. SecNEP-ACE was constructed by fusing in frame the stop-transfer and cytosolic domains of angiotensin I-converting enzyme (EC 3.4.15.1; ACE) [16] to the C-terminus of SecNEP. The results indicate that the transmembrane and cytosolic sequences at the C-terminus of the NEP ectodomain stopped further translocation of nascent polypeptides and anchored them into the ER membrane in a type I manner. However, the SecNEP-TMC and SecNEP-TMIC type I membrane proteins were found to be transport-incompetent, whereas SecNEP-ACE was transported out of the ER.

MATERIALS AND METHODS

DNA manipulations

DNA manipulations were performed by standard procedures [17]. Site-directed mutagenesis was performed as described by

Abbreviations used: ACE, angiotensin I-converting enzyme (EC 3.4.15.1); BFA, brefeldin A; endo F, endo- β -N-acetylglucosaminidase F (EC 3.2.1.96); endo H, endo- β -N-acetylglucosaminidase H (EC 3.2.1.96); ER, endoplasmic reticulum; MAbs, monoclonal antibody; NEP, neutral endopeptidase (EC 3.4.24.11; neprilysin); octyl glucoside, 1-O-n-octyl β -D-glucopyranoside; SA domain, signal-peptide/membrane-anchor domain.

* To whom correspondence should be sent. Present address: Laboratory of Immunopathology, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115, U.S.A.

Figure 1 (a) Schematic representation of the C-terminal extensions of SecNEP; (b) sequence of the transmembrane and cytosolic domains of the type I fusion proteins

(a) Details of the constructions are presented in the Materials and methods section. The black triangles point to the Glu → Val substitution. (b) The boxed amino acid residues were encoded by the DNA linkers used in the genetic manipulations.

Taylor et al. [18] using a kit for mutagenesis *in vitro* from Amersham. Cartridge-mutagenesis was done essentially as described by Murray et al. [19]. PCR conditions were as described by Scharf [20]. Oligonucleotides were purified by urea/PAGE, followed by desalting on Sep-Pak C₁₈ columns. In every case, the presence of the mutations in the expression vectors was confirmed by sequencing the mutated regions by the chain-termination method for double-stranded DNA templates, using T7 DNA polymerase [21].

Construction of the SecNEP C-terminal extension mutant

SecNEP is a soluble form of NEP [15]. Extension of the SecNEP C-terminus was achieved in three steps. First, site-directed mutagenesis was performed on a M13 subclone containing most of the NEP cDNA, in order to change the stop codon (TGA) to a serine codon (TCA). This extended the reading frame to the next in-frame stop codon, which was 66 bp (22 amino acid residues) downstream from the original stop codon. Second, another round of mutagenesis was made to change the cysteine codon located immediately in front of

the new stop codon into an alanine codon, in order to avoid the presence of a free thiol group in the recombinant protein. At the same time the mutation created a unique *Mlu*I restriction site, at the end of the coding sequence, in which subsequent insertions would be made. Finally, a fragment containing the mutated region was isolated from the replicating form of the M13 recombinant phage and substituted in pSVsecENK19, an expression vector using the simian-virus-40 early promoter to express SecNEP in COS-1 cells [15]. This new expression vector was called pSVsecENK19-E and encoded the protein SecNEP-E.

Construction of type I mutants of SecNEP-E

This was achieved by introducing DNA cartridges in the *Mlu*I site of pSVsecENK19-E. Three pairs of complementary oligonucleotides were synthesized. The first pair encoded the transmembrane domain of NEP, and the second encoded the cytosolic domain of NEP in the N → C orientation. The third pair also encoded the cytosolic domain of NEP, but in the C → N orientation. These oligonucleotides also had appropriate cohesive ends that would allow their assembly, in-frame, in the *Mlu*I

restriction site of pSVsecENK19-E. Two expression vectors were thus constructed. The first one, called pSVsecENK-TMC, contained the transmembrane cartridge (pair 1) in tandem with the cytosolic cartridge (pair 2) cloned in the *Mlu*I site of pSVsecENK-E. The second one, called pSVsecENK-TMIC, contained the transmembrane cartridge (pair 1) and the inverse sequence of the cytosolic domain (pair 3) also cloned in the *Mlu*I site of pSVsecENK-E. These vectors encoded the SecNEP-TMC and SecNEP-TMIC proteins respectively. The expression vector pSVsecENK-ACE was constructed by inserting in-frame in the *Mlu*I site of pSVsecENK-E a DNA fragment encoding the ACE stop-transfer and cytosolic domains. This DNA fragment was obtained by PCR amplification using a human ACE cDNA clone as a template (generously given by Dr. F. Alhenc-Gelas, Paris, France) with two primers having *Mlu*I sites at the 5' end. This vector promotes the synthesis of the fusion protein SecNEP-ACE. The structures of the fusion proteins are depicted in Figure 1(a), and the sequences of the fused protein domains are shown in Figure 1(b).

Culture of COS-1 cells and transfection

Culture conditions for COS-1 cells and transfection protocols have been described in detail elsewhere [22,23]. [³⁵S]Methionine labelling of COS-1 cells was performed for 30 min by the protocol published elsewhere [24], except that brefeldin A (BFA), at a concentration of 10 µg/ml, was added to the culture media 20 min before collecting the cells.

Isolation of intracellular membranes, Na₂CO₃ treatment and immunoprecipitation

At the end of the [³⁵S]methionine labelling, cells were lysed and the intracellular vesicular membranes isolated as described in [25]. Membranes were treated with Na₂CO₃ at pH 11.5 to release the soluble proteins [26] and were then pelleted by centrifugation. NEP-related proteins present in the supernatant were immunoprecipitated as described previously [27].

Protein extraction

At 28 h after transfection, the serum-containing medium in each Petri dish was replaced by 5 ml of synthetic medium as described previously [15]. The synthetic medium was recovered after 16 h of incubation and concentrated to 250 µl/dish by ultrafiltration using a Centricon-10 (Amicon). Transfected cells were rinsed twice with TBS (10 mM Tris, 150 mM NaCl, pH 7.5) and recovered by scraping with a rubber policeman. Cells were then solubilized in 250 µl of TBS containing 1 % octyl glucoside/dish.

Digestion with endo-β-N-acetylglycosaminidase H and F (endo H and endo F)

Portions of cell extracts or of Centricon-concentrated culture media were incubated for 20 h at 37 °C with 0.01 EC unit of endo H or 0.5 unit (1 unit = 1 µmol/60 min) of endo F (Boehringer-Mannheim, Canada) in sodium acetate buffer (50 mM sodium acetate, pH 5.5, 25 mM EDTA, 1 % β-mercaptoethanol, 0.02 % SDS and 0.5 mM PMSF).

SDS/PAGE and immunoblotting

These were performed essentially as described previously [15]. Two monoclonal antibodies (MAbs) were used for immunoblotting, either MAb 18B5, which is specific for the ectodomain of NEP (P. Crine, unpublished work), or MAb 23B11, which is specific for the cytosolic domain of NEP [28]. The domain

specificity of MAb 18B5 has also been clearly confirmed in the present experiments, since it recognizes SecNEP, which lacks the cytosolic and transmembrane domains of NEP (see lanes 3–4 in Figure 2 and lanes 7–12 in Figure 3). Detection of the ACE portion of the chimeric protein SecNEP-ACE was performed by immunoblotting using the antiserum 28A7 (generously provided by Dr. F. Alhenc-Gelas), which was raised against a synthetic peptide corresponding to the last 20 amino acid residues of the human ACE C-terminal cytosolic sequence [29].

RESULTS

Transport and enzymic activity of SecNEP-E

Arg-747 near the C-terminus of NEP was shown to be involved in substrate binding [30]. Therefore, it was a concern that the fusion of hydrophobic protein domains close to the enzyme active site might disrupt enzyme activity and/or transport to the cell surface. Thus, an extended form of SecNEP, SecNEP-E (Figure 1a), was created, and its expression compared with that of NEP and SecNEP after transfection of COS-1 cells and immunoblotting analyses of cellular extracts and culture media. As observed previously [15], NEP synthesized by the transfected COS-1 cells was present exclusively in the cell extract (Figure 2, lanes 1 and 2), whereas SecNEP was found in the cell extract and in the culture medium (Figure 2, lanes 3 and 4). SecNEP-E migrated with an apparent *M_r* larger than that of SecNEP, consistent with the presence of the 22-amino-acid extension at its C-terminus (Figure 2, lanes 5 and 6). The cell-associated forms of SecNEP and SecNEP-E had lower *M_r* values than that observed for their soluble forms. To seek an explanation for this observation, samples of cell extracts and culture media of transfected cells were treated with endo H and endo F, and analysed by immunoblotting. The cellular form of NEP (Figure 3, lanes 4–6) was resistant to digestion by endo H, but was sensitive to digestion by endo F as expected for a cell-surface protein. In contrast, the cell-associated forms of both SecNEP (Figure 3, lanes 10–12) and SecNEP-E (Figure 3, lanes 16–18) were sensitive to both enzymes. This observation suggests that the cell-associated forms of these proteins are present in an early compartment of the exocytic pathway, most probably the ER. The pulse-chase labelling indicated that SecNEP and SecNEP-E only temporarily stayed in the ER (results not shown). It is noteworthy, however, that the soluble forms of SecNEP (Figure 3, lanes 7–9) and SecNEP-E (Figure 3, lanes 13–15) were resistant to endo H digestion, suggesting true secretion of these mutant proteins rather than release from lysed cells. Furthermore,

Figure 2 Immunoblot analysis of extracts (Cellular) and culture media (Soluble) from transfected COS-1 cells

At 44 h after transfection, cell extracts and concentrated media were prepared as described in the Materials and methods section. Samples (40 µl) of cell extracts or concentrated media were loaded on the gel. The proteins were detected with the NEP-ectodomain-specific MAb 18B4. The position of an *M_r* marker is indicated on the right.

Figure 3 Sensitivity to endoglycosidases

Samples (40 μ l) of extracts or concentrated culture media from transfected cells were digested with endo H or endo F before loading on the gel, and proteins were detected by immunoblotting with the NEP-ectodomain-specific MAb 18B5. The position of an M_r marker is indicated.

Table 1 Kinetic parameters of NEP, SecNEP and SecNEP-E

Kinetic parameters were determined as described in the Results section, with the substrate [Tyr-3,5- 3 H][D-Ala 2 , leu 5] enkephalin. The results are means $\pm$ S.D. of three different determinations.

Enzyme	K_m (μ M)	K_{cat} (min^{-1})	K_{cat}/K_m ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$)
NEP	74.6 ± 16.5	155.1 ± 11.0	2.1
SecNEP	43.1 ± 4.5	152.1 ± 4.3	3.5
SecNEP-E	76.4 ± 7.8	298.6 ± 10.0	3.9

enzymic studies using [Tyr-3,5- 3 H][D-Ala 2 , Leu 5]enkephalin (50 Ci/mmol; Commissariat à l'Energie Atomique, Saclay, France) as a substrate [31] showed that the soluble forms of SecNEP and SecNEP-E had kinetic parameters similar to those determined for NEP (Table 1). Thus the results suggest that extending the C-terminus of SecNEP by 22 amino acid residues had limited effects on protein transport and enzymic activity. These observations make SecNEP-E a good protein model with which to attempt transformation into a type I integral membrane protein.

Expression and characterization of SecNEP-TMC and SecNEP-TMIC

In order to transform SecNEP-E into a type I integral membrane protein, the transmembrane and cytosolic domains at the N-terminus of NEP were fused in-frame at its C-terminus, creating SecNEP-TMC (Figure 1). Since charged amino acid residues have been shown to be important to determine membrane topology of proteins (see the Discussion section), mutant SecNEP-TMIC was also constructed, in which the orientation of the cytosolic domain was inverted compared with that found in NEP or SecNEP-TMC. This was done in order to bring a group of positively charged amino acid residues close to the inside surface of the plasma membrane, as found in NEP (Figure 1b). When expressed in COS-1 cells the type I mutants gave both cellular and soluble forms (Figure 2, lane 7–10). The cell-

associated forms of the fusion proteins had larger M_r values than that of SecNEP-E, which was expected, due to the presence of the transmembrane and cytosolic domains. For each mutant approx. 75% of the protein was found in the culture medium and 25% was associated with the cell pellet. The soluble forms of SecNEP-TMC and SecNEP-TMIC appeared as homogeneous bands which were resistant to digestion by endo H (Figure 3, lanes 21 and 27). In contrast with the single soluble form, two cell-associated forms of SecNEP-TMC and SecNEP-TMIC were observed. The abundant forms were sensitive to endo H digestion, whereas the minor higher- M_r forms were resistant (Figure 3, lanes 24 and 30). The presence of sugar moieties on NEP ectodomain in SecNEP-TMC and SecNEP-TMIC strongly suggests translocation of this protein domain across the ER membrane [32].

To test the hypothesis that SecNEP-TMC is inserted in the ER membrane as a type I membrane protein, COS-1 cells expressing either SecNEP-TMC or SecNEP were labelled with [3 S]methionine. BFA was added to the culture medium, since it is known to block rapidly intracellular transport of proteins from the ER to the Golgi apparatus [33]. Under these conditions both proteins accumulated as endo-H-sensitive species in the cell extracts (results not shown). Furthermore, the difference of 9200 in M_r observed between SecNEP and SecNEP-TMC is consistent with the presence of the extended regions in the latter protein (Figure 4, lanes 1 and 2). Isolated intracellular membranes were treated with Na_2CO_3 . This treatment is known to release luminal proteins from vesicles, while the transmembrane proteins remain associated with the vesicular membranes. As shown in Figure 4, treatment of equal samples of membranes with Na_2CO_3 resulted in the release of SecNEP into the supernatant (lane 3), whereas no SecNEP-TMC could be detected (lane 4). Similar experiments showed that the ER-associated form of SecNEP-TMIC is also resistant to this treatment (results not shown). Since SecNEP-TMC and SecNEP-TMIC are glycosylated (see Figure 3), our results strongly suggest that these fusion proteins are first inserted in the ER membrane as type I membrane proteins with the NEP ectodomain exposed in the lumen of the ER.

It is noteworthy that the difference in mobility between soluble forms of SecNEP-TMC and SecNEP-E is much smaller than the

Figure 4 Immunoprecipitation of [^{35}S]methionine-labelled SecNEP and SecNEP-TMC

COS-1 cells expressing SecNEP (lane 1 and 3) or SecNEP-TMC (lanes 2 and 4) were labelled with [^{35}S]methionine. Samples of transfected cells were extracted with 1% octyl glucoside, and the proteins in the cell extracts were immunoprecipitated with an NEP-specific polyclonal antibody (lanes 1 and 2). Intracellular membranes were isolated from equivalent samples of transfected cells and pelleted after treatment with Na_2CO_3 , pH 11.5. The proteins in the supernatant were immunoprecipitated with the same NEP-specific antibody (lanes 3 and 4). The position of an M_r marker is indicated on the right.

Figure 5 Immunoblot analysis of extracts (Cellular) or culture media (Soluble) from transfected COS-1 cells

Proteins were detected by immunoblot with the NEP-cytosolic-domain-specific MAb 23B11 without or with treatment with endo H. The position of an M_r marker is indicated on the right.

difference in mobility between the cell-associated forms (see Figure 2). One explanation could be that the soluble form of SecNEP-TMC has undergone some proteolytic cleavage of the cytosolic and transmembrane domains. In order to test this possibility, extracts and culture media from transfected cells expressing NEP and SecNEP-TMC were analysed by immunoblotting using the MAb 23B11, which specifically recognizes the cytosolic tail of NEP [28]. As expected, MAb 23B11 detected a protein in the extract of cells expressing NEP (Figure 5, lane 4). This MAb did not detect the presence of a NEP-related protein in the culture medium of cells expressing SecNEP-TMC (Figure 5, lane 1). Furthermore, only an endo-H-sensitive species was detected in the cell extract by MAb 23B11 (Figure 5, lanes 2 and 3), whereas the ectodomain-specific MAb 18B5 (see Figure 3, lane 24) detected two species. These results suggest that both the soluble form and the endo-H-resistant intracellular species of SecNEP-TMC have lost the cytosolic domain. Thus it appears that SecNEP-TMC loses its cytosolic tail before it exits from the ER. The absence of endo-H-resistant cellular forms of SecNEP-TMC containing the cytosolic domain suggests that this type I topological mutant is transport-incompetent, and that transport can be achieved only after cleavage of the cytosolic and transmembrane domains.

Figure 6 Immunoblot analysis of extracts (Cellular) and culture media (Soluble) from transfected COS-1 cells expressing SecNEP-ACE

Samples of cell extracts or concentrated media from an equal number of cells were loaded on the gel. The proteins in lanes 1–6 were detected with the NEP-ectodomain-specific MAb 18B5, and those in lanes 7–10 were detected with ACE-cytosolic-domain-specific antibody 28A7. The position of an M_r marker is indicated on the right.

Expression and characterization of SecNEP-ACE

The transport-incompetence of SecNEP-TMC could be due to the reverse topology of the NEP ectodomain or to the presence of 'unnatural' transmembrane and cytosolic domains in the C-terminus of the NEP ectodomain. To distinguish between these two possibilities, the chimeric protein SecNEP-ACE was constructed. SecNEP-ACE in transfected COS-1 cells was found in approximately equivalent proportions in the culture medium and in the cell extract (Figure 2, lanes 11 and 12; Figure 6, lanes 1–6). The soluble form of SecNEP-ACE was resistant to endo H digestion, suggesting that it had reached the cell surface through the exocytotic pathway. Two species found in approximately equal amounts were observed in the cell-associated forms of SecNEP-ACE (Figure 6, lanes 4–6). The low- M_r species was sensitive to endo H digestion, whereas the higher- M_r species was resistant. We next analysed by immunoblotting the cellular extract and the culture medium from transfected cells expressing SecNEP-ACE using the antiserum 28A7, which specifically recognizes the cytosolic tail of ACE [29]. Antiserum 28A7 revealed very little protein in the culture medium (Figure 6, lane 7), suggesting that SecNEP-ACE soluble form had lost its cytosolic domain. On the other hand, both the endo H-sensitive and endo H-resistant cell-associated species were detected by the antiserum 28A7 (Figure 6, lanes 8–10). Furthermore, the ratio was very similar to that observed with the NEP ectodomain-specific MAb 18B5 (Figure 6, lanes 4–6). This observation suggests that both intracellular species are membrane bound. This hypothesis was confirmed by Na_2CO_3 treatment of intracellular membranes of cells expressing SecNEP-ACE (results not shown).

Thus it appears that SecNEP-ACE is inserted in the ER membrane as a type I membrane protein, transported as such to

the plasma membrane, where the NEP ectodomain is released through an uncharacterized proteolytic event. This model is supported by the following observations. First, SecNEP-ACE is resistant to Na_2CO_3 extraction (results not shown). Second, the NEP ectodomain of SecNEP-ACE is glycosylated (Figure 6, lanes 2, 3, 5 and 6). Third, there is no intracellular form of SecNEP-ACE lacking the ACE cytosolic domain (Figure 6, lanes 6 and 10). Fourth, the soluble form of SecNEP-ACE lacks the ACE cytosolic domain (Figure 6, lane 7). Fifth, fluorescence-activated-cell-sorting analysis of cells expressing SecNEP-ACE showed the presence of some NEP epitope at the plasma membrane (results not shown).

DISCUSSION

In the present study we showed that both NEP and ACE transmembrane and cytosolic domains could act as stop-transfer sequences when placed in the C-terminus of the NEP ectodomain. These results emphasize the functional similarity between the SA domain and the stop-transfer domain over their differences. Charged residues on either side of the membrane have been shown to be important in determining the topology of integral membrane proteins [34–41]. In particular, SA domains of type II membrane proteins are oriented with their most positive flanking region on the cytosolic side of the membrane [34]. In contrast, a recent statistical study showing that the exact location and number of basic amino acid residues in the C-terminal region of type I membrane proteins are not critical [42]. Our results are consistent with this analysis, since both SecNEP-TMC and SecNEP-TMIC adopted a type I topology, despite differences in the position of the positively charged residues in the regions flanking the transmembrane domain (see Figure 1). Introduction of charged residues on the luminal side of the transmembrane domain in SecNEP-TMC and SecNEP-TMIC had no observable counteractive effect against the positively charged residues in the cytosolic domain on determining the topology of these mutants.

Although SecNEP-TMC and SecNEP-ACE were both initially inserted as a type I membrane protein in the ER membrane of COS-1 cells, further analysis suggested a different subsequent fate for these proteins. The membrane-bound intracellular species of SecNEP-TMC stayed in the ER unless it lost its cytosolic domain. On the other hand, SecNEP-ACE can be transported from the ER to the plasma membrane as a type I membrane protein. This suggests that the type I SecNEP-TMC is transport-incompetent, which indicates that transmembrane and/or cytosolic domains of type I membrane proteins are important in determining transport-competence. This observation is consistent with similar reports which also emphasized the roles of stop-transfer domains of type I membrane proteins in intracellular transport [43,44]. The lack of appropriate antibodies prevented us from performing similar studies with SecNEP-TMIC. However, based on the electrophoretic patterns and its resistance to Na_2CO_3 extraction, we suggest that a biosynthetic pathway similar to SecNEP-TMC applies.

Another important finding of this study is that a large proportion of the chimeric proteins was recovered in the culture media of transfected cells. The soluble forms of these proteins lacked their cytosolic domain, but showed full enzymic activity, indicating that the ectodomain was intact. We thus propose that a proteolytic event in the C-terminal region of the type I NEP fusion proteins is responsible for the secretion of NEP ectodomain. The nature of the enzyme and the location of the proteolytic cleavage resulting in the loss of the cytosolic tails are unknown. However, as mentioned previously, Arg-747 is present in the active site of NEP [30], suggesting that the C-terminal

extension in the fusion proteins may be in the vicinity of the active site. To test the possibility that cleavage of the cytosolic tail is an autocatalytic event, we replaced the catalytic Glu-584 residue by a Val residue in both SecNEP-TMC and SecNEP-ACE (see Figure 1a). It has been shown previously that this mutation completely abolished NEP activity [23]. This mutation did not prevent the formation of the soluble form of SecNEP-TMCE584V and that of SecNEP-ACEE584V (results not shown). It should also be noted that the cleavage of the cytosolic tail is not due to putative proteolytic cleavage sites in the 22-amino-acid residue spacer present between the NEP ectodomain and the transmembrane segment. Indeed, similar results were obtained with a chimeric protein in which the transmembrane and cytosolic domains of ACE were fused directly to the ectodomain of NEP. However, the overall expression of the mutant protein was decreased by a factor of approx. 10 as compared with the mutants presented in this study (results not shown).

Proteolytic cleavage of the cytosolic domains does not occur in the same intracellular compartment for SecNEP-TMC and SecNEP-ACE. Our results suggest that cleavage in the C-terminal domain of SecNEP-TMC is required for the protein to pass the ER quality control, as discussed by Hurtley and Helenius [45]. A similar situation has been observed with the α -subunit of endopeptidase-24.18 (EC 3.4.24.18) [46]. It is not clear whether the recently described ER proteolytic system [47,48] is responsible for the C-terminal cleavage observed in the type I fusion proteins SecNEP-TMC and SecNEP-TMIC. In contrast, cleavage of SecNEP-ACE occurs late in the exocytotic pathway and most probably at the cell surface. It is noteworthy that the mechanism of proteolytic release of human ACE characterized in transfected CHO cells may not be contributing in the C-terminal cleavage of SecNEP-ACE, since the identified cleavage site of ACE in transfected CHO cells [49] was 92 amino acid residues away from the hydrophobic stop-transfer and cytosolic domains (amino acid residues 1230–1277) used in this study.

Our use in the present report of a cellular system, which has allowed not only the study of membrane protein anchorage, but also the monitoring of protein transport, has indicated that the NEP cytosolic and SA domains can also function as stop-transfer sequences *in vivo* when fused to the C-terminus of the NEP ectodomain. These observations also suggest that chimeric type I membrane proteins behave differently during intracellular transport, depending on the nature of the anchoring sequences. It will be interesting in the future to determine whether the transmembrane domain and/or the cytosolic domain is responsible for the behaviour observed.

We thank Ms. M.-L. Faure and Ms. L. Cournoyer for technical assistance, and Dr. H. K. Withers and Dr. S. Howell for critical reading of the manuscript and discussions. This work was supported by a grant from the Medical Research Council of Canada to G. B. G. B. is the recipient of a 'Chercheur-Boursier' award from the Fonds de la Recherche en Santé du Québec.

REFERENCES

- 1 Blobel, G. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1496–1500
- 2 Singer, S. J. (1990) Annu. Rev. Cell Biol. **6**, 247–296
- 3 Puddington, L., Machamer, C. E. and Rose, J. K. (1986) J. Cell Biol. **102**, 2147–2157
- 4 McQueen, N. L., Nayak, D. P., Stephens, E. B. and Compans, R. W. (1987) J. Biol. Chem. **262**, 16233–16240
- 5 Kundu, A., Jabbar, M. A. and Nayak, D. P. (1991) Mol. Cell. Biol. **11**, 2675–2685
- 6 Coleman, J., Inukai, M. and Inouye, M. (1985) Cell **43**, 351–360
- 7 Finidori, J., Rizzolo, L., Gonzales, A., Kreibich, G., Adesnik, M. and Sabatini, D. D. (1987) J. Cell Biol. **104**, 1705–1714
- 8 Wessels, H. P. and Spiess, M. (1988) Cell **55**, 61–70

- 9 Zerial, M., Huylebroeck, D. and Garoff, H. (1987) *Cell* **48**, 147–155
- 10 Lipp, J., Flint, N., Haeuptle, M.-T. and Dobberstein, B. (1989) *J. Cell Biol.* **109**, 2013–2022
- 11 Rothman, R. E., Andrews, D. W., Calayag, M. C. and Lingappa, V. R. (1988) *J. Biol. Chem.* **263**, 10470–10480
- 12 Spiess, M., Handschin, C. and Baker, K. P. (1989) *J. Biol. Chem.* **264**, 19117–19124
- 13 Gafvelin, G. and von Heijne, G. (1994) *Cell* **77**, 401–412
- 14 Devault, A., Lazure, C., Nault, C., Le Moual, H., Seidah, N. G., Chrétien, M., Kahn, P., Powell, J., Mallet, J., Beaumont, A., Roques, B. P., Crine, P. and Boileau, G. (1987) *EMBO J.* **6**, 1317–1322
- 15 Lemay, G., Waksman, G., Roques, B. P., Crine, P. and Boileau, G. (1989) *J. Biol. Chem.* **264**, 15620–15623
- 16 Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G. and Corvol, P. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9386–9390
- 17 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- 18 Taylor, J. W., Ott, J. and Eckstein, F. (1985) *Nucleic Acids Res.* **13**, 8764–8785
- 19 Murray, M. G., Bradley, J., Yang, X.-F., Wimmer, E., Moss, E. G. and Racaniello, V. R. (1988) *Science* **241**, 213–215
- 20 Scharf, S. J. (1990) in *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J., eds.), pp. 84–91, Academic Press, New York
- 21 Tabor, S. and Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4767–4771
- 22 Noël, G., Zollinger, L., Larivière, N., Nault, C., Crine, P. and Boileau, G. (1987) *J. Biol. Chem.* **262**, 1876–1881
- 23 Devault, A., Nault, C., Zollinger, M., Fournie-Zaluski, M.-C., Roques, B. P., Crine, P. and Boileau, G. (1988) *J. Biol. Chem.* **263**, 4033–4040
- 24 Jalal, F., Lemay, G., Zollinger, M., Bertaloot, A., Boileau, G. and Crine, P. (1991) *J. Biol. Chem.* **266**, 19826–19832
- 25 Evans, W. H. (1987) in *Biological Membranes: A Practical Approach* (Findlay, J. B. C. and Evans, W. H., eds), pp. 1–35, IRL Press, Oxford
- 26 Fujiki, Y., Hubbard, A. L., Fowler, S. and Lazarow, P. B. (1982) *J. Cell Biol.* **93**, 97–102
- 27 Corbeil, D., Boileau, G., Lemay, G. and Crine, P. (1992) *J. Biol. Chem.* **267**, 2798–2801
- 28 Aubry, M., Zollinger, M., Fortin, S., Venien, C., LeGrimellec, C. and Crine, P. (1988) *Biochim. Biophys. Acta* **967**, 56–64
- 29 Wei, L., Alhenc-Gelas, F., Soubrier, F., Michaud, A., Corvol, P. and Clauser, E. (1991) *J. Biol. Chem.* **266**, 5540–5546
- 30 Beaumont, A., Le Moual, H., Boileau, G., Crine, P. and Roques, B. P. (1991) *J. Biol. Chem.* **266**, 214–220
- 31 Lemay, G., Zollinger, M., Waksman, G., Roques, B. P., Crine, P. and Boileau, G. (1990) *Biochem. J.* **267**, 447–452
- 32 Wessels, H. P., Beltzer, J. P. and Spiess, M. (1991) in *Laboratory Methods in Vesicular and Vectorial Transport* (Tartakoff, A. M., ed.), pp. 3–18, Academic Press, New York
- 33 Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L. and Klausner, R. D. (1991) *Cell* **67**, 601–616
- 34 Hartmann, E., Rapoport, T. A. and Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5786–5790
- 35 von Heijne, G. (1990) *J. Membr. Biol.* **115**, 195–201
- 36 von Heijne, G. (1994) *Annu. Rev. Biophys. Biomol. Struct.* **23**, 167–192
- 37 von Heijne, G. (1995) *BioEssays* **17**, 25–30
- 38 Parks, G. D. and Lamb, R. A. (1991) *Cell* **64**, 777–787
- 39 Parks, G. D. and Lamb, R. A. (1993) *J. Biol. Chem.* **268**, 19101–19109
- 40 High, S. and Dobberstein, B. (1992) *Curr. Opin. Cell Biol.* **4**, 581–586
- 41 Sakaguchi, M., Tomiyoshi, R., Kuroiwa, T., Mihara, K. and Omura, T. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 16–19
- 42 Landolt-Marticorena, C., Williams, K. A., Deber, C. M. and Reithmeier, R. A. F. (1993) *J. Mol. Biol.* **229**, 602–608
- 43 Adams, G. A. and Rose, J. K. (1985) *Mol. Cell. Biol.* **5**, 1442–1448
- 44 Hennecke, S. and Cosson, P. (1993) *J. Biol. Chem.* **268**, 26607–26612
- 45 Hurtley, S. M. and Helenius, A. (1989) *Annu. Rev. Cell Biol.* **5**, 277–307
- 46 Milheit, P. E., Corbeil, D., Simon, V., Kenny, A. J., Crine, P. and Boileau, G. (1994) *Biochem. J.* **300**, 37–43
- 47 Klausner, R. D., Lippincott-Schwartz, J. and Bonifacio, J. S. (1990) *Annu. Rev. Cell Biol.* **6**, 403–431
- 48 Bonifacio, J. S., Lippincott-Schwartz, J. and Klausner, R. D. (1991) in *Intracellular Trafficking of Proteins* (Steer, C. J. and Hanover, J. A., eds.), pp. 549–574, Cambridge University Press, Cambridge
- 49 Beldent, V., Michaud, A., Wei, L., Chauvet, M. T. and Corvol, P. (1993) *J. Biol. Chem.* **268**, 26428–26434