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MODULATION OF A NOVEL THERMOLYSIN-LIKE METALLO-ENDOPEPTIDASE ACTIVITY DURING RETINOIC ACID-INDUCED DIFFERENTIATION OF HUMAN NEUROECTODERMAL TUMOR CELL LINES

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ABSTRACT: Neuroectodermal tumours express hormones which are post-translationally processed and inactivated by the action of specific proteases and peptidases. The data reported here show the presence of a novel thermolysin-like metallo-endopeptidase activity in several human cell lines. The soluble fractions of neuroblastoma, melanoma and a glioblastoma tumour cell lines are able, with different degrees, to cleave the Ser^{12}-Phe^{13} bond of a $DVDERDVRGFAS\downarrow FL_{NH2}$ substrate. The inhibition pattern suggests a metallo-endopeptidase thermolysin-like character, with the involvement of thiol group(s), clearly distinct from neutral endopeptidase (NEP; EC 3.4.24.11). This metallo-endopeptidase activity is down regulated during retinoic acid(RA)-induced neuronal differentiation in the RA-sensitive SK-N-BE(2) cells but not in the RA-resistant BE(2)-M17 cells, suggesting that the down regulation is related to neuronal differentiation and not a direct effect of RA on the enzymatic activity.

Neuroectodermal tumours include a variety of malignancies derived from the neurocrest, such as melanoma, C-thyroid carcinoma, central brain tumours, schwannoma and neuroblastoma. Melanomas exhibit some of the fastest growing incidences among adult tumours in the western world. Neuroblastomas and central brain tumours are the most common solid tumours of childhood, accounting for over 40% of neoplasms, with a very severe prognosis depending on age, site and stage of the tumour. All three tumours grow in culture and are commonly used as models to study both tumour development and therapy, and development and differentiation of neural cells. Both glioblastomas [1] and melanomas [2] can be induced to differentiate upon retinoic acid (RA) treatment towards a more mature neural or melanocytic phenotype respectively. Human neuroblastoma cell lines often exhibit two morphologically distinct cell types. One is neuroblastic (N-type) with neuritic processes and the biochemical features of neuronal cells. The other one is highly substrate-adherent (S-type) and somewhat epithelial-like, with the biochemical

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characteristics of immature Schwannian, glial or melanocytic cells. A third phenotype is intermediate (I) in morphology and has the biochemical properties of either N or S cells. I-type cells are thought to represent multipotent precursors that give rise to either N- or S-type cells; alternatively, they are transitional cells occurring during phenotypic interconversion (transdifferentiation) between N and S [3]. Human neuroblastoma SK-N-BE(2) cells differentiate toward a neural phenotype upon RA-treatment [4-6]. However, we have recently demonstrated that, during the RA-treatment, a S-type subset of SK-N-BE(2) cells undergoes apoptosis; these specifically express transglutaminase (TG; EC 2.3.2.13) [7]. Indeed, by cloning the parental SK-N-BE(2) cell line we could separate the neuronal differentiation action of RA on the parental line (enriched in N-type cells) from the induction of apoptosis on BE(2)-C cell clone (enriched in S-type) [8]. Furthermore one of the clones, BE(2)-M17, showed a marked resistance to the action of RA which can be evaluated as reduction of cell growth, induction of neuronal differentiation or apoptosis, which was mediated by a sharp induction of the insulin-like growth factor 2 (IGF-2) [9].

A vast number of neuropeptides have been detected on neuroectodermal tumour cell lines. These include the above mentioned IGF-2 [10-11], proopiomelanocortin (POMC) [12], vasoactive intestinal peptide (VIP) [13], growth hormone-releasing hormone (GHRH) [9], neuropeptide Y (NPY) [14], and substance P related peptides [15-16]. Even though the main biological effects seem related to the control of cell proliferation, these neuropeptides elicit a broad spectrum of biological actions, yet to be elucidated. Their presence implies the involvement of defined peptidases involved in the posttranslational activation/inactivation of peptide messengers [17-18]. Indeed the action of the enkephalin-degrading enzyme neutral endopeptidase (NEP; enkephalinase, EC 3.4.24.11) can be prolonged by selective inhibitors [19-20]. We have recently identified, purified and characterized in *Xenopus laevis* skin secretion a new thermolysin-like metallo-endopeptidase that cleaves at the Ser-Phe bond situated on the carboxyl side of the RXVRG sequence in a synthetic peptide [20]. as well as the Ser-Phe bond (or the Phe-Phe, Ala-Phe, His-Phe related motifs) which can be found in several neuropeptides, of the somatostatin, neuromedin, atrial natriuretic factor (ANF), and tachykinin families. More recently we detected a similar metallo-endopeptidase activity in human neuroblastoma cell line extracts, NB-OK-1 [21], as well as in the culture media which was clearly distinct from NEP and angiotensin converting enzyme (ACE; EC 3.4.15.1).

In this paper we compare the thermolysin-like metallo-endopeptidase activity in several neuroectodermal tumour cell lines established in our laboratory. Since retinoids have a well documented ability to reduce neoplastic progression, due in part to neuronal differentiation and in part to apoptosis, we also investigated the effect of RA on the endopeptidase activity in two different neuroblastoma cell lines.

MATERIALS AND METHODS

Reagents. Plastics, tissue culture media, trypsin, EDTA, Hepes, L-glutamine, Na bicarbonate, PBS, FBS and non-essential amino acids were all obtained from Flow Laboratories Ltd. (Herts, UK). DABTC-[DR^8]-Kermit was prepared by solid phase synthesis on a Multisynthetizer NPS 4000 (Neosystem, Strasbourg, France), purified by HPLC and checked as described [20-21]. All-*trans* retinoic acid, and other reagents were from Sigma Chemical Co. Ltd. (St Louis, MO, USA). The purified recombinant neutral-endopeptidase (NEP; EC 3.4.24.11) was a generous gift from G. Boileau (Montreal, Quebec, Canada).

Cell culture and differentiation. The human neuroblastoma cell line SK-N-BE(2) and the derived clones, BE(2)-C and BE(2)-M17, have been described by [3]. All experiments were performed using cells at the 60-66th, 29-32th and 21-25th passage respectively; the other cell lines were over the 100th passage. Glioblastoma cell line LI was established and characterized in our laboratory [1]. CHP100 and SH-SY5Y human neuroblastoma cell lines were a kind gift from Dr. June Biedler (Memorial Sloane-Kettering Cancer Hospital, New York, NY USA); M14, MNT3 and IR1 human melanoma cell lines were kindly given by Dr. PG Natali (Istituto Regina Elena, Rome, Italy). Cells were grown in monolayer culture in a 1:1 mixture of MEM and Hams F-12 media supplemented with 15% heat-inactivated FBS, sodium bicarbonate (1.2 mg ml^{-1}), Hepes buffer (15 mM), L-glutamine (2 mM) and non-essential aminoacids (1% v/v). Cells were fed every 3-4 days and were split weekly at a ratio of 1:5 to 1:10 using trypsin (0.025%)-EDTA (0.02%). Cells were routinely fed 24 hours before harvest for experiments. Differentiation experiments were performed by plating 5,000 to 10,000 cell·cm^{-2} and was induced with 5 μM RA (5 mM stock solution in 70% ethanol); 0.07% ethanol was added to the control cultures. Medium was replaced every two days. Indirect immunofluorescence was performed according to standard tecniques using polyclonal antibodies raised against purified NEP/CALLA [7-8].

Enzyme extraction. After removal of the culture medium, cells were washed in the flask with the serum free medium, then detached and lysed by freezing-thawing in distilled water. the soluble and membrane fractions were separated by centrifugation at 25000x g for 1 hour. the membrane-bound pellet was washed two times in 50 mM pH 7,4, Tris-HCl and centifuged at 25000 x g for 1 hour, before analysis.

Enzyme activity. Endopeptidase activity was evaluated by HPLC, as already reported [20-21]. This thermolysin-like activity cleaves the peptide bond on the N-terminus of an aromatic aminoacid on a synthetic peptide protected at both C- and N-termini. DABTC-[DR^8]-Kermit (DVDERDVRGFASFL$_{NH2}$), which undergoes a Ser^{12}-Phe^{13} cleavage, was used as substrate. Briefly, 1-2 nmoles of peptide were incubated 60-120 min in 25 mM Tris-HCl buffer pH 7.4 in the presence of 30 μl aliquots of the cell samples or 0.1μg of NEP in a final volume of 60 μl. The reaction was stopped by heating for 5 min at 100°C, and the resulting mixture was applied on HPLC using Nucleosil 5 μ C18 column (146x4.5 mm). Fragments were identified both by reference standards and by amino acid composition on a picoTag station (Waters, UK). Results were normalized on a reference standard curve. Inhibitions were expressed as percentages of the reference activity in the absence of chemical reagents under the same experimental conditions.

RESULTS

Metallo-endopeptidase activity in human neuroectodermal tumour cell lines. Our previous report [21] demonstrated the activity of a metallo-endoprotease in a human neuroblastoma cell line NB-OK-1, clearly distinct from NEP and ACE. This thermolysin-like activity was routinely tested using a tetradecapeptide, DABTC-[DR^8]-Kermit (DVDERDVRGFASFL$_{NH2}$) [20-22]; since this substrate is substituted and protected both at the amino- and carboxyl-termini, it is not cleaved by amino- and carboxy-peptidases, therefore it is particularly suitable to study endopeptidases. We are now expanding this observation to several cell lines derived from neuroectodermal tumours. Table I reports the activity found in the soluble fraction of 5 neuroblastoma, 3 melanoma, and 1 glioblastoma cell lines. The substrate (DABTC-[DR^8]-Kermit) was shown by HPLC and aminoacid analysis to be hydrolysed at the Ser^{12}-Phe^{13} bond as reported previously [21]. All lines showed the presence of the enzymatic activity, with a 20-fold difference in activity. There was no obvious difference among the tumour type.

The five neuroblastoma cell lines differ in culture by the presence of two morphologically and biochemically distinct phenotypes, N- and S-type. Whereas BE(2)-C is I-type (rich in S-cells), showing flat cells with only rare neuritic processes, and is similar to the pure I-type parental SK-N-BE(2) cells and CHP100 cells; BE(2)-M17, SH-SY5Y cells are N-type, with smaller, round cell bodies and short neuritic processes. Even though SK-N-BE(2) cells show an intermediate enzymatic activity between its derived clones SK-N-BE(2)-M17 (N-type) and SK-N-BE(2)-C (I-type rich in S-cells) the data comparing all neuroblastoma cell lines reported in Table I indicated no obvious correlation with phenotype.

Table I. Metallo-endopeptidase activity in human neuroectodermal tumour cell lines

Cell line		Activity			
		Soluble Fraction		Membrane Fraction	
		Ser^{12}-Phe^{13}	Gly^{9}-Phe^{10}	Ser^{12}-Phe^{13}	Gly^{9}-Phe^{10}
Neuroblastoma cell lines					
CHP 100	(I-type)	67.1	nd	nd	nd
SK-N-BE(2)	(I-type)	65.5	nd	nd	nd
SK-N-BE(2)-C	(I-type rich in S-cells)	49.7	nd	nd	nd
SK-N-BE(2)-M17	(N-type)	108.7	nd	nd	nd
SH-SY5Y	(N-type)	30.5	nd	nd	nd
Melanoma cell lines					
M 14		60.0	nd	35.0	35.3
MNT 3		5.6	nd	66.0	14.1
IR 1		29.4	nd	37.4	35.0
Glioblastoma cell line					
LI		107.0	nd	nd	nd

Results are expressed in pmoles of substrate cleaved/mg protein/hour. Both Ser^{12}-Phe^{13} and Gly^{9}-Phe^{10} cleavages of the DABTC-[DR^{8}]-Kermit (DVDERDVRGFASFL$_{NH2}$) peptide are shown. After removal of the culture medium, cells were washed in the flask, then detached and lysed by freezing-thawing in distilled H_2O. The soluble and membrane fractions were separated by centrifugation of the cellular extracts at 25000 x g for 1 hour. nd=not detectable.

Table II compares the inhibition patterns in 5 neuroectodermal tumour cell lines. EDTA and 1,10-*o*-phenanthroline inhibited enzyme activity, suggesting its metallo-endopeptidase character. N-ethyl malemide (NEM), a reagent of thiols and an inhibitor of cysteinyl-peptidases, also inhibited the metallo-endopeptidase activity, indicating the involvement of thiol(s) in enzyme activity. A specific inhibitor of NEP, phosphoramidon, was unable to inhibit metallo-endopeptidase activity.

Activity present in the membrane fraction of melanomas. Finally, we also investigated the possibility that this metallo-endopeptidase activity could be secreted, i.e. present in the cell media, or also present in the cell membrane fraction. The media of all tumour cell lines were free of specific activity, indicating that the soluble enzyme was not secreted into the culture media (data not shown); this result is in contrast with the previous report on an other neuroblastomas cell line [21]. The data reported in Table I show the endopeptidase activity of both the soluble and

Table II. Inhibition pattern of metallo-endopeptidase activity in neuroectodermal cell lines

	o-phenantroline 10mM	EDTA 10mM	NEM 1mM	phosphoramidon 0.01mM
Neuroblastoma cell lines				
SK-N-BE(2)	97.0%	70.0%	71.4%	41.0%
SK-N-BE(2)-M17	100.0%	69.0%	100.0%	0.0%
SK-N-BE(2)-C	100.0%	74.0%	44.6%	46.0%
NB-OK-1 [21]	95.0%(*)	91.0%	88.0%	0.0%(*)
Melanoma cell line				
M 14	100.0%	33.8%	100.0%	11.2%
M 14, membrane fraction: Ser^{12}-Phe^{13}($)	100.0%	35.5%	100.0%	10.1%
M 14, membrane fraction: Gly^{9}-Phe^{10}($)	100.0%	100.0%	0.0%	100.0%
Glioblastoma cell line				
LI	96.6%	42.0%	98.8%	0.0%
Xenopus laevis				
skin secretion [20]	98.0%(*)	67.0%	0.0%	41.0%(*)

The results shown, expressed as % of control cells in the absence of inhibitors, refer only to the soluble fractions, unless otherwise stated. All values were calculated with reference to the amount of cleavage on 200 pmol of DABTC-[DR^{8}]-Kermit. (*) *o*-phenantroline was 1 mM and phosphoramidon was 0.001 mM. ($) inhibition of the Ser^{12}-Phe^{13} cleavage, or of the Gly^{9}-Phe^{10} cleavage of DABTC-[DR^{8}]-Kermit (DVDERDVRGFASFL$_{NH2}$). The membrane-bound pellet was washed two times in 50 mM pH 7.4 Tris-HCl and centrifuged at 25000 x g for 1 hour before analysis.

membrane fractions. The membrane-bound activity was absent in all neuroblastoma and glioblastoma cell lines; yet, in the membrane-bound fraction of all three melanomas we detected two different activities: (1) a Ser^{12}-Phe^{13} cleavage in DABTC-[DR^8]-Kermit (DVDERDVRGFAS↓FL$_{NH2}$), identical to the activity detected in the soluble fraction of all neuroecodermal tumours, and (2) a Gly^9-Phe^{10} cleavage in DABTC-[DR^8]-Kermit (DVDERDVRG↓FASFL$_{NH2}$). The inhibition pattern of the latter activity was different, as shown in Table II: (1) it is not inhibited by NEM, suggesting that thiol group(s) are not involved in the enzyme activity, and (2) it is inhibited by the NEP specific inhibitor phosphoramidon at 10^{-5} M. As a matter of fact this activity was still inhibited 100% by 10^{-6} M, and 80% by 10^{-7} M phosphoramidon. Moreover, panel B in Figure 1 shows that purified NEP cleaves at Gly^9-Phe^{10} on the DABTC-[DR^8]-Kermit peptide, evaluated both by retention time and subsequent aminoacid analysis. These data indicate a NEP-like activity present only in the membrane-bound fraction of melanomas. NEP has been identified also as CD10/CALLA, common acute lymphoblastic leukaemia antigen; indeed CALLA surface expression and NEP activity are correlated in several leukaemic lines [23], where it seems to regulate neuropeptide activity [24]. It is noteworthy that

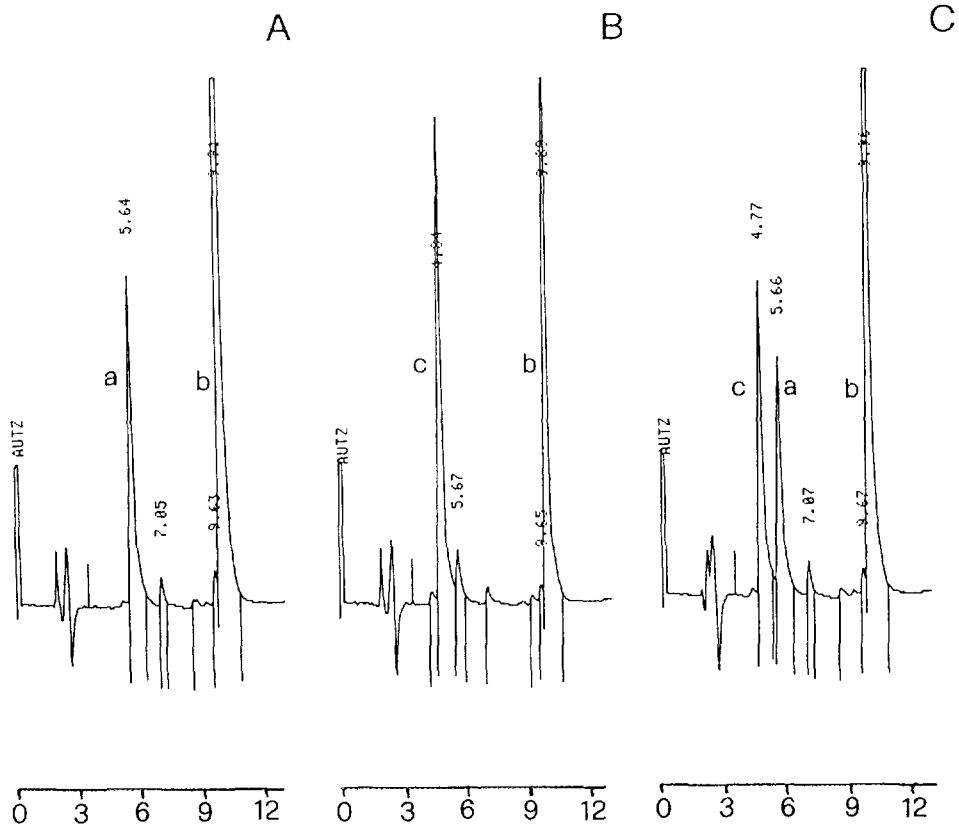

Figure 1. HPLC elution profiles of DABTC-[DR^8]-Kermit (DVDERDVRGFASFL$_{NH2}$) fragments generated by the action of neuroblastoma soluble fraction (A), purified NEP (B), and melanoma membrane fraction (C). The 5.7 min retention time (a) was identified by aminoacid composition analysis as the Ser^{12}-Phe^{13} cleavage; while the 4.7 retention time (c) was confirmed as the Gly^9-Phe^{10} cleavage. Uncleaved substrate was eluted at 9.9 min (b).

CALLA has also been reported on 1 of 12 neuroblastoma cell lines [25-27] and on melanoma [28]. Direct evidence for NEP protein expression was provided by immunofluorescence performed on all neuroectodermal cell lines. By using antibodies raised against purified NEP/CALLA, the immunoreactivity was found exclusively on the cell membrane of all melanoma cell lines (data not shown). Indeed the magnitude of staining followed the amplitude of enzyme activity shown in Table I and defined as the amount of DABTC-[DR^8]-Kermit fragments generated by the Gly^9-Phe^{10} cleavage. In contrast the neuroblastomas were devoid of immunoreactivity (results not shown) in keeping with the absence of corresponding activity (Table I).

Modulation of metallo-endopeptidase activity by retinoic acid in neuroblastoma cells. RA causes BE(2)-C cell migration and extension of neuritic processes, discernable on day 2 and more marked by day 5. RA induces only minor morphological changes in BE(2)-M17 cells; cells are slightly more neuroblastic with a slight increase in length of processes on days 4-5. Thus, SK-N-BE(2) respond dramatically to RA, whereas BE(2)-M17 cells do not. Indeed we reported that BE(2)-M17 cells are markedly resistant to the action of RA, evaluated as reduction of cell growth, induction of neuronal differentiation or apoptosis [9]. In order to evaluate a possible modulation of metallo-endopeptidase activity in parallel to neuronal differentiation, we treated both cell lines, SK-N-BE(2) RA-sensitive and BE(2)-M17 RA-insensitive, with 5 μM RA for 8 days.

Figure 2 indicates that the thermolysin-like metallo-endopeptidase is down regulated by RA only in the RA-sensitive cell line. After 2-4 days of treatment the enzyme activity was reduced about 50% of the control and after 6-8 days it was only 20% of the control.

DISCUSSION

Several hormones and neurotransmitters are synthesized from larger precursor proteins, which are then post-translationally processed to active peptide hormones. Similarly the degradation of such

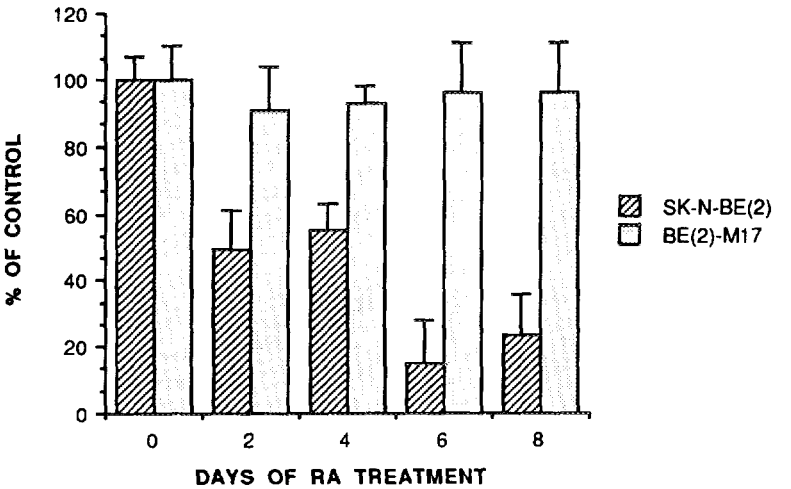

Figure 2. Modulation of metallo-endopeptidase activity upon retinoic acid in human neuroblastoma cells. Results, pmoles of substrate cleaved/mg protein/hour, are expressed as percentage of controls. Fresh media and RA, at the final concentration of 5 μM, were replaced every 48 hours, and, at the time indicated, cells were lysed to evaluate the enzyme activity in the soluble fractions. Figure shows one of two experiments; bars indicate SEM of duplicate measurements.

peptides occurs by the action of specific peptidases. Hitherto most processing endoproteases are unknown, hence it has been difficult to distinguish and characterize these enzymes during the developmental and mature functions. The data reported here show the presence of a new metallo-endopeptidase activity [21] in several human neuroectodermal tumour cell lines. We also attempt to evaluate this activity during neuronal differentiation.

The soluble fractions of neuroblastoma, melanoma and a glioblastoma tumour cell lines are able, to different degrees, to cleave the Ser^{12}-Phe^{13} bond of DABTC-[$D R^{8}$]-Kermit ($DVDERDVRGFAS\downarrow FL_{NH2}$) substrate. The inhibition spectra suggest a metallo-endopeptidase character, with the involvement of thiol group(s), clearly distinct from NEP. This metallo-endopeptidase shows a very similar activity to that already described by our group in the neuroblastoma cell line NB-OK-1 and in skin secretion of *Xenopus laevis* [20-21]. This activity has also been shown to be able to cleave ANF, somatostatin, substance P, neuromedin [20-21], and is strongly inhibited by Met- and Leu- enkephalins [20]. Its ability to inactivate such hormones by net truncations seems particularly important in neuroectodermal cells, known to produce several neuropeptides and neurotransmitters, such as preprotachykinin, its derived substance P and neurokinin, NPY, VIP, GHRH, IGFs, POMC and endorphins [9-16].

SK-N-BE(2) cells can differentiate towards a neuronal phenotype in response to RA, whereas BE(2)-M17 has a rather weak response to RA treatment, mainly with a slight increase in the length of neurites, a minor growth inhibition and a reduction of apoptosis [9]. The metallo-endopeptidase activity is clearly down regulated during RA-induced neuronal differentiation in the RA-sensitive SK-N-BE(2) cells but not in the RA-resistant BE(2)-M17 cells showing that the down regulation is correlated to neuronal differentiation and is not a direct effect of retinoic acid on the enzyme(s). Thus, the physiological role of this metallo-endopeptidase, which at present is not known, might be related to the inactivation of a number of neuropeptides, so its down regulation during differentiation may be correlated to the increased production of neuropeptide(s) and/or neuromediator(s) that occurs during neuronal differentiation. Further work is warranted to purify the enzyme, hence clarify this hypothesis, as well as to identify the natural substrate(s).

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