Recombinant neutral endopeptidase-24.11 expressed in mouse neuroblastoma cells is associated with neurite membranes

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Neutral endopeptidase-24.11 (EC 3.4.24.11) (NEP) is a transmembrane metallo-endopeptidase that has been shown to be involved in the degradation of several mammalian neuropeptides, including enkephalins. The enzyme has recently been found to be specifically associated with the axonal and synaptic membranes of neurons in the globus pallidus of the pig brain. This result suggests that neurons must possess mechanisms for targeting NEP to particular membrane domains. Study of these mechanisms would greatly benefit from the existence of an established neuron-like cell line capable of expressing and targeting NEP to specific membrane domains. For this reason we have used a retroviral vector containing the cDNA for rabbit kidney NEP to express this enzyme in a mouse neuroblastoma cell line (Neuro2A). Labelling of the cell surface with an antibody coupled to colloidal gold particles and examination of the cells by electron microscopy revealed a non-uniform distribution of NEP at the surface of the cells, the protein being preferentially associated with the membrane of neurites compared with the cell body. This observation suggests that Neuro2A cells possess a mechanism for targeting NEP to specific domains of the plasma membrane. This cell line could thus constitute a good model for studying the mechanisms responsible for targeting this enzyme to specialized regions of the plasma membrane.

INTRODUCTION

Neutral endopeptidase-24.11 (EC 3.4.24.11) (NEP) is a membrane-bound enzyme present at the surface of many different cell types. NEP preferentially cleaves small peptides rather than proteins. Cleavage occurs at the amino side of hydrophobic amino acids (Kerr & Kenny, 1974). The presence of the enzyme at the plasma membrane coupled with its broad specificity suggest that it may be involved in the inactivation of a variety of small regulatory peptides (Matsas *et al.*, 1984). NEP is present in the brain (Malfroy *et al.*, 1978), where it has been shown to be involved in the inactivation of several neuropeptides, including the enkephalins (Roques *et al.*, 1980; Almenoff *et al.*, 1981).

Lesion studies have shown that NEP is mainly associated with neurons rather than glial cells (Waksman *et al.*, 1986, 1987). Moreover a recent ultrastructural study at the electron-microscopic level in the globus pallidus of a newborn pig has shown that immunoperoxidase staining for NEP is associated with axonal membranes, including some synapses (Barnes *et al.*, 1989). This important observation strengthens the view that NEP may play a role in inactivating some neuropeptides after their synaptic release. It also suggests the existence, in neuronal cells, of a mechanism responsible for the targeting of NEP to specific membrane domains.

Although neurons in primary culture do express NEP at their surface (Matsas & Kenny, 1989), established neuroblastoma cell lines are devoid of NEP (Lentzen *et al.*, 1983; Endo *et al.*, 1985). A permanent cell model for the study of NEP transport in neuronal cells was thus lacking. As a first step towards the understanding of the molecular mechanism responsible for the

expression of NEP at the surface of neuron cells, we have expressed the protein encoded by rabbit kidney NEP cDNA (Devault *et al.*, 1987) in mouse neuroblastoma cells (Neuro2A). These cells can be differentiated, under certain conditions, into pseudo-neuronal cells extending long neurites (Ross *et al.*, 1975). Our results show that the protein expressed from the cDNA is normally transported to the cell surface and has enzymic properties identical with those of the kidney enzyme. Immuno-electron-microscopy analysis of differentiated cells revealed a non-uniform distribution of NEP at the plasma membrane, most of the protein being associated with the membrane of neurites rather than cell body. These results suggest the existence in Neuro2A cells of mechanisms that preferentially target NEP at the membrane of neurites and establish this cell line as a potentially useful model for the study of targeting mechanisms in neuronal cells.

MATERIALS AND METHODS

Vector construction

All DNA manipulations were performed by using described procedures (Maniatis *et al.*, 1982; Ausubel *et al.*, 1987). The cloning of rabbit NEP cDNA and the construction of the SV40-based expression vector pSVENK19 have been previously published (Devault *et al.*, 1987, 1988). The expression vector pN2-NEP was constructed by blunt-end ligation of an *SphI*–*AvaI* DNA fragment from pSVENK19 (encompassing NEP coding sequences downstream from the SV40 early promoter) into the unique *XhoI* site of pN2 (Armentano *et al.*, 1987; generously given by Dr. Eric Rassart, Université du Québec à Montréal).

Abbreviations used: NEP, neutral endopeptidase-24.11; LTR, long terminal repeat; PBS, phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4); TBS, Tris-buffered saline (0.15 M-NaCl/10 mM-Tris/HCl buffer, pH 7.4); SV40, simian virus 40.

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The retroviral sequences are derived from the Moloney murine leukaemia virus. It contains both LTRs and the sequences required for the production of infectious virus. In such a construct, the expression of NEP is driven by the SV40 early promoter whereas the bacterial gene conferring the resistance to the neomycin analogue G418 is under the control of the retrovirus 5'-LTR. The transcription-termination/poly(A)-addition signals are provided by the virus 3'-LTR (Fig. 1).

Cell culture and retroviral infections

All cells were grown in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% (v/v) fetal bovine serum and antibiotics; cells were kept at 37 °C under an atmosphere of 5% CO₂ in air. The Psi-2 cell line (Mann *et al.*, 1983) was a gift from Dr. Eric Rassart. The mouse neuroblastoma cell line (Neuro2A) was originally obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.). These cells were routinely grown in a medium containing 10% (v/v) fetal bovine serum and they exhibited minimal outgrowth of neurites under these conditions. Neurite outgrowth, typical of differentiated cells, was stimulated by an incubation of 4 days in medium containing 0.2 M-sucrose (Ross *et al.*, 1975). Infectious recombinant retrovirus was obtained from Psi-2 cells and used to infect Neuro2A cells by following standard procedures. Briefly, Psi-2 cells were transfected with pN2-ENK by the calcium phosphate co-precipitation procedure (Graham & van der Eb, 1973) followed by a 1 min shock with 15% (v/v) glycerol. The transfected cells were incubated in Petri dishes (75 cm²) containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) serum for 48 h with one medium change after 24 h. The medium was then replaced with fresh one containing 400 µg of G418 sulphate/ml and replaced every 3 days until G418-resistant colonies appeared. The resistant cells were then trypsinized, pooled and divided into several Petri dishes. When cells reached semi-confluency the medium was replaced with 3 ml of G418-free medium. This medium, containing the recombinant virus N2-NEP, was collected every 3 h (rapid harvest procedure). The viral suspension thus obtained was kept frozen at -70 °C. Before being used to infect neuroblastoma cells the viral suspension was passed through a 0.45 µm-pore sterile nitrocellulose filter and 10 µg of sterile Polybrene was added/ml. For infection, the medium of Neuro2A cultures in exponential growth was replaced with 2 ml of this viral suspension (in a 75 cm² Petri dish) and incubated for 2 h at 37 °C; 10 ml of fresh medium was then added; 48 h later, infected cells were placed under selection in G418-containing medium.

Antibodies

A guinea-pig polyclonal antibody raised against rabbit NEP has already been described (Lemay *et al.*, 1989). Monoclonal 2B12 antibody recognizes a conformational epitope on the extracellular domain of rabbit NEP (Aubry *et al.*, 1988). Peroxidase-conjugated rabbit anti-(guinea-pig immunoglobulin) antibody was purchased from Dako Immunoglobulins. Coupling of the 2B12 antibody to colloidal gold particles of size 8 nm was done according to published procedures (Frens, 1973; De Mey *et al.*, 1981; Roth, 1985).

Binding of radiolabelled antibody to infected cells

The 2B12 monoclonal antibody was radioiodinated by the chloramine-T method (Stahli *et al.*, 1983); we routinely obtained specific radioactivities of 5–10 µCi/µg of protein. Binding of antibody to live cells was performed essentially as described previously (Devault *et al.*, 1988). Briefly, cells were scraped from Petri dishes with a rubber 'policeman' after dissociation with PBS containing 4 mM-EGTA/KOH and incubated (10⁶ cells/ml)

for 30 min at room temperature in PBS containing 1% (w/v) BSA. Radiolabelled 2B12 monoclonal antibody was added at 2 µCi/ml and incubated for 30 min at room temperature with slow agitation. Cells were then pelleted by centrifugation, resuspended in PBS/BSA and repelleted. This washing procedure was repeated twice before measurement of bound radioactivity by direct counting of the cell pellet in a γ-radiation counter.

Immunoblotting

Proteins were resolved by electrophoresis in SDS/7.5%-polyacrylamide slab gels (Laemmli, 1970), then electrophoretically transferred on to 0.45 µm-pore nitrocellulose filters (Schleicher and Schuell) by using standard procedures (Towbin *et al.*, 1979). For immunological detection of NEP, filters were preincubated for 1 h in TBS containing 1% (w/v) BSA. Guinea-pig polyclonal antiserum was then added at a 1/500 dilution and incubated at room temperature for another 1 h. After three washes of 10 min in TBS containing 0.1% Tween 20, the peroxidase-conjugated anti-(guinea-pig immunoglobulin) antibody was added in TBS/BSA at 1/500 dilution and incubated for 1 h, and the filters were washed again. The peroxidase substrate was prepared as follows: 0.1 g of substrate (4-chloro-1-naphthol) was dissolved in 20 ml of ethanol, then 100 ml of TBS and 0.15 ml of 30% (v/v) H₂O₂ were slowly added. Filters were immediately incubated in this solution for 15 min at room temperature and in the dark. The reaction was stopped by extensive rinses in distilled water.

Protein solubilization and endoglycosidase digestions

NEP was solubilized from cells with 1% (w/v) n-octyl β-D-glucoside in TBS as described previously (Devault *et al.*, 1988); solubilization was done at a cell concentration of 2 × 10⁷ cells/ml. Endoglycosidase digestions were performed by adding directly 0.3 unit of endo-β-N-acetylglucosaminidase F or 0.005 unit of endo-β-N-acetylglucosaminidase H to 50 µl of the protein solution corresponding to 5 × 10⁶ cells or approx. 100 µg of cellular proteins. Incubations were for 16 h at 37 °C.

Assay of enzyme activity

The enzyme activity of solubilized NEP was assayed by degradation of the tritiated substrate [Tyr-3,5-³H][p-Ala²,Leu⁵]enkephalin (50 Ci/mmol; Commissariat à l'Energie Atomique, Saclay, France). Degradation products were separated from unchanged substrate on Sep-Pak C₁₈ cartridges as previously described (Crine *et al.*, 1985). The K_m values were determined by the isotopic dilution method with unlabelled substrate (Peninsula Laboratories, U.S.A.); calculations were done by using Eadie-Hofstee plots and were facilitated by using the microcomputer program ENZFITTER (Elsevier-Biosoft, Cambridge, U.K.). The effect of phosphoramidon [N-(α-rhamnopyranosyloxy-hydroxyphosphinyl)-L-leucyl-L-tryptophan] (Sigma Chemical Co.) was determined by serial dilution of the inhibitor in enzyme assays at a substrate concentration of 50 nM.

Electron microscopy

Live cells still attached to Petri tissue-culture dishes were washed with PBS and preincubated for 60 min in PBS containing 1% (w/v) BSA and 20 mM-NaN₃. The gold-conjugated 2B12 monoclonal antibody (8 nm particles) was directly applied on the cells after dilution in PBS/BSA/azide at an absorbance of 0.3 at 525 nm. After 60 min at room temperature the cells were washed in the same buffer, then in PBS/azide, and fixed for 1 h on ice with 2.5% (v/v) glutaraldehyde in PBS. Cells were washed with PBS containing 4.5% (w/v) sucrose and post-fixed for 1 h on ice

with 1 % OsO₄ in PBS. They were then washed with PBS and dehydrated with increasing ethanol concentrations. The fixed cells were embedded directly in Petri dishes with Epon B resin. This was achieved by overlaying the cells with a thin layer of resin, then after an overnight incubation at 40 °C interesting regions of the Petri dish were enclosed within small plastic cylinders and the cylinders were filled with resin. After polymerization at 60 °C, resin cylinders could be oriented and cut with the ultramicrotome so as to obtain longitudinal sections of the enclosed cells.

RESULTS

Expression of NEP in Neuro2A cells

The rabbit NEP cDNA was previously shown to encode an authentic functional enzyme when transiently expressed in COS-1 cells (Devault *et al.*, 1988). To promote the expression of the rabbit NEP cDNA in Neuro2A cells on a permanent basis, a retrovirus-based vector containing the neomycin-resistance gene was constructed (Fig. 1 and the Materials and methods section) and the recombinant retrovirus was used to establish clones of Neuro2A cells resistant to the neomycin analogue G-418. The presence of NEP at the surface of Neuro2A cells infected with the recombinant virus was first detected by binding of a radiolabelled antibody (see the Materials and methods section). The binding measured with transfected Psi-2 cells or infected Neuro2A cells was 30–90-fold over the background observed with the control cells (Table 1). The total amount of NEP present at the surface of Neuro2A cells as measured by antibody binding does not seem to be significantly affected after differentiation of Neuro2A cells

Table 1. Binding of radiolabelled antibody to cells

Cell line (10 ⁶ cells)	10 ⁻³ × Radioactivity bound (c.p.m.)
Psi-2	8
Psi-2 (NEP)	230
Neuro2A	10
Neuro2A (NEP)	900
Neuro2A (differentiated)	15
Neuro2A (NEP) (differentiated)	400

by incubation in medium containing sucrose (Table 1). The presence of NEP at the plasma membrane does not significantly interfere with cell growth, since a stable cell line was readily established and has now been maintained in culture for over a year.

Analysis of proteins by immunoblotting

To characterize NEP expressed in Neuro2A cells, the proteins solubilized from these cells were resolved by SDS/PAGE and analysed by immunoblotting (Fig. 2). The recombinant protein detected in Neuro2A-cell extracts had a slightly lower apparent molecular mass (92 kDa) than the authentic kidney enzyme (94 kDa) (Fig. 2, lanes 3 and 6). Removal of *N*-linked sugar moieties with endo- β -*N*-acetylglucosaminidase F from both the kidney and the recombinant enzymes produced polypeptides that migrated with similar mobility on SDS/PAGE (Fig. 2, lanes 2 and 5), suggesting that the sugar moieties are responsible for most of the observed differences in apparent molecular mass. Both NEP extracted from Neuro2A cells and authentic kidney NEP were found to be largely resistant to endo- β -*N*-acetylglucosaminidase H digestion (Fig. 2, lanes 1 and 4), indicating that the infected cells could add complex sugars to the recombinant enzyme in the Golgi apparatus. However, minor species with lower apparent molecular mass could be observed when the recombinant NEP extracted from infected cells was treated with endo- β -*N*-acetylglucosaminidase H (Fig. 2, lane 1). These species may represent proteins in transit in the Golgi apparatus that have not totally acquired complex sugars. These lower-molecular-mass species are not present in endo- β -*N*-acetylglucosaminidase H-treated kidney NEP (Fig. 2, lane 4), since it was purified from brush-border membrane and thus represents mature enzyme. Alternatively, these minor species may reflect a slightly different glycosylation pattern in Neuro2A cells. The lower apparent molecular mass of the recombinant

Fig. 1. Retroviral vector for expression of NEP

Thin lines represent pBR322 sequences, wavy lines are mouse cellular sequences adjacent to retrovirus proviral sequences. Retroviral LTRs are indicated by boxes, the black portion corresponding to the R-U5 region; the arrow indicates direction of transcription starting from the 5'-LTR and responsible for *Neo* gene transcription. Thick lines adjacent to LTRs are viral sequences required for reverse transcription and packaging of the viral genome. The NEP-containing fragment was subcloned at the unique *Xho*I site of pN2. The fragment encompasses the complete NEP coding sequences indicated by the stippled box, the *Aval* site being in the 3'-untranslated region. The hatched box represents the SV40 early promoter.

Fig. 2. Analysis of NEP by immunoblotting

Neuro2A cells infected with retrovirus carrying NEP were lysed with octyl glucoside. One-fifth of a Petri dish (roughly 10⁶ cells) was analysed by immunoblotting as described in the Materials and methods section. Samples were either left untreated (-) or digested with endo- β -*N*-acetylglucosaminidase F (F) or endo- β -*N*-acetylglucosaminidase H (H). NEP purified from rabbit kidney cortex (200 ng per lane) was analysed in parallel on the same gel.

Fig. 3. Enzymic activity of NEP expressed in Neuro2A cells compared with kidney NEP

The enzymic activity of NEP obtained by octyl glucoside solubilization of infected Neuro2A cells (○) was compared with NEP purified from rabbit kidney cortex (●). (a) Eadie-Hofstee plots obtained by the isotopic dilution method as indicated in the Materials and methods section. (b) Inhibition of activity by phosphoramidon.

enzyme compared with that of the authentic kidney enzyme also supports the idea that subtle differences exist between Neuro2A and kidney epithelial cells in the addition of complex sugars. This conclusion is also consistent with our own recent observation that MDCK cells, which are widely used as a model for kidney epithelial cells (Simons, 1987), apparently glycosylate recombinant NEP to the same extent as normal kidney cells (G. Lemay, G. Boileau & P. Crine, unpublished work). A similar hypothesis had previously been proposed by Relton *et al.* (1983), who showed that the enzyme extracted from pig kidney has a higher apparent molecular mass than the enzyme from pig brain and that this difference is mainly due to differential glycosylation of the same polypeptide. All these data strongly suggest that kidney cells add more or different complex sugars to NEP than neuronal cells do. The significance, if any, of this differential glycosylation pattern is unknown.

Enzymic activity of NEP expressed in Neuro2A cells

We then verified the enzymic activity of NEP expressed in Neuro2A cells. These cells, like other neuroblastoma cells (Endo

Fig. 4. Immunolocalization of NEP in Neuro2A cells by electron microscopy

Neuro2A cells expressing NEP were differentiated by incubation in hyperosmotic medium. Gold-conjugated 2B12 monoclonal antibody was applied on living cells. Fixation and processing for electron microscopy were as described in the Materials and methods section. A low magnification of a portion of a cell is presented. Inset represents a higher magnification of extensions. Bar = 0.5 μm.

et al., 1985; Lentzen *et al.*, 1983), do not normally express NEP (result not shown). In contrast, cells infected with the recombinant retrovirus N2-NEP showed a phosphoramidon-sensitive activity that degraded the substrate [D-Ala²,Leu⁵]-enkephalin. The K_m of the recombinant enzyme was estimated to be 66 μ M compared with 59 μ M found for the enzyme purified from rabbit kidney (Fig. 3a). The concentration of phosphoramidon (an NEP inhibitor) necessary to achieve 50% inhibition (IC_{50}) was found to be 12 nM for the recombinant enzyme, in good agreement with the value of 8 nM found with the authentic kidney enzyme (Fig. 3b). The recombinant enzyme expressed in Neuro2A cells has thus catalytic properties similar to those of the normal kidney enzyme.

Localization of NEP at the cell surface

Cells were labelled with the gold-conjugated 2B12 monoclonal antibody and prepared for transmission electron microscopy as described in the Materials and methods section. This technique, which allows the observation of discrete regions of the cell membrane, revealed the preferential presence of NEP on the neurites compared with cell bodies (Fig. 4). There is no obvious patching but instead a rather uniform distribution of the protein along the length of the neurites. Short extensions close to the cell body can also be seen labelled with gold particles. Since the cells were incubated in the presence of NaN₃ before the labelling, internalization of specific regions of the plasma membrane should have been prevented (Loor, 1977). We therefore believe that the non-uniform immunogold labelling observed in this study is not due to a selective internalization of some regions of the plasma membrane following the incubation of the cells with the antibody coupled to colloidal gold particles, and does represent the normal distribution of the enzyme at the surface of Neuro2A cells.

DISCUSSION

One of the central problem of modern biology concerns the mechanisms responsible for the distribution of proteins to specific subcellular compartments. In addition to well-characterized compartments such as lysosomes, nuclei, mitochondria and secretory granules, it is becoming increasingly evident that plasma membranes can be subdivided into distinct functional regions. The best-known example of such membrane domains is the epithelial cell, where the plasma membrane can be divided into an apical and a basolateral domain. NEP is known to have a polarized distribution at the surface of many different cell types (Johnson *et al.*, 1985; Lorkowski *et al.*, 1987). For instance, NEP is present exclusively at the apical membrane in the polarized epithelial cells of the kidney proximal tubules (Kenny, 1986). Furthermore, NEP has recently been found to the predominantly associated with the axonal and synaptic regions of neuronal cells (Barnes *et al.*, 1989), which also constitute a typical example of highly polarized cells.

Targeting of newly synthesized proteins to specific organelles or plasma membranes is widely believed to be directly by specific address sequences encoded in the structure of the protein (Garoff, 1985). One of the most promising strategies for investigating the role of protein structural domains in targeting mechanisms is to induce the synthesis of these proteins in heterologous cells and look whether these cells can target proteins to specific plasma-membrane domains. Recombinant DNA technology can then be used to change the protein sequence and test directly the functional importance of various domains or specific amino acid residues in directing the routing of these proteins. As a first step to gain more information concerning mechanisms of transport, membrane insertion and targeting of NEP to specific membrane domains in neuronal cells, we have used a recombinant retrovirus

as vector to express this enzyme in the neuroblastoma cell line Neuro2A. Expression of NEP in Neuro2A cells does not appear to be detrimental to cell growth, or to interfere with their morphological differentiation. Furthermore, NEP expressed in these cells has conserved its structural and functional properties. Analysis of the proteins extracted from infected Neuro2A cells and sensitivity to endo- β -N-acetylglucosaminidases F and H indicated that most of the recombinant NEP acquired complex sugar moieties and was therefore most probably transported to the Golgi apparatus. Finally, binding of radioiodinated antibodies to the surface of non-permeabilized cells showed that the protein had reached the cell surface. These observations are consistent with previous results obtained during transient expression of NEP in a fibroblastic cell type (COS-1 cells) (Devault *et al.*, 1988; Lemay *et al.*, 1989).

Differentiated Neuro2A cells project neurites, which can be compared with dendritic and axonal extensions of neuronal cells (Ross *et al.*, 1975). The extensions observed in our transformed Neuro2A cells were found to contain the characteristic neurofilaments of neuronal extensions described by Ross *et al.* (1975). When Neuro2A cells expressing NEP were differentiated and examined with the electron microscope, NEP was found preferentially on neurite membranes and in short extensions projecting from the cell bodies. The same distribution was found for acetylcholinesterase (results not shown), an enzyme that had previously been shown to be specifically associated with neuronal extensions in primary cultures of neurons (Rotundo & Carbonetto, 1987). These different observations reinforce the similarity between neuronal extensions and Neuro2A neurites.

In the central nervous system neuropeptides are released from nerve endings and are believed to act in the vicinity of synapses (Hamel & Beaudet, 1984). NEP has been shown to be localized to nerve terminals of striatal efferent neurons in adult rats (Waksman *et al.*, 1986, 1987). More recently, the association of the enzyme, with axonal membranes in the pig brain, including regions of synaptic contact (Barnes *et al.*, 1989), might reflect its proposed physiological role as a bioactive-peptide-degrading enzyme. Indeed, if the role of NEP is to terminate the physiological action of bioactive peptides, one can propose that it must be located near their site of release or their target receptors. The results presented in this paper showing the preferential localization of recombinant NEP on neurites of infected Neuro2A cells are also consistent with this hypothesis. Furthermore they establish Neuro2A cells as a potentially useful system for further probing the mechanism responsible for the targeting of proteins to specific domains of the plasma membrane of neurons.

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