Cell-specific activity of neprilysin 2 isoforms and enzymic specificity compared with neprilysin

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Neprilysin (NEP) 2 is a recently cloned glycoprotein displaying a high degree of sequence identity with neprilysin (EC 3.4.24.11), the prototypical member of the M13 subfamily of metalloproteases. Whereas NEP is involved in the metabolism of several bioactive peptides by plasma membranes of various cells, the enzymic properties and physiological functions of NEP2 are unknown. Here we characterize the cell-expression modalities and enzymic specificity of two alternatively spliced isoforms of NEP2 in Chinese hamster ovary and AtT20 cells. In the two cell lines, both isoforms are type II glycoproteins inserted in the endoplasmic reticulum as inactive precursors. Maturation detected by Western-blot analysis of glycosidase digests was cell-specific and more efficient in the endocrine cell line. The enzymic activity of both isoforms semi-purified from AtT20 cells reveals comparable specificities in terms of model substrates, pH optima and inhibitory patterns. NEP2 activity was compared with that of NEP regarding potencies of transition-state inhibitors, modes of hydrolysis, maximal hydrolysis rates and apparent affinities of bioactive peptides. Although all transition-state inhibitors of NEP inhibited NEP2 activity, albeit with different potencies, and many peptides were cleaved at the same amide bond by both peptidases, differences could be observed, i.e. in the hydrolysis of gonadotropin-releasing hormone and cholecystokinin, which occurred at different sites and more efficiently in the case of NEP2. Differences in cleavage of bioactive peptides, in cell-trafficking patterns and in tissue distribution indicate that NEP and NEP2 play distinct physiological roles in spite of their high degree of sequence identity.

Key words: enkephalin, M13 subfamily, metallopeptidase, neuropeptide inactivation.

INTRODUCTION

A number of peptidases exert critical roles in the metabolism of biologically active peptides. Among these, the structure, catalytic properties and/or functional roles of few members of the M13 family of zinc-containing metallopeptidases are well characterized [1]. This family comprises neprilysin (NEP; also known as neutral endopeptidase or enkephalinase; EC 3.4.24.11) [2–4], endothelin-converting enzymes (ECEs) 1 (EC 3.4.24.71) [5] and 2 [6], the Kell-blood-group antigen [7], phosphate-regulating neutral endopeptidase on the X chromosome (PEX) [8] and X-converting enzyme (XCE) [9]. All these peptidases are type II integral membrane glycoproteins with a large extracellular domain containing an active site characterized by its highly conserved consensus sequence of a zinc-binding motif, HEXXH [10].

NEP, the most extensively studied member of this family [11], was first identified as a 'kidney brush-border neutral proteinase' slowly hydrolysing [^{125}I]iodoinsulin B chain [12] and rediscovered independently as an 'enkephalinase' of cerebral membranes hydrolysing the Gly^{3}–Phe^{4} amide bond of enkephalins [13]. NEP was thereafter found to hydrolyse into inactive fragments a number of other biologically active peptides, such as tachykinins [14], bradykinin [15] and natriuretic peptides [16,17]. The design and use of potent inhibitors, e.g. thiorphan [18], has confirmed the role of NEP in the inactivation of endogenous enkephalins [19], tachykinins [20] and natriuretic peptides [21]. NEP inhibitors were recently introduced in human therapeutics in the gastroenterologic [22] and cardiovascular fields [23].

Using degenerate PCR technology with primers based upon conserved sequences of NEP, ECE1, ECE2 and Kell-blood-group antigen, three laboratories independently cloned the cDNAs encoding a potentially novel peptidase. It was termed NEP2 in rat, a name reflecting its high sequence identity with NEP [24], whereas it was named soluble secreted endopeptidase (SEP) [25] or neprilysin-like peptidase (NL1) [26] in mouse. The NEP2 cDNA encodes a 774-amino acid protein with a short N-terminal cytoplasmic tail, followed by a transmembrane domain and a large C-terminal core. Rat NEP2 displays 66 % overall amino acid sequence similarity with rat NEP, and 74 % over their C-terminal cores, which contain the catalytically important residues Arg^{102}, Asn^{542}, Ala^{543}, Glu^{646} and Arg^{747}, identified in NEP by site-directed mutagenesis [11] and which are all conserved in NEP2 [24–26].

Whereas NEP is distributed ubiquitously, NEP2 seems localized only in the central nervous system and testis [24]. Among the multiple splice variants of NEP2 [24,27], the two most abundant seem to lead to either a membrane-bound or a secreted protein [25,26]. The largest mRNA, expressed mainly in testis, encodes a protein containing two basic residues located at the beginning of the extracellular domain (Lys^{62}–Arg^{63} in mouse) which constitute a site of cleavage for subtilisin-like prohormone convertases, e.g. furin [28]. When expressed in Chinese hamster ovary (CHO)-K1 or HEK-293 cells, cleavage of this isoform produces a secreted soluble enzyme, here designated NEP2(s), which was only characterized preliminarily in terms of substrate specificity [25,26]. Another splice form, which has lost a 23 amino acid sequence comprising the prohormone convertase cleavage site, is expressed heterogeneously in brain regions and represents a membrane-associated protein whose activity is yet to be demonstrated [24,25].

Abbreviations used: NEP, neprilysin; NEP2(m), membrane-bound form of NEP2; NEP2(s), secreted soluble form of NEP2; ECE, endothelin-converting enzyme; SP, substance P; GnRH, gonadotropin-releasing hormone; CHO, Chinese hamster ovary; Endo-H, endoglycosidase H; AMC, amidomethylcoumarin; Suc, succinyl; MeOSuc, 3-methoxysuccinyl; ER, endoplasmic reticulum; CCK, cholecystokinin.

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The high degree of identity between the NEP and NEP2 sequences and the observation that [^{3}H](D-Ala^2,Leu^5)enkephalin, a known NEP substrate, is also cleaved by NEP2 [26] suggest that the two peptidases may display similar enzymic properties. We present here data from expression studies of the two major isoforms in two mammalian cell lines, CHO cells and mouse pituitary AtT20 cells, and show that the membrane-bound form of NEP2, NEP2(m), displays a cell-specific trafficking pattern. In AtT20 cells, its activity is similar to that of the secreted isoform NEP2(s). We also describe a detailed study of the enzymic properties of NEP2 based mainly on the activity of the secreted form purified from conditioned media of NEP2(s)-transfected AtT20 cells using a large series of artificial and natural peptides, and show a comparison to those of NEP.

EXPERIMENTAL

Materials

The various fluorogenic substrates, i.e. peptide amidomethylcoumarin (AMC) derivatives and biological peptides, were from Bachem (Voisins le Bretonneux, France) or Sigma-Aldrich (Saint Quentin Fallavier, France). Thiorphan, omapatrilat and fasidotrilat were generous gifts from D. Danvy and T. Monteil (Bioprojet, Paris, France). Pure recombinant human NEP (described in [28a]) was a generous gift from B. Malfroy.

Expression of NEP2 splice variants in mammalian cell lines

Total RNA from rat brain and testis were reverse transcribed using the random primer p(dN)$_6$ (Roche Molecular Biochemicals, Meylan, France) and amplified by PCR using 5′-GGGGTACCCATGGGGAAGTCGGAGAGCTCAGTG-3′ and 5′-CGGAATTCGGCTACCAGATGCGACATCG-3′ as sense and antisense primers, respectively (the underlined sequences are the enzyme-recognition sites plus two or three amino acids in the 5′ direction as necessary for each enzyme). The generated full-length cDNAs for secreted isoform NEP2(s) or its membrane-bound isoform, NEP2(m) [24,25], were digested with *Kpn*I and *Eco*RI and ligated in-frame into the pcDNA3.1 expression vector (Invitrogen, Groningen, The Netherlands), which contains an N-terminal polyhistidine tag and an anti-Xpress tag antibody epitope. Each construct was verified by automated sequencing (Licor). CHO-K1 and AtT20 cells were maintained in Ham's F-12 medium or Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum respectively. pcDNA3.1 vector (control), NEP2(m) or NEP2(s) constructs were transiently transfected using Superfect (Qiagen, Courtaboeuf, France). After 4 h at 37 °C, the transfection medium was replaced by a low-protein, serum-free medium, CHO-S-SFM II (Life Technologies, Cergy Pontoise, France). Stable AtT20 clones expressing either NEP2 isoform or the vector alone were selected after 2 weeks in medium supplemented with 800 μg/ml Geneticin, and maintained in culture medium containing 200 μg/ml of the antibiotic. Routinely, cells and conditioned media were collected 48–72 h post-transfection, or 5–6 days after cell seeding for stable transfectants. While the media were retained for purification, cells were scraped and harvested by centrifugation (1000 ***g*** for 10 min) in ice-cold Hepes buffer (50 mM, pH 7.0/0.1 mM PMSF) and resuspended in 20 vol. of the same buffer before disruption with a Polytron (Kinematica, Luzern, Switzerland). Membranes were obtained by centrifugation (twice at 50000 ***g*** for 30 min each), resuspended in Hepes buffer at a final protein concentration of 10 mg/ml and solubilized in Hepes buffer with 2% Brij-58/0.5% Triton X-100 for 16 h at 4 °C.

Antibody production and immunoblotting

A 151-amino acid fragment (residues 212–363), common to NEP2(m) and NEP2(s) and located 156 amino acids after the predicted transmembrane domain, was amplified by PCR using the primers 5′-GGGGATCCCGGAGCGGCAGTTGGCTGTGTTGAACTC-3′ and 5′-GCGAATTCATCATGCCTTTTCTCCTGGGG-3′, which contained sites for *Bam*HI and *Eco*RI, respectively (underlined). The obtained band was digested, subcloned into the corresponding sites of the polycloning site of the expression vector pGEX5-X1 (Amersham Bioscience, Orsay, France) in-frame with glutathione S-transferase and expressed in JM109 cells (Stratagene, Amsterdam, The Netherlands). After induction with isopropyl β-D-thiogalactoside, bacteria were lysed and the protein purified on a glutathione–Sepharose column. Rabbits were immunized by three subcutaneous injections of 200 μg of purified fusion protein at 8-day intervals. Animals were then injected regularly with 100 μg of the fusion protein and bled weekly for 4 weeks.

For immunoblotting, samples were diluted in buffer (0.1 M Tris/HCl, pH 6.8, 15% glycerol, 3% SDS, 3% β-mercaptoethanol and 0.02% Bromophenol Blue), subjected to SDS/PAGE on 7.5% polyacrylamide gels and transferred on to PVDF membranes (Amersham Bioscience). After 1 h in Tris-buffered saline (0.1 M Tris/HCl/0.9% NaCl, pH 7.4) containing 0.2% Tween 20 and 5% BSA, blots were incubated with either NEP2-specific antibody (diluted 1/2000) or the monoclonal anti-Xpress tag antibody (1/5000) for 16 h at 4 °C. Immunoblots were revealed after a 1 h incubation with the appropriate horseradish peroxidase-coupled IgG (1/3000), using Supersignal (Perbio Science France, Bezons, France).

Endoglycosidase digestions

Solubilized membranes or wheatgerm fractions (see the following section) were heat-denatured in SDS and incubated with either recombinant N-glycosidase F (2 units) or endoglycosidase H (Endo-H; 2.5 m-units) for 16 h at 37 °C according to the supplier's instructions (Roche Biochemicals). Controls were incubated without enzyme, and samples were then immunoblotted.

Partial purification of NEP2(s) and NEP2(m) activities

Suc-AAF-AMC (where Suc is succinyl) was selected to follow the purification of NEP2 activity from solubilized membranes and culture media of CHO-K1 or AtT20 cells. Culture media from between two and eight 140 mm dishes of control and NEP2(s)-transfected cells were concentrated 10-fold using the centrifugal filter device Centriprep 30 (Amicon), and passed on to between one and four columns of 1 ml of Hi-trap wheatgerm lectin (Amersham Bioscience), equilibrated with 20 mM Tris/HCl buffer (pH 7.4) containing 0.5 M NaCl. After two 20 ml washes with the buffer, each column was eluted with 5 ml of the same buffer containing 0.5 M *N*-acetylglucosamine. Solubilized membranes of NEP2(m)-transfected or control cells were subjected to the same purification step.

The eluate was diluted 2-fold with 40 mM Tris/HCl buffer, pH 7.4, containing 2 M $(NH_4)_2SO_4$ and 20% glycerol and loaded on to a phenyl-Sepharose Hi-Prep 16/10 column (5 ml/min; Amersham Bioscience) equilibrated with 20 mM Tris/HCl buffer, pH 7.4, containing 1 M $(NH_4)_2SO_4$ and 10% glycerol. After a 10 min wash with the same buffer, the column was eluted using a linear $(NH_4)_2SO_4$ gradient (1.0–0.0 M over 30 min), and fractions were assayed for enzyme activity. Active fractions were pooled, concentrated with Centriprep 30 and diluted 20-fold

with a 20 mM Tris/HCl buffer, pH 7.4, containing 10 % glycerol; the diluted fraction was loaded on to a DEAE–Sepharose Hi-Prep 16/10 column (5 ml/min; Amersham Bioscience) equilibrated with Tris/HCl buffer, washed with the same buffer for 10 min and eluted with a linear NaCl gradient (0.0–1.0 M over 30 min). Fractions (5 ml) were assayed for enzyme activity. After the phenyl-Sepharose purification step, NEP2(m)-active fractions were pooled and concentrated on a Centricon 100 device. Enzyme concentration was quantified by SDS/PAGE with BSA standards and detection by silver staining.

Enzyme activity assays and inhibitory potencies

NEP and NEP2 activities were assayed essentially as described in [29–31]. For determination of inhibitory potencies, the incubation mixture comprised 20 μM Suc-AAF-AMC in 100 μl of 100 mM Hepes buffer, pH 7.2, containing 0.15 M NaCl, 0.01 % Triton X-100 and 0.01 pmol/ml of pure recombinant human NEP or 200 μg/ml of total protein of the wheat-germ fraction of NEP2(s) (first step of purification), corresponding to 0.06 pmol/ml of pure NEP2. Incubations were for 60 min at 37 °C. No Suc-AAF-AMC-hydrolysing activity could be detected in parallel incubations performed with either culture media or solubilized membranes of control cells submitted to the same purification procedure as NEP2(s)- or NEP2(m)-transfected cells. Inhibitors were tested in concentrations ranging from 0.01 nM to 10 μM, whereas inhibitory potencies of natural peptides were determined between 0.1 and 100 μM. K_i values of inhibitors were calculated from the IC_{50} values using the Cheng and Prussoff equation [32], competitive inhibitions being verified in some cases by Lineweaver–Burk plots, which yielded comparable K_i values.

HPLC analysis of the hydrolysis products of peptides generated by NEP2(s) or NEP

The partially purified preparation of NEP2(s) from transfected AtT20 cells (third step of the purification procedure; final concentration of 2 μg of total protein/ml, corresponding to 0.3 pmol/ml of pure enzyme) or the pure recombinant NEP (final concentration, 0.2 pmol/ml) were used to establish their substrate-specificity patterns against a variety of peptides. Incubations were performed in 0.1 M Hepes buffer, pH 7.2, containing 0.15 M NaCl, 0.01 % Triton X-100 and peptides at concentrations corresponding to three times their K_i values, established previously against Suc-AAF-AMC hydrolysis, or at a maximal final concentration of 0.1 M. Control incubations were performed in each case using 2 μg of total protein/ml of culture media from control AtT20 cells submitted to the same three-step purification procedure as media from NEP2(s)-transfected cells. Incubations were performed at 37 °C for 30–120 min according to the rates of appearance of detectable peptide fragments for each substrate and stopped by addition of 10 μl of 1 M HCl. The intact substrates were separated from generated fragments by HPLC on a C_{18} μBondapak column (Waters Associates) using a mobile phase consisting of 0.1 % trifluoroacetic acid adjusted to pH 2.5 and a linear gradient of acetonitrile (0–50 % over 20 min). Detection was by UV absorption at 210 nm. Hydrolysis rates (k_{cat}) were calculated from the decrease of the height of the substrate peak under linear time conditions. Generated fragments were identified and quantified by UV analysis of relevant synthetic peptides using parallel HPLC or, when required, sequence analysis. For some peptides [i.e. (Met)enkephalin, substance P (SP) and gonadotropin-releasing hormone (GnRH)] K_m values were also obtained by HPLC analysis and found to be comparable with their K_i values with the model substrate.

RESULTS

Expression of NEP2(s) and NEP2(m) in endocrine and non-endocrine eukaryotic cells

In order to characterize NEP2 enzymic activity, its two major isoforms, NEP2(m) and NEP2(s), were transfected in either non-endocrine CHO cells or endocrine AtT20 cells. Solubilized membranes and media were semi-purified on a wheatgerm column. Western blots of crude solubilized membranes, the lectin flow through and the corresponding purified product from NEP2(m)-transfected AtT20 cells revealed that this isoform is membrane-bound and present mainly as a 110 kDa protein with an additional faint band of approx. 130 kDa (Figures 1A and 1B). Only the latter was retained on the lectin column, the major 110 kDa form being recovered in the column flow through (as well as in the washing fluid; results not shown). NEP2(m) was never detected in the culture medium using either the N-terminal anti-Xpress tag antibody or the C-terminal NEP2-specific antibody (Figures 1A and 1B). In contrast, although membranes of AtT20 cells transfected with the NEP2(s) isoform also contained the protein, detected as a 110 kDa band using either N- or C-terminal antibodies (Figures 1C and 1D), the majority of NEP2(s) was found in the conditioned-cell culture media as a higher-molecular-mass form of approx. 125 kDa (Figure 1D). Purification of NEP2(s)-containing membrane preparations on a lectin column did not yield any other membrane-bound form and, as for NEP2(m), the 110 kDa form was found in the column

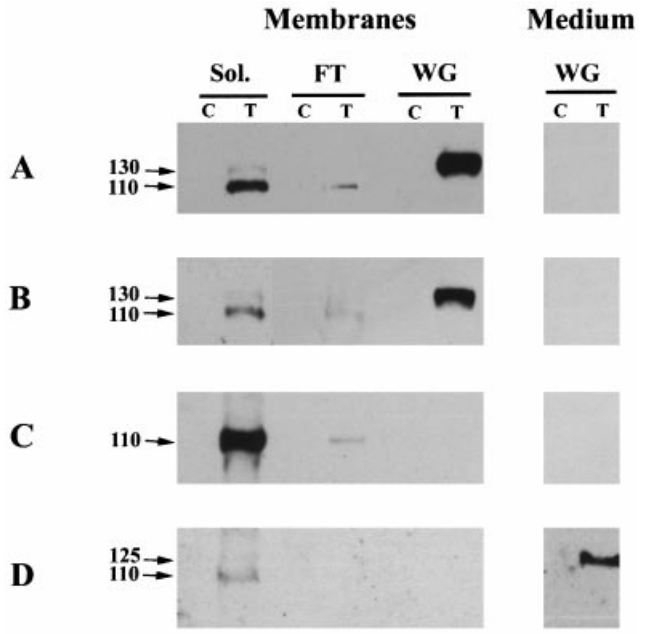

Figure 1 Immunoblot analysis of semi-purified fractions from crude solubilized membranes and culture media from NEP2(m)- and NEP2(s)-transfected AtT20 cells

Proteins from crude solubilized membrane preparations (Sol.), their wheatgerm flow-through fraction (FT), wheatgerm semi-purified fraction (WG) and culture media semi-purified on a wheatgerm lectin column (WG; about 20 μg of protein/lane) from control (C) and NEP2(m)- or NEP2(s)-transfected (T) AtT20 cells, were separated by SDS/PAGE (7.5% gel) under reducing conditions and immunoblotted. Protein fractions of NEP2(m)-transfected AtT20 cells were revealed using either the N-terminal anti-Xpress tag antibody (**A**) or the C-terminal NEP2-specific polyclonal antibody (**B**). The immunoblot in (**C**) represents NEP2(s) fractions revealed with the anti-Xpress tag antibody, and (**D**) was revealed with the NEP2-specific polyclonal antibody.

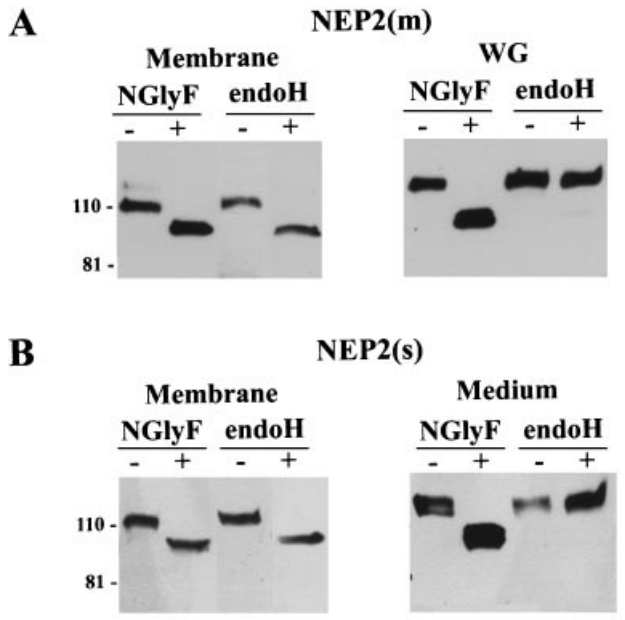

Figure 2 Endoglycosidase digestion of crude solubilized membranes and wheatgerm products from NEP2(m)- and NEP2(s)-transfected AtT20 cells

(**A**) Crude solubilized membranes (left-hand panel) of NEP2(m)-transfected AtT20 cells and the corresponding wheatgerm (WG) semi-purified fraction (right-hand panel) were digested with either N-glycosidase-F (NGlyF) or Endo-H (endoH) and immunoblotted with the anti-Xpress tag antibody. (**B**) Crude solubilized membranes (left-hand panel) of NEP2(s)-transfected AtT20 cells and the corresponding culture media semi-purified on a wheatgerm lectin column (right-hand panel) treated with the same endoglycosidases and immunoblotted with the NEP2-specific antibody.

flow through (Figure 1C). Whereas transfection of CHO cells with NEP2(s) produced the same results as in AtT20 cells, the 130 kDa form of NEP2(m) detected in solubilized AtT20 cell membranes and after lectin purification was never detected (results not shown).

In order to differentiate the various forms by their glycosylation state, solubilized membranes and wheatgerm products of AtT20-transfected cells were treated with either N-glycosidase F or Endo-H (Figure 2). Whereas N-glycosidase F removes high mannose and most complex N-linked oligosaccharides added only in the Golgi apparatus, Endo-H only digests oligosaccharides of the high-mannose type found on proteins of the endoplasmic reticulum (ER). Resistance to Endo-H is therefore conferred to glycoproteins that have exited the ER and travelled through the Golgi apparatus. Digestion of membrane preparations containing either isoform produced a shift in molecular mass from 110 kDa to about 95 kDa by both endoglycosidases. Treatment of the lectin semi-purified products, however, revealed that these forms had acquired resistance to Endo-H digestion. In CHO cells, only the semi-purified conditioned media of NEP2(s)-transfected cells were resistant to Endo-H digestion (results not shown). These results suggest that the shift in the apparent molecular mass observed after lectin purification of either isoform of NEP2 in AtT20 cells reflects acquisition of complex oligosaccharides in the Golgi apparatus.

Characterization of the enzymic activity of NEP2(m) and NEP2(s) using model substrates

The substrate currently used to monitor NEP activity, Suc-AAF-AMC, was found to be a model substrate for NEP2 as well (Table 1). Whereas NEP2(m) activity was never detected in CHO membranes, NEP2(m)-transfected AtT20 cells contained a slight activity (10 pmol/min per mg of protein). In addition, while the 110 kDa band present in the lectin column flow through was inactive, the purified 130 kDa form possessed enzymic activity (545 pmol/min per mg of protein). No activity was detected in conditioned media of NEP2(m)-transfected CHO or AtT20 cells. These results suggest that NEP2(m) is inactive in the ER and needs to reach the Golgi apparatus to become active, a process

Table 1 Hydrolysis of synthetic substrates by NEP2: comparison with NEP

Incubations were performed at 37 °C for 60 min followed by 60 min of incubation in the presence of aminopeptidase M to release fluorescent AMC. Arrows indicate cleavage sites by NEP2 (black arrows) and NEP (white arrows) as determined by separate HPLC analysis. Values for NEP are given in parentheses. Data are mean values from at least two independent determinations. Specificity constant values represent the ratio of k_{cat} to K_m for NEP2(s). The following synthetic substrates were not hydrolysed by either isoform of NEP2: Suc-GP-AMC, MeOSuc-AAPM-AMC (where MeOSuc is 3-methoxysuccinyl), MeOSuc-DYM-AMC, Z-PR-AMC (where Z is benzyloxycarbonyl), Boc-VGR-AMC (where Boc is *t*-butyloxycarbonyl), Z-R-AMC, Boc-RVRR-AMC and Z-GPR-AMC.

Substrates	k_{cat} [min^{-1}]	K_M [μM]		Specificity constant [$min^{-1}.\mu M^{-1}$]
		NEP2(m)	NEP2(s)	
Suc AA↓-F-AMC Suc AA↑-F-AMC	510 *(2200)*	27	50 *(70)*	10.2 *(31.4)*
Suc AA↓-V-AMC Suc AA↑-V-AMC	830 *(1400)*	40	100 *(100)*	8.3 *(14)*
MeOSuc G↓-LF-AMC MeOSuc G↑-LF-AMC	690 *(1300)*	23	35 *(80)*	19.7 *(16.2)*

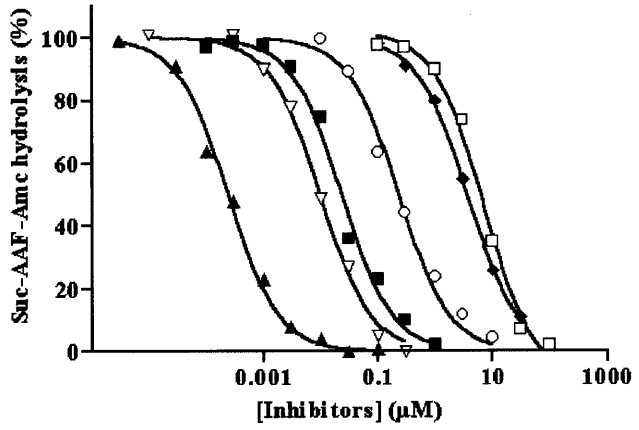

Figure 3 Inhibitory potencies of various compounds against NEP2(s)

Suc-AAF-Amc (20 μM) was incubated in the presence of NEP2(s) and increasing concentrations of phosphoramidon (▲), omapatrilat (▽), fasidotrilat (■) or thiorphan (○). The K_i values obtained are reported in Table 2. Examples of inhibition by natural peptides are presented for SP (□) and bradykinin (◆), and their K_i values are reported in Table 5 and 6 respectively.

Table 2 Potency of various metallopeptidase inhibitors against NEP2(s) and NEP(m) activities: comparison with NEP

The fluorogenic substrate Suc-AAF-AMC (20 μM) was used to evaluate the activities of both isoforms of NEP2 and of NEP [K_i values for NEP2(s) were determined from data shown on Figure 3]. Enzyme activities were abolished completely by 1 mM EDTA.

	K_i		
Inhibitor	NEP2(s)	NEP2(m)	NEP
Phosphoramidon	0.8 nM	2 nM	2 nM
Omapatrilat	8 nM	17 nM	3 nM
Fasidotrilat	16 nM	19 nM	5 nM
Thiorphan	120 nM	250 nM	4 nM
Captopril	> 100 μM	> 100 μM	10 μM

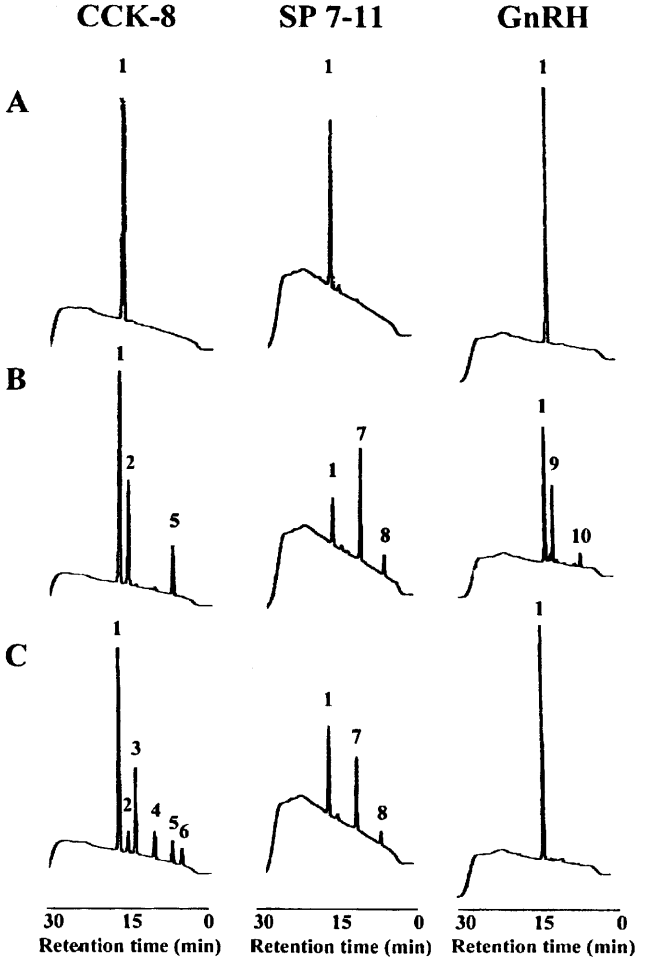

Figure 4 HPLC analysis of NEP and NEP2(s) hydrolysis products of CCK octapeptide (CCK-8), SP [SP-(7–11)] or GnRH

Substrates (100 μM) were incubated for 2 h [SP-(7–11)], 3 h (CCK-8) or 4 h (GnRH) in the presence of semi-purified proteins from cell-culture medium of control (**A**) or NEP2(s)-expressing AtT20 (**B**) cells and pure recombinant NEP (**C**). Hydrolysis products were analysed by HPLC using a linear acetonitrile gradient (0–50% in 20 min) and detection at 210 nm. Peaks were identified by comparison with standard synthetic peptides and found to correspond to unchanged substrates (1), WMDF-NH_2 (2), DYMGWMD (3), WMD (4), DYMG (5), F-NH_2 (6), FFG (7), LM-NH_2 (8), pEHWSYG (9) and LRPG-NH_2 (10).

which does not occur in CHO cells. In the case of NEP2(s)-transfected cells, the enzymic activity was also detectable exclusively in the Endo-H-resistant fraction, i.e. in media of CHO cells (56 pmol/min per mg of protein), and more so from AtT20 cells (420 pmol/min per mg of protein).

Suc-AAF-AMC hydrolysis reflected an endopeptidase activity, since omission of aminopeptidase M from the assays abolished the release of fluorescence. HPLC analysis also confirmed the formation of F-AMC by NEP2(s) (Table 1). The hydrolysis displayed typical saturation kinetics with apparent K_m values of approx. 30 and 50 μM for NEP2(m) and NEP2(s), respectively (Table 1), and a pH optimum of 6.5 for both isoforms (results not shown). Their activity was completely abolished by metal-chelating agents (1 mM EDTA or *o*-phenanthroline) as well as, in a concentration-dependent manner, by transition-state NEP inhibitors containing a zinc-chelating group (Figure 3 and Table 2). Whereas the various inhibitory agents studied differed by no more than a factor of 2 for the two NEP2 isoforms, thiorphan remained 30 times more potent against NEP than NEP2(s).

A large variety of artificial fluorogenic substrates were not hydrolysed by NEP2, but two other NEP substrates were cleaved at the same bonds (Table 1). In order to differentiate the two peptidase activities, a specificity constant (k_{cat}/K_m) was calculated. Whereas this index was reduced by about 2-fold for NEP in the case of either Suc-AAV-AMC or MeOSuc-GLF-AMC (where MeOSuc is 3-methoxysuccinyl) as compared with Suc-AAF-AMC, this was not the case for NEP2 (Table 1).

Since both isoforms displayed similar enzymic activities, with identical substrates, pH optima and kinetics, NEP2(s) was selected to characterize in detail NEP2 activity against natural peptides and compare it with NEP.

Hydrolysis of natural peptides

To avoid interference by contaminating peptidases, AtT20 cell-culture media were subjected to a three-step purification procedure leading to 35-fold purification and a pure enzyme concentration estimated at 10 pmol/ml. Since the enzyme was not purified to homogeneity, the absence of cleavage by a parallel preparation derived from control AtT20 cell-culture media was verified for each substrate (see Figure 4 for example). The hydrolysis of several natural peptides by a purified preparation

Table 3 Hydrolysis of enkephalins and related opioid peptides by NEP2: comparison with NEP

Linear hydrolysis rates were evaluated from incubations in the presence of peptides in concentrations corresponding to three times their K_i values, followed by HPLC analysis. The black and white arrows represent cleavage sites by NEP2 and NEP respectively. Values for NEP are given in parentheses. Specificity constant values represent the ratio of k_{cat} to K_i. The K_m value of NEP2 for (Met^5)enkephalin is 19 μM. The enkephalin heptapeptide (YGGFMRF), endomorphin I (YPYF-NH_2) and β-endorphin (YGGFM…, 31 amino acids) were not hydrolysed detectably ($k_{cat} < 20$). Peaks were identified by retention times of corresponding synthetic marker peptides and quantified by their absorption at 210 nm.

Compound	Sequence	k_{cat} [min^{-1}]	Ki [μM]	Specificity constant [$min^{-1}.\mu M^{-1}$]
[Met^5]enkephalin	YGG-FM	540 *(1300)*	10 *(44)*	54 *(29.5)*
[Met^5]enkephalin-NH_2	YGG-FM-NH_2	20 *(800)*	25 *(>100)*	0.8 *(<8)*
[Des-Tyr^1,Met^5]enkephalin	GG-FM	510 *(1000)*	14 *(80)*	36.4 *(12.5)*
[L-Ala^3, Met^5]enkephalin	YGA-FM	2500 *(1600)*	30 *(50)*	83.3 *(32)*
[D-Ala^3,Met^5]enkephalin	YG(dA)FM	0 *(0)*	50 *(>100)*	0 *(0)*
[Leu^5]-enkephalin	YGG-FL	160 *(1200)*	3 *(100)*	53.3 *(12)*
[Leu^5]-enkephalin-NH_2	YGG-FL-NH_2	20 *(200)*	21 *(>100)*	0.95 *(<2)*

Table 4 Hydrolysis of CCK-8 and related peptides by NEP2: comparison with NEP

CCK-(1–5), i.e. DYMGW, was not hydrolysed detectably ($k_{cat} < 10$) by either NEP2 or NEP. The black and white arrows represent major cleavage sites by NEP2 and NEP, respectively, whereas the hatched arrow represents a minor cleavage site by NEP. The asterisk corresponds to the sulphation site.

Compound	Sequence	k_{cat} [min^{-1}]	Ki [μM]	Specificity constant [$min^{-1}.\mu M^{-1}$]
CCK-8 (sulphated)	*DYMG-WMD-F-NH_2	370 *(530)*	21 *(15)*	17.6 *(35.3)*
CCK-8 (non sulphated)	DYMG-WMD-F-NH_2	790 *(750)*	50 *(>100)*	15.8 *(<7.5)*
CCK (1-6)	DYMG-WM	2070 *(1400)*	18 *(>100)*	115 *(<14)*
CCK (4-8)	G-WMD-F-NH_2	240 *(500)*	>100 *(>100)*	<2.4 *(<5)*
CCK (5-8)	WMD-F-NH_2	<10 *(400)*	39 *(>100)*	<0.2 *(<4)*

of NEP2(m) was studied in parallel and found to be identical to that of NEP2(s).

A specificity constant (k_{cat}/K_i) was determined for both peptidases, which reflects the efficiency of the enzyme to cleave a given peptide at low substrate concentrations. K_i values used for these determinations were derived from IC_{50} values, which were comparable with the values derived from Lineweaver–Burk plots (i.e. 12.6, 23.5 and 2.5 μM for SP, GnRH and bradykinin, respectively). These constants of the various substrates can be compared directly for each enzyme but not for the two enzyme

Table 5 Hydrolysis of SP and related peptides by NEP2: comparison with NEP

SP-(9–11), i.e. GLM-NH_2, was not hydrolysed ($k_{cat} < 10$) by either NEP2 or NEP. K_m values of NEP2 for SP-(1–11) and SP-(7–11) were 13 and 81 μM, respectively. Arrows are as for Table 4.

Compound	Sequence	k_{cat} [min^{-1}]	Ki [μM]	Specificity constant [$min^{-1}.\mu M^{-1}$]
SP (1-11)	RPKPQQ-FFG-LM-NH_2	360 *(800)*	4 *(9)*	90 *(90)*
SP (7-11)	FFG-LM-NH_2	3000 *(1800)*	90 *(>100)*	33.3 *(<18)*
SP (8-11)	FG-LM-NH_2	1370 *(700)*	>100 *(>100)*	<13.7 *(<7)*

preparations, since they depend upon the specific activity of each preparation. The specificity constants reported for NEP are in agreement with published data [33].

Regarding enkephalins and related opioid peptides (Table 3), the two peptidases cleaved the same Gly–Phe bond in all susceptible peptides, and hydrolysis by NEP2 and NEP was strongly reduced when the C-terminal carboxylate was amidated. However, differences were found: Ala instead of Gly in P1 significantly enhanced hydrolysis by NEP2 but not NEP, whereas Leu instead of Met in P′2 had an opposite influence (whereas affinity was enhanced) on NEP2; in contrast, neither affinity nor hydrolysis was significantly affected by the latter change in the case of NEP. The efficient hydrolysis by NEP2 of [^{3}H](D-Ala^2,Leu^5)enkephalin, a known substrate for NEP, was reported by Ghaddar et al. [26].

In the case of cholecystokinins (CCKs) and derivatives (Table 4), clear differences could be observed: NEP2 did not attack the Asp^7–Phe^8-NH_2 bond, a preferential cleavage site for NEP [34], but cleaved the Gly^4–Trp^5 amide bond. There was also a marked decrease in specificity for CCK-(4–8) and an enhanced hydrolysis rate when the Tyr^2 residue of CCK-8 was not sulphated.

Hydrolysis of SP and derivatives (Table 5) occurred predominantly at the Gly^9–Leu^{10} bond in the case of NEP and, in addition, at the Gln^6–Phe^7 bond with NEP2. The latter hydrolysis was underestimated because its product, SP-(7–11), is approx. 10 times more rapidly hydrolysed at its Gly–Leu bond than its parent compound SP-(1–11).

For a large variety of other bioactive peptides (Table 6), similar hydrolysis patterns were found for NEP2 and NEP. For instance, the long list of barely detectable hydrolysed peptides ($k_{cat} < 100$ min^{-1}) comprised in both cases nociceptin, pituitary adenylate cyclase-activating polypeptide, neurotensin, somatostatin, big-endothelin-$1_{19–38}$, corticotropin-releasing hormone, galanin, oxytocin, vasopressin, angiotensin II, growth hormone-releasing hormone, neuropeptide Y and gastrin. Hydrolysis of the bond involving the amino group of a Phe residue occurred with comparable specificity constants for both enzymes in angiotensin I or bradykinin. In the case of GnRH, however, hydrolysis by NEP2 was rapid at the level of the Gly^6–Leu^7 bond, whereas it was much slower and had a very low specificity constant with NEP, and occurred primarily at the Ser^4–Tyr^5 bond.

Table 6 Hydrolysis of various bioactive peptides by NEP2: comparison with NEP

The K_m value of NEP2 for GnRH was 32 μM. The following peptides were hydrolysed by NEP2 at rates < 100 μmol/min per μmol of enzyme: vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide (1–27) and (1–38), orphanin FQ, somatostatin 14 and 28, neurotensin, adrenocorticotropin hormone (1–24) and (1–39), α-melanocorticotropin hormone, big-endothelin-$1_{19–38}$, corticotropin-releasing hormone, galanin, oxytocin, vasopressin, angiotensin II, growth hormone-releasing hormone, neuropeptide Y and gastrin. Arrows are as for Table 4. rANP, rat atrial natriuretic peptide.

Compound	Sequence	k_{cat} [min^{-1}]	K_i [μM]	Specificity constant [$min^{-1}.\mu M^{-1}$]
GnRH	pEHWS-YG-LRPG-NH_2	510 *(120)*	18 *(>100)*	28.3 *(<1.2)*
Angiotensin I	DRVY-IHP-FHL	180 *(420)*	6 *(23)*	30 *(18.2)*
Bradykinin	RPPGFSP-FR	150 *(1500)*	2 *(34)*	75 (44.1)
rANP (1-28)	....NS-FRY	120 *(400)*	12 *(9)*	10 *(44)*

DISCUSSION

Expression of the two major isoforms of NEP2, i.e. NEP2(m) and NEP2(s), in two mammalian cell lines, one endocrine (AtT20) and one non-endocrine (CHO), reveals that transport and enzymic activity of NEP2 are tightly linked and cell-specific. In agreement, our results show that the ER-associated 110 kDa glycoproteins of both NEP2 isoforms are inactive, and that their transport out of the ER is most effective in endocrine AtT20 cells. This was never demonstrated for any other member of the M13 family of metalloproteases and, for instance, NEP has successfully been expressed in non-endocrine COS cells [35]. Furthermore, mutation of glycosylation sites of NEP resulting in some ER retention did not significantly alter NEP enzymic activity [36]. The ER localization of NEP2(m) in CHO cells was also observed by Raharjo et al. [37], and we show here that NEP2(m) and NEP2(s) are only active in their Endo-H-insensitive forms that have exited the ER and reached the Golgi apparatus, for NEP2(m), or been secreted, in the case of NEP2(s). The absence of a membrane-bound form of NEP2(s) insensitive to Endo-H further confirms that NEP2(s) is secreted as a consequence of its cleavage beyond its transmembrane domain, most probably at the subtilisin site [26]. Moreover, our results suggest that this maturation occurs early in the secretory pathway. Taken together, our data suggest, that unlike other members of the M13 family, NEP2(m) and NEP2(s) are synthesized as inactive enzymes, requiring post-translational modifications to both be transported out of the ER and become active. Transport out of the ER in AtT20 cells may require post-translational modifications specific to a secretory pathway, or a chaperone protein present in this secretory cell. It may therefore be that expression of NEP2 in an enzymically active form is limited to homologous systems, and it is noteworthy that *in situ* hybridization detects the NEP2 mRNA only in secretory (neuronal or endocrine) cells [24].

The enzymic specificity of NEP2 shares common features with its closest homologue, NEP. Both peptidases cleaved the same fluorogenic substrates at the same amide bonds, particularly Suc-AAF-AMC that is currently used to assay membrane-bound or soluble NEP activity in tissues, blood plasma or urine [29–31]. In tissues where both peptidases are expressed, MeOSuc-GLF-AMC constitutes a more favourable assay for NEP2, minimizing the contribution of NEP. The specificity of inhibitors also overlapped since the prototypical transition-state inhibitors of NEP, i.e. phosphoramidon [12] or thiorphan [18], and the dual NEP/angiotensin-converting enzyme inhibitors, i.e. fasidotrilat [38] or omapatrilat [39], inhibited both peptidases. However, they had distinctly apparent affinities, thiorphan being thirty times as potent against NEP than against NEP2. Phosphoramidon was equipotent against NEP2 and NEP, casting doubts on the identity of the peptidase characterized previously as NEP in complex biological systems containing both enzymes (NEP and NEP2), using inhibition by this compound as the sole criterion.

The substrate specificities of NEP and NEP2 also displayed overall similarities inasmuch as both metallopeptidases appear to hydrolyse a variety of bioactive oligopeptides of a size < 3 kDa, often at the same amide bond, preferentially comprising the amino group of neutral bulky aliphatic or aromatic amino acid residues. Hence enkephalins and derivatives were cleaved at their Gly–Phe bonds [13], SP cleaved at its Gly–Leu bond [40] and bradykinin or angiotensin I at their Pro–Phe bond [15]. This preference of both peptidases is also shown by the nature of the susceptible fluorogenic substrates and that of active transition-state inhibitors, which all display a hydrophobic P′1 residue [41]. NEP2 also resembles NEP in its preference for a non-substituted CO–NH linkage between P′1 and P′2, as shown by the low affinity of captopril (which contains a Pro residue in P′2), and by the absence of cleavage of the Xaa–Pro bond in Suc-GP-AMC or MeOSuc-AAPM-AMC.

Finally, the importance of a free C-terminal carboxylate to confer affinity to substrates (or inhibitors), explaining the preferential dipeptidyl carboxypeptidase over endopeptidase activity of NEP [42], also applies to NEP2, as shown by the marked reduction in affinity and hydrolysis rates of enkephalins upon amidation of this carboxylate. In addition, bioactive peptides with a free carboxylate were hydrolysed preferentially at their C-terminal ends, e.g. bradykinin, which was cleaved by both NEP and NEP2 at the Pro^7–Phe^8 rather than at the Gly^4–Phe^5 bond.

Nevertheless, several distinct features of NEP2 activity have been disclosed here. Whereas NEP prefers aromatic residues in P′1, bulky aliphatic residues are preferred by NEP2, e.g. the hydrolysis of GnRH occurs preferentially at P′1 Tyr and Leu residues for NEP and NEP2, respectively. The marked preference of NEP2 for phosphoramidon over thiorphan, in which the P′1 residues are Leu and Phe, respectively, could also reflect the same feature. Another difference is the higher importance of the P1 substite for recognition of substrates or inhibitors in the case of NEP2. In agreement, the Asp^7–Phe^8-NH_2 bond of CCKs is cleaved by NEP but not NEP2, presumably reflecting a detrimental role of the dicarboxylic amino acid residue on the P1 subsite of the latter.

Although this study of the enzymic specificity of NEP2 is the most in-depth to date, it remains difficult to infer a physiological role for the enzyme based only on these data. Indeed, substrate selectivity under physiological conditions results from a combination of enzymological and cell-localization factors which must all be taken into account [43]. Differences in their distribution [24], together with those in cell-trafficking-associated changes in activity and in specificity towards natural peptides (e.g. GnRH or CCK), indicate that NEP and NEP2 display distinct physiological roles.

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