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A NEW METALLO- ENDOPEPTIDASE FROM HUMAN NEUROBLASTOMA NB-OK-1 CELLS WHICH INACTIVATES ATRIAL NATRIURETIC PEPTIDE BY SELECTIVE CLEAVAGE AT THE SER^{123}-PHE^{124} BOND

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Abstract : A novel metallo-endopeptidase from human neuroblastoma NB-OK-1 cells was partially purified and characterized. This enzyme activity was detected in the culture medium and could be detached from intact cells by gentle washing, suggesting a peripheral localization of the enzyme. This endopeptidase inactivated Atrial Natriuretic Peptide (ANP) by a unique and selective cleavage of the Ser^{123}-Phe^{124} bond. It also produced hydrolysis at the Xaa-Phe, Xaa-Leu, or Xaa-Ile bonds of other peptide hormones such as bradykinin, somatostatin 14, litorin, substance P, neuromedin C and angiotensin II. The substrate selectivity and inhibition profile of the enzyme showed obvious similarities with the peptide hormone inactivating endopeptidase (PHIE) recently purified from *Xenopus laevis* skin secretions and indicated a thermolysin-like activity distinct from neutral endopeptidase (EC 3.4.24.11) and from angiotensin converting enzyme (EC 3.4.15.1). © 1992 Academic Press, Inc.

The human neuroblastoma NB-OK-1 cell line is a good model to study Atrial Natriuretic Peptide (ANP) metabolism at the receptor level since these cells possess high affinity R_1-type receptors for ANP-(99-126) but no R_2-type receptors. Occupancy of these ANP receptors is coupled with cyclic GMP accumulation (1). It was recently observed that human neuroblastoma NB-OK-1 cells inactivate ANP-(99-126) by a Ser^{123}-Phe^{124} bond cleavage (2). The activity responsible for this ANP degradation was sensitive to divalent metal cation-chelators and was detected in the incubation medium (2). This endopeptidase

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activity is of particular interest since both amino-terminal and carboxy-terminal fragments of ANP do not exhibit any affinity for the R_1-type ANP receptors (1).

Partial purification and characterization of this endopeptidase allowed to compare its functional properties with those of neutral endopeptidase (NEP, EC 3.4.24.11), angiotensin converting enzyme (ACE, EC 3.4.15.1) and of a peptide hormone inactivating enzyme (PHIE) isolated from *Xenopus laevis* skin secretions which produces a selective Ser^{123}-Phe^{124} bond cleavage of ANP, and hydrolysis of Xaa-Phe, Xaa-Leu ou Xaa-Ile bonds in a number of other peptide hormones (3).

We demonstrate that the ecto-endopeptidase present at the periphery of human neuroblastoma NB-OK-1 cells, possesses a thermolysin-like character clearly distinct from NEP (4) and from ACE (5), and exhibits obvious similarities with the frog enzyme (PHIE) (3). We hypothesize that this enzyme might play an important role in ANP metabolism at the cell surface.

MATERIALS AND METHODS

Purification procedure. Neuroblastoma NB-OK-1 cells (70 mg proteins) were suspended and incubated according to Delporte et al. (2). The supernatant was submitted to ion exchange chromatography on DEAE MemSep 1000 cartridge (Millipore). The sample was eluted stepwise with 0.05 N Tris-HCl (pH 7.4), from 0 to 1M NaCl. The active fractions were concentrated ten times by ultracentrifugation (Unisep™ Ultracent-30 membrane cartridges, Bio-Rad) to 0.7 ml . This sample was then applied to an hydroxyapatite column (2x5cm) eluted with a 10-500 mM phosphate buffer (pH 7.3) gradient. Proteins were determined using the Bradford assay (6).

Enzyme assay. Endopeptidase activity was monitored by HPLC using ANP as substrate (see below) as in (3). DABTC-[DR^8]-Kermit, a derivative of Kermit (i.e; $DVDERDVRGFASFL_{NH2}$) which undergoes cleavage at the Ser^{12}-Phe^{13} bond, was also used as substrate (7). Alternatively, when ANP, substance P, bradykinin, somatostatin 14, angiotensin II, litorin and neuromedin C were used as substrate, the conditions were as follows: 1-2 nmol of peptide were incubated 30-180 min in 100 mM phosphate buffer (pH 7.4) in the presence of a 10 µl aliquot of the active fractions in a final volume of 20µl. The reaction was stopped by heating 10 min at 100 °C, and the resulting products applied on HPLC using a Nucleosil™ 5 µ C18 column (146x4.5mm) eluted under conditions described in Fig. 1B. The remaining substrate and resulting product(s) were monitored at 220 nm. Fragments produced by endoproteolytic cleavage(s) were analysed for their amino-acid composition using a picoTag station (Waters). Inhibitors were tested (as shown in Table I) in routine conditions of the endopeptidase assay (see above) and ANP-(103-126) as substrate. Inhibitions were expressed as percentages of the reference activity in the absence of chemical reagents under the same experimental conditions. The pH profile of endopeptidase activity was obtained using ANP-(103-126) as substrate (2 nmol per assay) incubated 60 min with 10 µl aliquot of the active fractions in a 100 mM phosphate buffer adjusted to cover a pH range of 5.8 to 8.0, in a final volume of 20 µl. Under these conditions, enzyme kinetics remained linear. Both enzyme assays and inhibition experiments were run two or three times for each given peptide. Analysis of peptide products was performed at least in duplicate.

Peptides and chemicals. [DR^8]-Kermit was prepared by solid phase synthesis (8) on a Multisynthetizer NPS 4000 (Neosystem, Strasbourg, France), purified by HPLC and checked as in (9). Chemicals were reagent grade from Sigma (Saint Louis, Mo, USA) and the other peptide substrates or fragments were from Neosystem or Sigma.

RESULTS

Partial purification of an endopeptidase from human neuroblastoma NB-OK-1 cell line. Endopeptidase activity was monitored by HPLC using both ANP and DABTC-[DR^8]-Kermit as substrates (Methods). Enzyme activity was detached from intact suspended cells (70 mg protein) by gentle washing. The concentrated enzyme activity was submitted to a diethylaminoethyl (DEAE) chromatography and a major peak of activity was eluted with 0.05 N Tris (pH 7.5) -0 mM NaCl (Fig. 1A). Fractions containing this enzyme activity were concentrated and applied on a hydroxyapatite column where it was eluted with 10 mM phosphate buffer (pH 7.3). This allowed to eliminate some of the contaminant proteolytic activities. HPLC profile of ANP hydrolysis demonstrated a unique selective cleavage at the Ser^{123}-Phe^{124} bond by showing unequivocally the production of both amino terminal ANP-(103-123) and carboxy terminal Phe^{124}-Arg^{125}-Tyr^{126} fragments (Fig.1B).

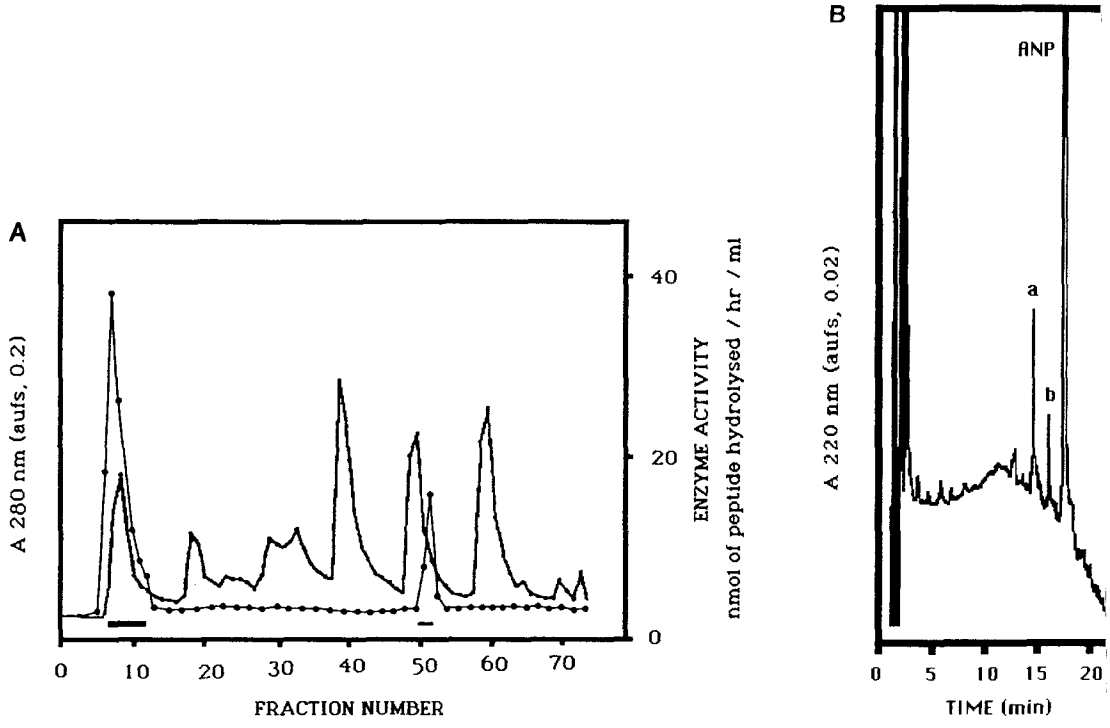

Figure 1 . (A) Enzyme was submitted to ion exchange chromatography and eluted stepwise with 0.05 N Tris-HCl (pH 7.4) from 0 to 1M NaCl. Absorbance of the column effluent was monitored at 220 nm. 1 ml fractions were collected and aliquots of 40 µl assayed for enzymatic activity. Two different substrates were used : DABTC-[DR^8]-Kermit and ANP-(103-126) and the results expressed in nmol of DABTC-[DR^8] -Kermit hydrolysed / hr / ml (•) and by the zones showing enzyme activity on ANP- (103-126)(▬). (B) Elution profile of fragments of ANP-(103-126) generated by the action of human neuroblastoma NB-OK-1 endopeptidase after ion exchange chromatography. The ANP-(103-123) (b) and the ANP-(124-126) (a) fragments were identified both by reference to standards and by amino acid composition.

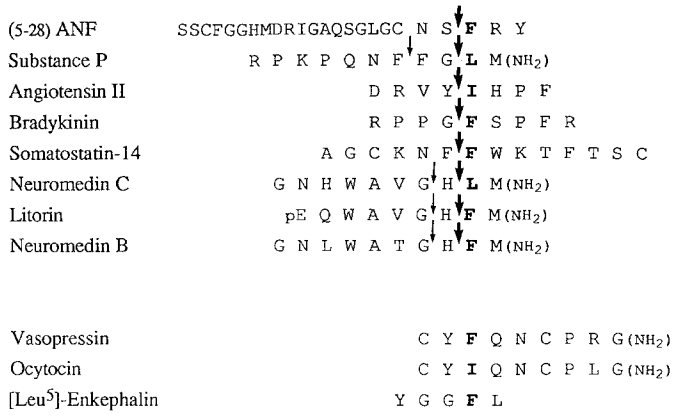

Figure 2 . Amino acid sequences of the peptide hormones used as substrates for human neuroblastoma NB-OK-1 endopeptidase. Plain arrows indicate the major cleavage sites. Dashed arrows correspond to minor cleavage sites. All corresponding fragments were identified unambiguously by amino acid composition.

Substrate selectivity. The partially purified enzyme hydrolysed Xaa-Phe, Xaa-Leu or Xaa-Ile bonds in ANP, bradykinin, somatostatin 14, litorin, substance P, neuromedin C and angiotensin II (Fig. 2). ANP-(103-126) hydrolysis produced the amino terminal ANP-(103-123) and the carboxy terminal tripeptide Phe^{124}-Arg^{125}-Tyr^{126} . Km measured toward ANP was 19 µM . Bradykinin was cleaved at the Gly^4-Phe^5 bond with a minor cleavage at the Pro^7-Pro^8 bond. The hydrolysis of somatostatin 14 at the Phe^6-Phe^7 bond, within the disulfide bridged segment, led to a single product. Litorin was submitted to a major cleavage at the His^7-Phe^8 bond and a minor one at the Gly^6-His^7 bond. Hydrolysis of substance P resulted in a major cleavage at the Gly^9-Leu^{10} bond and a minor cleavage at the Phe^7-Phe^8 doublet. Neuromedin C was principally cleaved at the His^8-Leu^9 bond and at the Gly^7-His^8 bond. Angiotensin II was hydrolysed at the Tyr^4-Ile^5 bond. However, analogous bonds were not cleaved in ocytocin, vasopressin or [Leu^5]-enkephalin.

Optimal pH and inhibitor profile. The optimal pH was 7.0 (not shown). EDTA, EGTA and 1,10-o-phenanthroline inhibited enzyme activity, suggesting its metallo-endopeptidase character (Table I). N-ethyl maleimide (NEM) and p-cloromercuriphenylsulfonic acid (PCMPS), inhibitors of cysteinyl-peptidases, at the mM concentration, inhibited the metallo-endopeptidase activity, suggesting either a direct or indirect involvment of thiol group(s) in enzyme activity. Table I compares the metallo-endopeptidase from human neuroblastoma NB-OK-1 cells versus the peptide hormone inactivating endopeptidase from *Xenopus laevis* skin exudate (3). Specific inhibitors of either NEP, like phosphoramidon and thiorphan, or of ACE, like captopril, were unable to inhibit endopeptidase activity at concentrations up to 0.1, 0.001 and 1 mM respectively. Serine- and carboxyl- protease inhibitors had no effect in the 0.1-1 mM range.

Table I . Effect of protease inhibitors on human neuroblastoma NB-OK-1 endopeptidase

PEPTIDASE CLASS	INHIBITOR	CONCENTRATION (mM))	INHIBITION (%)	
			NB-OK-1	X. laevis (ref.3)
METALLO-	o-PHENANTHROLINE	1	95	98
		0.1	0	5
	EDTA	10.	91	67
		1	64	59
	EGTA	10.	66	63
		1	27	51
CYSTEINYL-	PCMPS	1	93	0
	IODACETAMIDE	1	5	1
	NEM	1	88	0
SERINE-	PMSF	1	0	0
	STI	1	0	0
	APROTININ	1	2	0
	TPCK	1	21	2
CARBOXYL-	PEPSTATIN	1	0	0
	GEMSA	0.1	0	1
NEP	PHOSPHORAMIDON	0.001	0	41
		0.005	0	52
	THIORPHAN	0.001	0	0
ACE	CAPTOPRIL	1	0	0
ASPECIFIC	BENZAMIDINE	0.1	10	0
	TAME	1.	12	0

Enzyme activity was assayed as described in Materials and Methods in the presence of a single inhibitor at a time. All values were calculated with reference to the amount of Ser-Phe cleavage observed on 2 nmol of ANP-(103-126) in 60 min under standard conditions (taken as 0 % inhibition).

DISCUSSION

NB-OK-1 cells possess, at their periphery, ANP receptors of the R_1-type whereas no ANP receptors of the R_2-type, implicated in ANP clearance, could be detected (1). Thus, the presently described metallo-endopeptidase, which performed a selective cleavage at the Ser^{123}-Phe^{124} bond, might be of particular importance in the physiological regulation of ANP concentration at the receptor level in this cell model. Since this enzyme cleaved other peptide hormones at Xaa-Phe, Xaa-Leu or Xaa-Ile, a more general physiological role for this enzyme is postulated (3).

It is the first time that such a metallo-endopeptidase has been partially purified and characterized from a human cell type tissue. This novel human metallo-endopeptidase showed obvious similarities with the peptide hormone inactivating endopeptidase isolated from *Xenopus laevis* skin exudate (3). Both

enzymes cleaved Xaa-Phe, Xaa-Leu or Xaa-Ile in ANP, bradykinin, somatostatin 14, litorin, substance P, angiotensin II and neuromedins B and C, but not in ocytocin, vasopressin or [Leu^5]-enkephalin. This suggested that the topography of the conserved motif in the peptide substrates is an important and limiting factor for their hydrolysis by either human or frog enzymes. The minimal requisite for hydrolysis seemed to be the presence of four amino acids on the amino terminal and one amino acid on the carboxy terminal side of the conserved motif (3, Carvalho et al., unpublished results). Both enzymes exhibited the metallo-endopeptidase character, but human endopeptidase was inhibited by some cysteinyl-protease inhibitors, suggesting a direct or indirect involvment of thiol group(s) for its activity.

The present study establishes that the metallo-endopeptidase was capable of a single and selective cleavage on the ANP molecule, a conclusion only suggested previously (2,10,11). The metallo-endopeptidase from human neuroblastoma NB-OK-1 cells as well as the frog enzyme shows some similarities with the atrial peptide degrading enzyme from bovine kidney (12). However, further relationship can not be established between both enzymes because of the restricted number of substrates studied in the latter case. Moreover, since the complementary fragments ANP-(103-126) and Phe^{124}-Arg^{125}-Tyr^{126} resulting from ANP-(103-126) cleavage at the Ser^{123}-Phe^{124} bond, could not be demonstrated, Km determination on kidney enzyme was unfeasible (12). Both human and frog metallo-endopeptidases exhibit a thermolysin-like character and clearly appeared distinct from NEP and ACE since specific inhibitors for these endopeptidases (thiorphan and phosphoramidon or captopril, respectively) had no effect on their activity. Both human and frog enzymes, which hydrolysed substance P principally at Gly^9-Leu^{10} bond, appeared to be different from a substance P degrading endopeptidase from human brain and which cleaves at Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9 bonds (5).

This study demonstrates a novel ecto-metallo-endopeptidase at the cell surface of human neuroblastoma NB-OK-1 cells with a thermolysin-like character, clearly distinct from NEP and ACE. This human metallo-endopeptidase is very similar to the *Xenopus laevis* endopeptidase (PHIE) and might have a physiological importance in both ANP and other peptide hormone metabolism at the cell surface i.e. near the receptor level.

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