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Molecular and Immunochemical Evidences Demonstrate That Endooligopeptidase A Is the Predominant Cytosolic Oligopeptidase of Rabbit Brain

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Oligopeptidases are tissue endopeptidases that do not attack proteins and are likely to be involved in the maturation and degradation of peptide hormones and neuropeptides. The rabbit brain endooligopeptidase A and the rat testes soluble metallopeptidase (EC 3.4.24.15) are thiol-activated oligopeptidases which are able to generate enkephalin from a number of opiod peptides and to inactivate bradykinin and neurotensin by hydrolyzing the same peptide bonds. A monospecific antibody raised against the purified rabbit brain endooligopeptidase A allowed the identification of a 2.3 kb cDNA coding for a truncated enzyme of 512 amino acids, displaying the same enzymatic features as endooligopeptidase A. In spite of all efforts, employing several strategies, the full-length cDNA could not be cloned until now. The analysis of the deduced amino acid sequence showed no similarity to the rat testes metalloendopeptidase sequence, except for the presence of the typical metalloprotease consensus sequence [HEXXH]. The antibody raised against recombinant endooligopeptidase A specifically inhibited its own activity and reduced the thiol-activated oligopeptidase activity of rabbit brain cytosol to less than 30%. Analysis of the endooligopeptidase A tissue distribution indicated that this enzyme is mainly expressed in the CNS, whereas the soluble metallo EC 3.4.24.15 is mainly expressed in peripheral tissues.

Neuropeptides, displaying a multiplicity of neurochemical functions, have been found throughout the central nervous system (CNS). Modulation of their specific actions depends upon the activity of proteolytic enzymes present in the nervous tissues. These enzymes may cause the complete inactivation and/or conversion of neuropeptides into smaller, active homologues. This is the case for the thiol-activated endooligopeptidase A (EOPA) and the rat testes soluble metalloendopeptidase (EC 3.4.24.15 or EP 24.15), that effectively degrade bradykinin and neurotensin (1–3). Both enzymes convert enkephalin-containing peptides into enkephalins (4, 3) but only EP 24.15 generates a pentapeptide from the N-terminus of GnRH (5, 3), which is a potent inhibitor of the GnRH secretion (6). Based exclusively on the striking similarity between enzymatic properties of rabbit brain EOPA and rat testes EP 24.15, it was suggested that they should be the same enzyme (7). However, a few differences in their biochemical and immunochemical properties (8, 9), and in their subcellular localization in the CNS (10, 11), indicated that EOPA and EP 24.15 could not be the same enzyme.

Here, we describe a cDNA isolated after immunoscreening of a rabbit brain cDNA library, which allowed the expression of a truncated protein displaying the same enzymatic features as the purified rabbit brain EOPA. Analysis of the deduced amino acid sequence showed no similarity to the rat testes EP 24.15 sequence, except for the presence of the typical metallo protease consensus sequence HEXXH (12). The antibody raised against this recombinant enzyme (*r*EOPA) specifically inhibited its own activity, and also reduced the EOPA activity of rabbit brain crude cytosol to less than 30%. Analysis of the EOPA tissue distribution

The DNA sequence presented in this work received the Accession No. AF15037 in GenBank.

Abbreviations used: EOPA, endooligopeptidaseA; *r*EOPA, recombinant EOPA-GST-fusion; *r*EP 24.15, recombinant soluble metalloendopeptidase; QF-ERP$_7$, quenched fluorescence enkephalin-related peptide; pER-12 + MA-16, plasmid containing total cloned cDNA.

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indicated that this enzyme is mainly expressed in the CNS.

MATERIALS AND METHODS

Materials. The purified rabbit brain EOPA used in the present study was the same as described in Hayashi *et al.* (9). New Zealand rabbits were obtained from a local slaughterhouse and the male Balb/c mice were obtained from the local animal house. The peptide bradykinin [BK] and neurotensin [NT] were purchased from Peninsula Laboratories. The quenched fluorescent peptide substrate QF-ERP_7 [(orto-aminobenzoyl)-Gly^1-Gly^2-Phe^3-Leu^4-Arg^5-Arg^6-Val^7-(N-(2,4,dinitrophenyl-ethylenediamine)] was synthesized by classical solution method (13). The Cys(Npys) dynorphin-derived peptide [Tyr(D)Ala-Gly-Phe-(D)Leu-Cys(Npys)-NH_2] and the CPP-AAY-pAB [N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-(*p*-aminobenzoate)] were generous gifts of Dr. Rei Matsueda (New Lead Research Laboratories, Sankyo Co., Shinagawa-ku, Tokyo, Japan) and Dr. I. A. Smith (Baker Medical Institute, Melbourne, Australia), respectively.

Cloning and sequencing of the cDNA coding for the EOPA. A rabbit brain cDNA library constructed in λgt11 vector (Clontech) was screened after induction with IPTG using the anti-EOPA polyclonal antibody. Positive clones were identified using the ProtoBlot immunoscreening system (Promega) and were analyzed by sequencing the 5′ ends of the inserts. The longest cDNA insert (2 kb) was amplified by PCR using commercially available oligonucleotide primers for λgt11 (Gibco-BRL), and was subcloned into the vector pBluescript SK+ (Stratagene). This clone was named pER-12. Both strands of the cDNA insert were sequenced by the chain terminator dideoxy method (14), using Sequenase kit (USB), and by primer walking sequencing. The "fmol kit" (Promega) and the boiling method of DeShazer *et al.* (15) were employed for sequencing especially difficult regions. Amplification of sequences upstream of the cloned cDNA was performed using the "Marathon cDNA Amplification Kit" (Clontech) on mRNA of rabbit brain, following the manufacturer's instructions. A fragment overlapping with pER-12 (MA-16) was obtained and fused in frame to the 5′ end of the cDNA insert of pER-12, yielding the sequence coding for the truncated EOPA (see below).

Northern blot analysis. 10 μg of total RNA from each tissue of a male New Zealand rabbit were subjected to electrophoresis in denaturing agarose gel (1.7% formaldehyde), transferred to nylon membranes and hybridized as previously described (9). ^{32}P-labeled probes were prepared by random priming (Gibco BRL) using the complete sequence of the pER-12 cDNA insert.

Preparation of crude rabbit brain cytosol, rEP 24.15 and rEOPA. Rabbit brains were homogenized in 1:3 (w/v) of 0.32 M of sucrose solution and the cytosol was obtained by centrifugation at 4°C for 1 h at 100,000 × g (9). Recombinant EP 24.15 (*r*EP 24.15) was obtained by the method described by Glucksman and Roberts (16). The recombinant EOPA (*r*EOPA) was expressed in *Escherichia coli* as a gluthatione-S-transferase (GST) fusion-protein using the pGEX 4T-1 expression vector from Pharmacia (17). After linking the cDNA insert from pER-12 to its 5′ upstream fragment, MA-16, in frame (Fig. 2A), this sequence was inserted into the expression vector pGEX 4T-1 (Pharmacia), downstream of the sequence coding for GST, such that the transcription initiation site of the vector could be used. The expression of the fusion protein was conducted as previously described for the rat testis *r*EP 24.15 (16). The fusion protein was purified after incubation with the glutathione 4B–Sepharose resin (Pharmacia). The homogeneity of the fusion protein was verified by SDS-PAGE (18) and the protein concentration was determined by the method of Bradford (19).

Preparation of antisera. Male Balb/c mice, 7–8 weeks old and weighing 18–22 g, were immunized either with purified EOPA or with *r*EOPA. For each of four immunizations, 2 μg of purified EOPA or 3 μg of the fusion protein absorbed on $Al(OH)_3$ were injected intradermally at weekly intervals. Blood samples were collected one week after the last immunization and the sera were stored at −20°C. The animals were strictly maintained and manipulated under ethical conditions according to the International Animal Welfare Recommendations.

Immunoreaction assays. The anticatalytic activities of the antisera were monitored by HPLC using bradykinin as substrate. Antisera were pretreated at 55°C for 5 min to eliminate any endogenous peptidase activity. In the presence or absence of antisera, 20 μM bradykinin were incubated with 1.5 mU of enzyme in 50 mM Tris–HCl, pH 7.5, 20 mM NaCl, containing 0.5 mM DTT. The reactions were stopped by the addition of 10 μl of 10% TFA (v/v) and the formation of bradykinin products was determined by HPLC, as described below. The reactivity of the anti-EOPA and anti-*r*EOPA antibodies against the rabbit brain cytosol, purified EOPA, *r*EOPA and *r*EP 24.15 were evaluated by ELISA as previously described (20).

Enzyme assays. The fluorogenic substrate QF-ERP_7 was used to determine the thiol-activated oligopeptidase activity, as previously described (21, 9). One unit of the enzyme activity is the amount of enzyme which hydrolyses 1 μmol of QF-ERP_7 in 1 min. All the enzymatic assays were performed in triplicate.

Determination of the peptide cleavage sites. The sites of cleavage in bradykinin [BK], neurotensin [NT] and in the quenched fluorescent peptide substrate [QF-ERP_7] were determined by reverse-phase HPLC (22, 9). The peptides (20 μM) were incubated with 1.5 mU of the *r*EOPA in 50 mM Tris–HCl buffer, pH 7.5, containing 20 mM NaCl and 0.5 mM DTT. The reactions were carried out at 37°C and stopped by the addition of 10 μl of 10% (v/v) TFA. All enzymatic assays were performed in triplicate.

Effect of DTT and active-site directed inhibitors on rEOPA activity. The DTT activation was performed by incubating 1.5 mU of *r*EOPA in the presence [for the assays with CPP-AAY-pAB, 1 μM] (23) or absence of 0.5 mM DTT [for assays with Y(D)AGF(D)LC(Npys)-NH_2, 10 μM] (21) in 50 mM Tris–HCl, pH 7.5, 20 mM NaCl. After 10 min pre-incubation at 37°C with either compound, the inhibition of *r*EOPA was determined by the fluorimetric assay using the fluorogenic peptide QF-ERP_7 as substrate, as described above. Aliquots of 50 μl of the enzyme-inhibitor preincubation solutions were diluted in 500 μl of the same buffer containing 2 K_m of QF-ERP_7 [K_m = 5.4 μM].

RESULTS

Characterization of the antibody raised against rabbit brain EOPA. The polyclonal antiserum raised against the purified EOPA was able to inhibit more than 70% of the thiol-activated oligopeptidase activity of rabbit brain cytosol, but not of the *r*EP 24.15 (Fig. 1A). In addition it did not show cross-reactivity with *r*EP 24.15 (Fig. 1B). The ability of this antiserum to detect the antigen in the crude cytosol of rabbit brain was evaluated by ELISA, and showed strong immunoreactivity (up to the dilution of 1:16000) (Fig. 1B).

Cloning and sequence analysis of the isolated cDNA. The immunological screening of approximately 10^7 plaques of a rabbit brain cDNA library identified three positive clones, which were shown to correspond to the same cDNA by sequencing (data not shown). Complete sequencing of the longest cDNA insert (2 kb) showed that it was incomplete. The use of RT-PCR, 5′-RACE, cloning of 5 prime extension products, or either the screening of other rabbit brain cDNA libraries by hy-

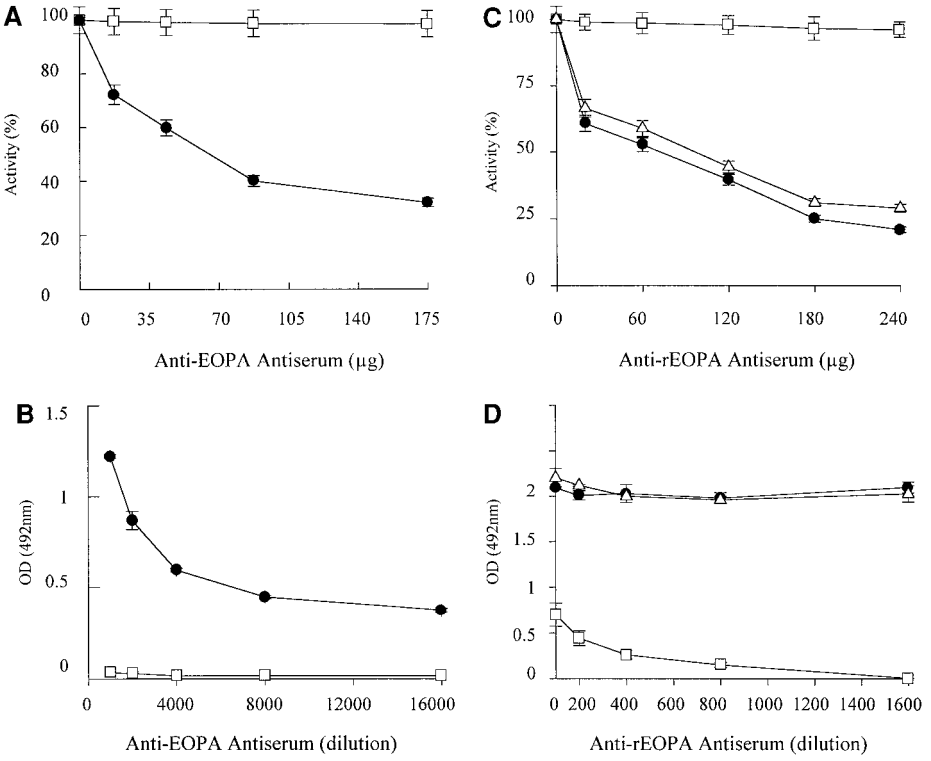

FIG. 1. Immunoreactivity of the anti-EOPA and the anti-*r*EOPA antisera. (A) The anticatalytic activity of anti-EOPA antiserum toward *r*EP 24.15 (□) and the thiol-activated oligopeptidase activity of rabbit brain cytosol (●). (B) The immunoreactivity detected by ELISA using the crude rabbit brain cytosol (●) and *r*EP 24.15 (□) as antigen sources. (C) The anticatalytic activity of the anti-*r*EOPA antiserum toward *r*EP 24.15 (□), *r*EOPA (△) and the thiol-activated oligopeptidase activity of rabbit brain cytosol (●). (D) The immunoreactivity detected by ELISA using *r*EOPA (△), the crude rabbit brain cytosol (●), and *r*EP 24.15 (□) as antigen sources. In A and C, data are presented as percent of enzymatic activity versus antibody concentration in incubation media.

bridization did not succeed in the cloning of the full-length cDNA. A fragment overlapping with the pER-12 cDNA insert (clone MA-16) was obtained only by using the "Marathon" system.

The total cloned cDNA sequence (pER-12 + MA-16) predicted an open reading frame of 1537 nucleotides which could code for a protein of 512 amino acids (Fig. 2B). The 3′ untranslated region was 755 bp long and presented a single AATAAA polyadenylation signal (24), 6 nucleotides upstream of the last residue. No poly(A)-tail was found. Neither the 5′ untranslated region nor the initial methionine (25) could be identified. Further several attempts at cloning the 5′ end with the "Marathon" system did not generate sequences which reached further upstream from clone MA-16. A human brain cDNA library was screened using the 2.3 kb cDNA (pER-12 + MA-16) as a probe, and the longest cDNA insert isolated (about 1.7 kb) was about 65% similar to the rabbit cDNA sequence but also lacked the 5′ end (data not shown).

Analysis of the deduced amino acid sequence of the rabbit brain total cloned cDNA sequence (pER-12 + MA-16) showed the presence of the typical metalloprotease consensus sequence [HEXXH] at the C-terminus, and a high content of cysteines (18 residues, 3.5%) and prolines (35 residues, 6.8%). Three putative phosphorylation sites for MAP kinase were also identified (Thr_{274}, Thr_{300} and Thr_{416}).

The hydropathic profile (26) showed no hydrophobic segment, typical of transmembrane domains or a putative signal peptide (data not shown).

Searches for nucleotide sequence homology identified a single sequence registered as *Homo sapiens* clone 23596, showing about 83% similarity for a region of about 1430 nucleotides. The function of this putative human protein is unknown. No significant homology with any known protein sequence was found for the deduced amino acid sequence, except for a segment of about 290 amino acid residues (positions 58–332 of the deduced amino acid sequence), which showed 70% similarity to a mitotic phosphoprotein identified in *Xenopus laevis* (27), whose function has still to be established. However, at the level of nucleotide sequences only very little similarity was observed between these two genes.

Northern blot analysis of rabbit tissues. The presence of mRNA corresponding to the pER-12 cDNA insert in different rabbit tissues was analyzed by Northern blot. A single mRNA transcript of approximately

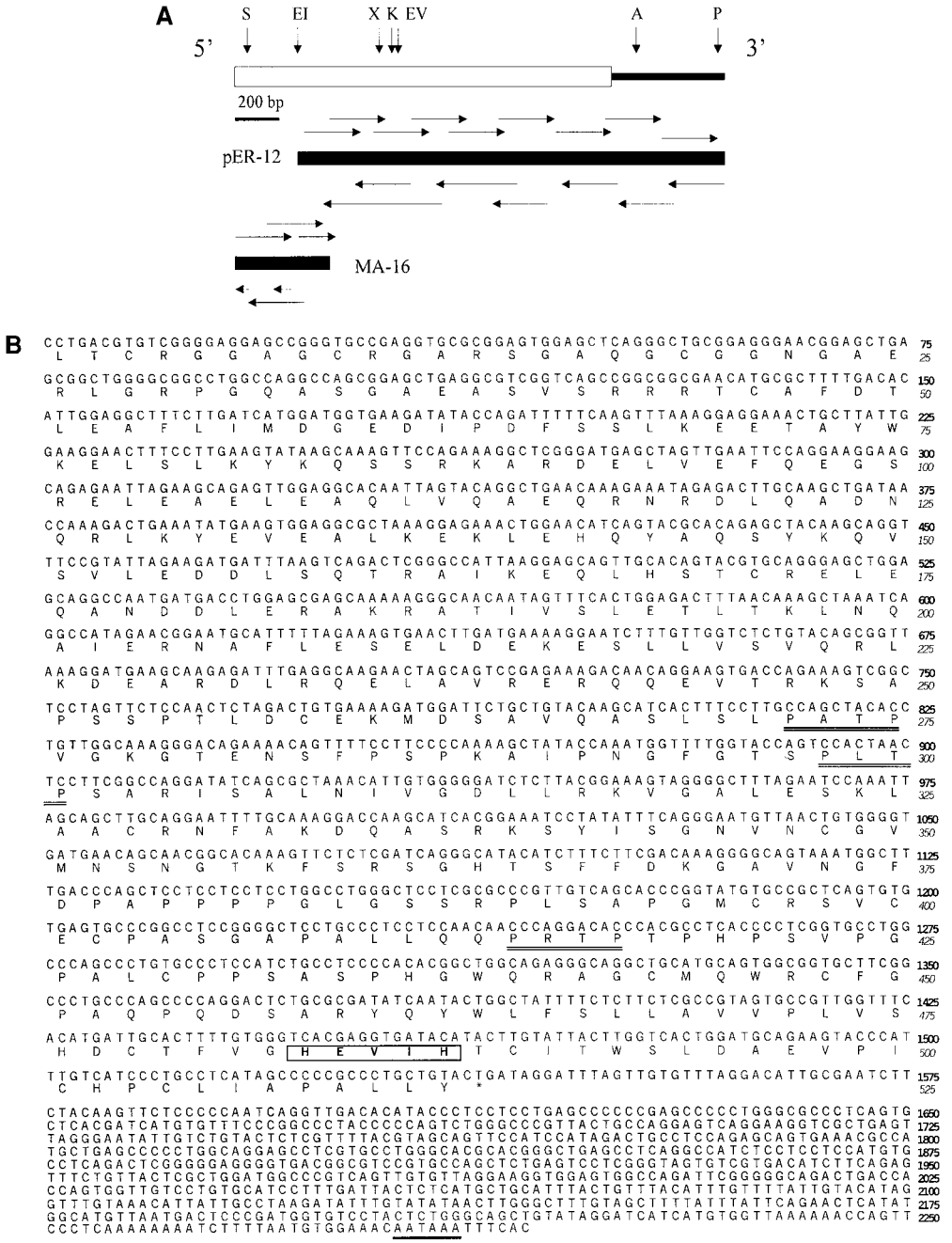

FIG. 2. Schematic representation of the isolated cDNA clones, their sequencing strategy, and the nucleotide and deduced amino acid sequences. (A) A solid line represents the 3′-untranslated region and an open bar indicates the open reading frame. The positions of some restriction enzymes are indicated. The entire sequenced cDNA was reconstituted from two independent overlapping clones, pER-12 and MA-16, as described in the text. Horizontal arrows indicate the direction and the extent of the sequences determined using internal oligonucleotides [EV, *Eco*RV; S, *Sac*I; EI, *Eco*RI; X, *Xba*I; A, *Apa*I; and P, *Pvu*II]. (B) Nucleotides and amino acid residues are numbered in the right column. Amino acids are numbered beginning at the first deduced residue and identified by the single letter code. Three putative MAP kinase phosphorylation sites are doubly underlined and the consensus sequence of metallopeptidases is boxed. An asterisk indicates the stop codon (TGA) of the open reading frame, and the polyadenylation site (AATAAA) is underlined with a single line.

2.8 kb was detected in the total RNA extracted from various rabbit tissues, such as testes, spleen, lung, heart, liver, stomach, hypothalamus, cortex, cerebellum, cerebral stem and striatum (Figs. 3A and 3B). Quantification of the signal intensities showed a clear predominance of transcripts in most of the brain regions, showing a weaker signal in the hypothalamus. In peripheral tissues, the highest abundance was observed in the testes, presenting much lower expression in other tissues (Fig. 3C).

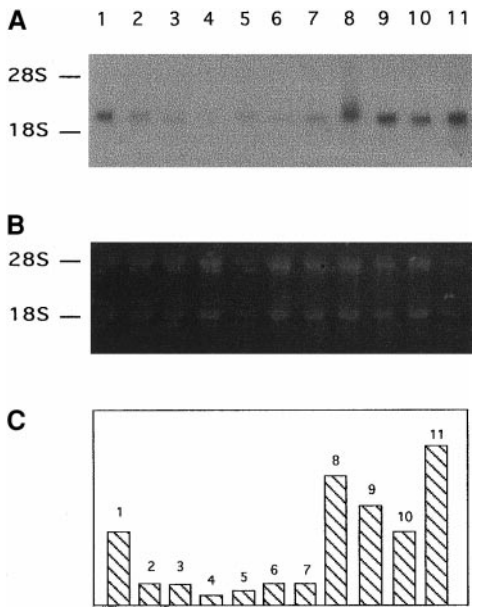

FIG. 3. Tissue distribution of the EOPA mRNA determined by Northern blot analysis. Total RNA (10 μg) from various rabbit tissues were hybridized with the radioactive pER-12 insert, as described under Materials and Methods. The analyzed tissues were: (1) testis, (2) spleen, (3) lung, (4) heart, (5) liver, (6) stomach, (7) hypothalamus, (8) cortex, (9) cerebellum, (10) cerebral stem and (11) striatum. (A) X-ray film after 24 h exposure, (B) ribosomal RNAs from each tissue in agarose gel stained with ethidium bromide and (C) relative intensity of each band quantified by densitometry of the X-ray film. The positions of ribosomal RNAs (28S and 18S) are indicated.

In a different set of analyses, hybridizations performed with total RNA from whole brain and testes of rabbit and rat confirmed the identification of a single band of about 2.8 kb (Fig. 4), as described above. In the testes of both animals, a new band of 1.5 kb was observed besides the 2.8 kb mRNA, suggesting the expression of a homologous mRNA in the testes of both animals. On the other hand, only a single band could be observed when the same membranes were hybridized with EP 24.15 cDNA probes (9). The intensity of the 2.8 kb bands, determined by densitometry, was 2.6 and 1.5 times stronger for the brain as compared to the testes for rabbit and rat, respectively.

Expression of the recombinant protein and enzyme characterization. Expression of the rabbit brain cDNA (pER-12 + MA-16) in *E. coli* generated a GST-fusion protein of about 81 kDa (*r*EOPA), from which the recombinant protein (55 kDa) could be released by digestion with thrombin. Both, the GST-fusion protein and the recombinant protein showed a typical pattern of EOPA/EP 24.15 specificity toward the fluorogenic substrate (QF-ERP$_7$), neurotensin and bradykinin. In fact, a single peptide bond, the Leu^4-Arg^5 of the QF-ERP$_7$, the Arg^8-Arg^9 of neurotensin and the Phe^5-Ser^6 of bradykinin, was hydrolyzed by the *r*EOPA showing a stoichiometric cleavage of these substrates (data not shown). The activity of the *r*EOPA was very unstable, but it could be partially stabilized by the addition of 5% glycerol to the buffer just after its elution from the gluthatione-4B-Sepharose resin.

The *r*EOPA was activated (95.5%) in the presence of low concentrations of DTT (0.5 mM) and was inhibited by either the Cys(Npys) dynorphin-related peptide (about 90% of inhibition with 10 μM) or the CPP-AAY-pAB inhibitor (about 85% of inhibition with 1 μM), similarly as previously observed for purified rabbit brain EOPA (9, 28).

Characterization of immune sera raised against the rEOPA. The polyclonal mouse anti-*r*EOPA antiserum was effective in inhibiting more than 70% of the thiol-activated oligopeptidase activity of both the rabbit brain cytosol and of the *r*EOPA (Fig. 1C). This antiserum did not inhibit the *r*EP 24.15 activity. The ELISA assays showed strong immunoreactivity with *r*EOPA as well as with the rabbit brain cytosol, but no cross-reactivity was detected with the *r*EP 24.15 (Fig. 1D).

DISCUSSION

The specific immunoreactivity of the antibody used to screen the rabbit brain cDNA library was the essential property to perform the isolation of the cDNA coding for the thiol-activated metallo-peptidase EOPA. This was assured by the lack of the antibody's cross-reactivity with EP 24.15. Surprisingly, the amino acid sequence deduced from the isolated cDNA showed that EOPA and EP 24.15 are two distinct metallopeptidases, sharing no sequence similarity. No similarity was found with any other known peptidase. This seems to be another typical example of enzymes displaying similar activities, although lacking homologies in their primary structures, as already described for UCH-L3 (ubiquitin C-terminal hydrolases-isozyme L3) and cathepsin B (29). This observation can be ex-

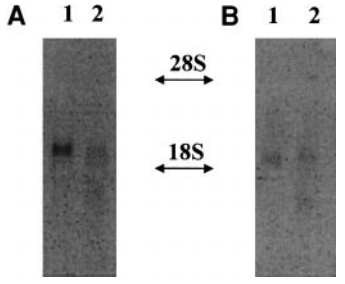

FIG. 4. Comparative Northern blot analysis of rabbit and rat tissues. Total RNA (10 μg) from brain (lane 1) and testes (lane 2) of rabbit (A) and rat (B) were hybridized with the radioactive pER-12 insert, as described under Materials and Methods. The bands observed in the X-ray films are present in (A) and (B). The positions of ribosomal RNAs (28S and 18S) are indicated.

tended to the metalloproteases thermolysin, matrixins, astacins and serralysins (30). Common features in the three dimensional structures of EOPA and EP 24.15 may explain why these two oligopeptidases share similar specificities toward the same substrates and inhibitors (9, 22, 31, 32).

Implying that a single enzyme was responsible for the whole thiol-activated oligopeptidase activity of the CNS, Healy and Orlowski (11) faced difficulties to explain why the EP 24.15 immunoreactivity was restricted to the nucleus of the nervous cells, while most of the thiol-activated oligopeptidase activity had been found in the cytosol (10). Using the anti-rEOPA antibody we clearly demonstrated that EOPA is responsible for over 70% of the thiol-activated neuropeptide-degrading activity of the rabbit brain cytosol. Another important difference between EOPA and EP 24.15 is concerned with the tissue distribution of both oligopeptidases. It was shown here that the EOPA specific mRNA was predominantly found in the CNS, while EP 24.15 mRNA prevailed in peripheral tissues (9, 12). Moreover, recently, Pierotti has shown that EP 24.15 gene is regulated differently in somatic versus germ cells and that the promoter contains a strong negative acting element important for suppressing transcription in non-germ cells, which determine a specific activity 3 to 5 fold higher in testes than in brain (unpublished data).

In spite of the use of several techniques and the screening of different libraries, it was not possible to clone de full-length cDNA coding for endooligopeptidase A. We concluded that the mRNA coding for this enzyme, expressed in both human and rabbit tissues, might present a strong secondary structure imposing the difficulties faced for the cloning of the respective 5′ ends. However, the main purpose of this work was to present structural data to confirm previous suggestions indicating that the oligopeptidase of the cytosol of nervous tissue is distinct from the cytosolic EP 24.15 of peripheral tissues. In fact, the expression of the truncated rabbit brain enzyme (pER-12 + MA-16) allowed us to assure that the enzymatic activity generated was the same as the previously described for the rabbit brain endooligopeptidase A (1–4). Furthermore, the antibody raised against this recombinant enzyme drastically reduced the EOPA activity of rabbit brain crude cytosol. Taken together, these results strongly suggest the existence, in rabbit tissues, of another oligopeptidase beside the soluble metallo-endopeptidase (EP 24.15) sharing similar enzymatic properties. Futhermore, it was also shown that the EOPA-specific mRNA was predominantly found in the CNS, while EP 24.15 mRNA prevailed in peripheral tissues.

The truncated recombinant enzyme (pER-12 + MA-16), displaying the same specificity of the rabbit brain EOPA (4), indicated that the full-length protein was not required to express the active enzyme. This was not surprising since truncated enzymes containing the catalytic domain were found to be able to generate active recombinant proteins (33, 34). Similarly to the natural EOPA and *r*EP 24.15, the fusion protein was activated by the thiol compound (DTT) and inhibited by the metallo- and thiol oligopeptidase inhibitors CPP-AAY-pAB and Y(D)AGF(D)LC(Npys)-NH_2, respectively. Taken together the results presented here demonstrate that not only the (pER-12 + MA-16) cDNA codes for EOPA but also that EOPA is the predominant thiol-activated enzyme in the rabbit brain cytosol able to perform the biotransformation and/or degradation of neuropeptides.

Analyses of the sequence obtained from the clone (pER-12 + MA-16), and its deduced amino acid sequence revealed the presence of a typical metalloprotease consensus motif [HEXXH] at the C-terminus (His_{483}, Glu_{484} and His_{487}). Besides the two histidine ligands of the metallo-protease consensus motif, Vallee and Auld (35) postulated the existence of a third ligand coordinating the catalytic zinc. This ligand is usually a glutamic acid frequently located 13 to 160 residues removed from the consensus sequence. In the case of the EOPA, the putative third ligand could be the glutamic acid residue located 10 residues removed from the second histidine residue of the consensus sequence (Glu_{497}).

The search for similarity in nucleic acid and protein data bases indicated that the (pER-12 + MA-16) cDNA was approximately 83% similar to a region of 1430 nucleotides of a clone from *Homo sapiens* brain coding for a protein with unknown function (36, 37). The deduced amino acid sequence of (pER-12 + MA-16) cDNA showed 70% of similarity for a segment of about 250 amino acid residue of a mitotic phosphoprotein 43 from *Xenopus laevis* (27).

Recently, we showed evidences for the involvement of EP 24.15 in the MHC class I antigen presentation in macrophages (38, 39), a role which might be extended to other peripheral tissues. In CNS, however, the EOPA might have its main physiological role related to the modulation of the action of neuropeptides although no direct evidences have yet been presented. The non-uniform brain distribution of EOPA, which is also a peculiar feature of neuropeptide localization in CNS, certainly favor this hypothesis. The cytosolic localization of EOPA may not limit its involvement in the modulation of neuropeptide metabolism, since it has been demonstrated that EOPA could be secreted by nervous cell (28). Among other possibilities, the enkephalin converting activity of EOPA is one of the most promising hypothesis. Accordingly, the level of EOPA mRNA evaluated by Northern blot analysis was higher in striatum as compared to the brain stem and hypothalamus. Coincidentally, an immunocytochemical survey of EOPA detected a strong immunoreactive "striasome like" structure in the striatum, which cor-

related with enkephalin-rich patches in adjacent sections (10).

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