Volume 309, number 2, 203–208 FEBS 11506 September 1992

Molecular cloning of the α-subunit of rat endopeptidase-24.18 (endopeptidase-2) and co-localization with endopeptidase-24.11 in rat kidney by in situ hybridization

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Received 20 July 1992

Endopeptidase-24.18 (endopeptidase-2, EC 3.4.24.18, E-24.18) is a Zn-ectoenzyme of rat renal and intestinal microvillar membranes exhibiting an oligomeric structure, α_2-β_2. The primary structure of the α-subunit of E-24.18 has been defined by molecular cloning and its expression mapped in rat kidney by in situ hybridization. A 2.9-kb cDNA coding for the α-subunit was isolated and sequenced. It had an open reading frame of 2,244 base pairs coding for a type I membrane protein of 748 amino acids. The deduced amino acid sequence showed 87% identity with that of meprin A, a mouse metallo-endopeptidase, sharing common properties with the rat enzyme, and 85% identity with the human intestinal enzyme, 'PABA-peptide hydrolase'. Northern blot analysis revealed the α-subunit to be encoded by a single mRNA species of 3.2-kb. In situ hybridization performed on rat kidney showed a co-localization of E-24.18 with endopeptidase-24.11 in proximal tubules of juxtamedullary nephrons, suggesting that the two enzymes have similar or complementary physiological functions in kidney.

Membrane protein; Endopeptidase-2; cDNA; Rat kidney; In situ hybridization

1. INTRODUCTION

The brush border membrane of rat kidney contains two well-defined metallo-endopeptidases: endopeptidase-24.11 (EC 3.4.24.11, E-24.11) and endopeptidase-24.18 (endopeptidase-2, EC 3.4.24.18., E-24.18) [1–3]. These enzymes are major constituents of the membrane, together constituting about 10% of the microvillar proteins [2]. E-24.11 is a 94 kDa ectoenzyme, a type II integral membrane protein. Its cDNA has been cloned and sequenced in rat [4], rabbit [5], mouse [6] and human [7]. E-24.11 appears to be a major enzyme in the inactivation of many peptides, including substance P and natriuretic peptides [8–12]. E-24.18 is a disulphide-linked oligomer composed of two non-identical subunits (α and β) [2]. Like E-24.11, it contains Zn at the active site and is strongly inhibited by chelating agents [2]. However, it is not sensitive to phosphoramidon [1] and can thus be distinguished from E-24.11. E-24.18 also hydrolyses a variety of peptides, including substance P, bradykinin, luliberin (LHRH) [13], neuropeptide Y [14] and transforming growth factor-α [15], the three last named being resistant to attack by E-24.11.

Here we report the cloning and sequencing of a cDNA encoding one of the E-24.18 subunits. The predicted amino acid sequence suggests that this protein is very similar in sequence to the α-subunit of mouse meprin A [16], which has recently been shown to be a new member of the astacin family of metallo-endopeptidases [17]. Using in situ hybridization techniques, we have compared the expression of E-24.18 and E-24.11 in rat kidney and suggest that they may have similar or complementary functions in the kidney.

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Abbreviations: E-24.18, endopeptidase-2; E-24.11, endopeptidase-24.11; PAH, *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase; bp, base pair; kb, kilobase; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

2. EXPERIMENTAL

2.1. *Amino acid sequencing*

The sequence at the N-terminus of the papain-released form [2] was determined using an Applied Biosystems 477A protein sequencer.

2.2. *Isolation of the cDNA clones and DNA sequencing*

The rat kidney λgt11 cDNA expression library (Clontech Co., Palo Alto, CA, USA) was screened following standard procedures [18] with a rabbit polyclonal antibody (RRt 151) directed against rat E-24.18 [3]. One positive clone was isolated from 1.0×10^5 recombinant lambda phages, subcloned as an *Eco*RI fragment in pUC19 plasmid, and sequenced by the dideoxy chain-termination method [19] using T7 DNA polymerase (Pharmacia, Canada) according to the manufacturer's protocol. The sequencing was performed by walking along the

cDNA with primers. An oligonucleotide (60-mer) was designed according to the determined sequence and used to probe a rat kidney λgt10 cDNA library (Clontech Co, Palo Alto, CA, USA) by plaque hybridization. Four positive clones were picked in the first round hybridization but only one survived through to second round screening. The purified λgt10 insert was subcloned into pUC19 and sequenced on both strands as described above. Procedures for the lambda phage purification and cDNA cloning have been described in detail [18]. Protein sequences were analyzed using the Fasta Programme [20].

2.3. *Northern blot analysis*

Total cellular RNA extracted from rat kidney and poly(A)⁺RNA from rat kidney, skeletal muscle and brain (Clontech Co., Palo Alto, CA, USA) were separated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose membranes. The membranes were hybridized under high stringency conditions [18] with the cDNA isolated from a rat kidney λgt11 cDNA library previously labelled with [^{32}P]dCTP (3,000 Ci/mmol; ICN Biomedicals, Canada) by random oligomer priming. Prior to use, the radiolabelled probe was purified on Nensorb 20 cartridges (NEN-Dupont, Boston, MA) to remove excess unincorporated radioactive nucleotides.

2.4. *cRNA probes*

Rat E-24.18 and E-24.11 cDNAs, cloned in PSPT18 plasmids, were used to generate, by in vitro transcription, cRNA probes of approximately 1,000 and 1,350 nucleotides, respectively. Probes were labelled with [^{35}S]UTP (1,409 Ci/mmol; NEN-Dupont, Boston, MA) to a specific activity of $1–4 \times 10^8$ cpm/μg. Probes were separated from unincorporated nucleotides on G-50 spin-columns and their sizes verified by denaturing polyacrylamide-urea gel electrophoresis.

2.5. *In situ hybridization*

Male Wistar rats (180–200 g) were perfused with freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). Pieces of kidney and brain were immersed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin and stored at 4°C. 5 μm sections were cut and mounted on microscope slides coated with 2% 3-aminopropyltriethoxysilane. Tissues were stored at 4°C until hybridization, which was essentially as described by Jaffe et al. [22]. Hybridized kidney sections were exposed under X-ray film (X-OMAT, Kodak, Toronto, Canada) for 2 days, dipped in liquid emulsion (K5, Ilford, Toronto, Canada) and developed after 2 weeks of exposure.

3. RESULTS AND DISCUSSION

3.1. *Cloning and sequence analysis of the α-subunit of E-24.18 cDNA*

Immunological screening of the rat kidney λgt11 cDNA expression library allowed the isolation and purification of one positive cDNA clone (clone 6.2). The cDNA fragment was excised, subcloned into the plasmid pUC19 and sequenced. The insert of clone 6.2 was 882 nucleotides long and contained an open reading frame of 294 amino acids, but lacked initiator and stop codons. A second rat kidney cDNA library was screened by plaque hybridization using a synthetic oligonucleotide probe based on the sequence of clone 6.2. This yielded clone 4 of 2,827 bp, including the 3′ region, the stop codon and the poly(A) tail but still lacking the initiator codon (Fig. 1). The polymerase chain reaction, using the RACE (rapid amplification of cDNA ends) protocol [23] yielded a cDNA which overlapped with the sequence of clone 4 and extended the 5′ end by 101 nucleotides (Fig. 1). An in-frame putative initiator methionine was found at nucleotide-33. The nucleotide sequence of the α-subunit of E-24.18 cDNA consists of 2,928 bp with an open reading frame of 2,244 bp ending with a TGA stop codon at nucleotide 2,277 (Fig. 2). The cDNA contains 32 nucleotide residues in the 5′ non-coding region and a long 3′ untranslated region of 652 nucleotide residues which includes a polyadenylation consensus sequence, AATAAA, located at 2,901, followed by a tract of 9 consecutive adenines (Fig. 2). The initiator Met codon at nucleotide 33 was found in a eukaryotic consensus sequence for initiation of translation with an adenine residue in position 3 relative to the AUG [24]. The length of the cDNA (2,928 bp) was consistent with the size of the mRNA (about 3.2 kb) as measured by Northern blot hybridization (Fig. 3, lanes 1 and 3). The small difference in size might be attributed to either a longer 5′ non-coding sequence, or a longer poly(A) tail in the mRNA, as compared to the cDNA isolated here. No signal was detected in poly(A)⁺ RNA from skeletal muscle and brain (Fig. 3, lanes 2, 4 and 5). When a similar blot was probed with the cDNA fragment of E-24.11, a 3.6-kb mRNA was detected in kidney (results not shown).

3.2. *Amino acid sequence of the α-subunit and comparison with other metallo-endopeptidases*

Translation of the open reading frame predicts a primary translation product of 748 amino acids (Fig. 2). Hydropathy plot analysis of this sequence identified a single putative hydrophobic membrane-spanning domain of 20 amino acids located near the C-terminus of the protein. Another hydrophobic sequence, located near the N-terminus, most likely corresponds to a cleavable signal peptide as already suggested by Jiang et al. for the mouse enzyme [16]. However, the initiator methionine found in our sequence is different from the one proposed by Jiang et al. [16]. No other methionine was found upstream from the ATG codon in our cDNA sequence, suggesting a difference between the sequence of the rat and mouse enzymes. Amino acid sequencing of the N-terminal region of the papain-released form of E-24.18 gave the sequence, NALRDPxxRWKPxI-PYILADNLDL, which was also found in both the papain- and detergent-solubilized forms of the mouse enzyme [16]. Taken together, these findings are consistent with the enzyme being a type-I transmembrane protein anchored by a C-terminal transmembrane domain of 20 amino acids, followed by a short, positively charged, cytoplasmic tail of 9 residues at the C-terminus (Fig. 2). The large extracellular domain starts at Asn-67 and results from the removal of the signal peptide and a pro-region by hydrolysis of the Arg-66–Asn-67 bond. The calculated M_r of the resulting 682-residue protein is 77,873. The extracellular domain contains six Asn residues, each located within the consensus sequence for *N*-glycosylation, (Asn-X-Ser/Thr). Glycosylation of one

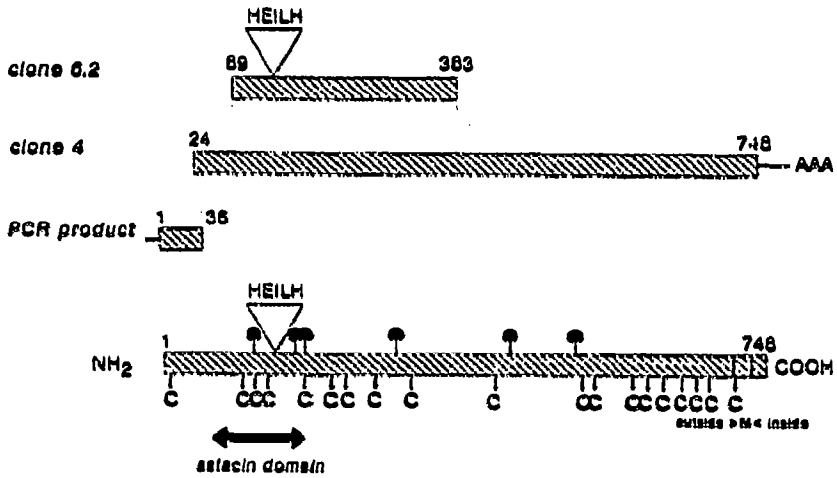

Fig. 1. Schematic model of the primary structure of the α-subunit of rat E-24.18 and the different cDNA clones isolated. Clones 6.2 and 4 refer to the clones isolated from λgt11 and λgt10 libraries, respectively. PCR product was obtained by the polymerase chain reaction according to the rapid amplification of cDNA ends. The boxed areas represent the coding region, while single lines represent the 5' and 3' non-coding regions. Numbers refer to the positions of amino acids starting at the N-terminal Met. The sites of possible *N*-glycosylation are indicated by 'lollipops'. Cysteine residues, C. The membrane spanning region, separating the large extracellular and small cytoplasmic domains is identified by M.

or more of these sites could account for the difference observed between the calculated M_r and that estimated for the native enzyme by SDS-PAGE under reducing conditions (80 kDa) [2,3]. There are 19 cysteine residues distributed throughout the molecule, of which one is located in the signal peptide and another in the transmembrane domain.

The His-Glu-Ile-Leu-His sequence, which corresponds to the consensus sequence H-E-X-X-H characteristic of the active site of most zinc metallo-endopeptidases [25], was found in the extracellular domain of the enzyme. Site-directed mutagenesis of E-24.11 has shown that the Glu and the two His residues of this sequence play an essential role in its catalytic activity [26,27]. The His residues act as Zn-binding ligands, while the Glu is involved in acid–base catalysis. It is likely that these residues play a similar role in E-24.18.

Comparison of the amino acid sequence of E-24.18 with that of mouse meprin A revealed 86.8% identity, suggesting a relatively high degree of interspecies conservation, as suggested in previous reports [3,16,28]. The rat enzyme showed 84.4% identity with the partial sequence of the human intestinal brush border 'PABA-peptidase' (*N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase, PPH) [17]. Recently, Dumermuth et al. [17] have reported that the partial cDNA sequences of PPH and meprin A showed 30% identity with astacin, a 200 residue zinc metalloproteinase isolated from the crayfish, *Astacus fluviatilis* [29]. Several cysteine residues, which have been shown to participate in the formation of disulphide bridges in astacin [29] were conserved in PPH and meprin A. These authors have suggested that the regions of PPH and meprin A which posssess a significant sequence identity with the crayfish protease be referred to as the 'astacin domain' and propose that these enzymes are members of an 'astacin family' [17]. Our sequence of E-24.18 exhibited 40% identity and 50% similarity (i.e. conservative amino acid substitutions) to astacin in a 129 residue stretch (from 92 to 221) that included the putative active site and several cysteine residues. Like PPH, meprin A and astacin, E-24.18 has an extended zinc binding site (HExxHxxGFxHE; Fig. 2) unique to this family [17]. Thus, E-24.18 is a member of the astacin family. The crystal structure of astacin has been recently solved [30]. The active site is at the bottom of a cleft, the Zn being liganded by His-92, His-96 and His-102 and a water molecule is anchored to Glu-93 and Tyr-149. Our sequence also contains a Tyr at approximately the same position downstream from the Zn-binding motif, possibly serving the same function in catalysis.

3.3. *Renal expression of E-24.18*

We have used in situ hybridization to compare the localization of E-24.18 and E-24.11 mRNA in the kidney. Strong staining was observed in the proximal tubules of the nephrons located in the juxtamedullary region of the cortex (Fig. 4a). The outermost region of the cortex was unstained, but small groups of stained tubules were observed in a radial pattern between the unstained outer and the highly stained juxtamedullary zones. This pattern is identical to that observed by immunohistochemistry using the rabbit polyclonal antibody, RRt151, in rat and mouse kidneys [3]. The same juxtamedullary distribution was obtained using a cRNA probe against E-24.11 mRNA (Fig. 4b). This distribution of the two enzymes may be specific to rodents, since E-24.11 seems to be located in all cortical

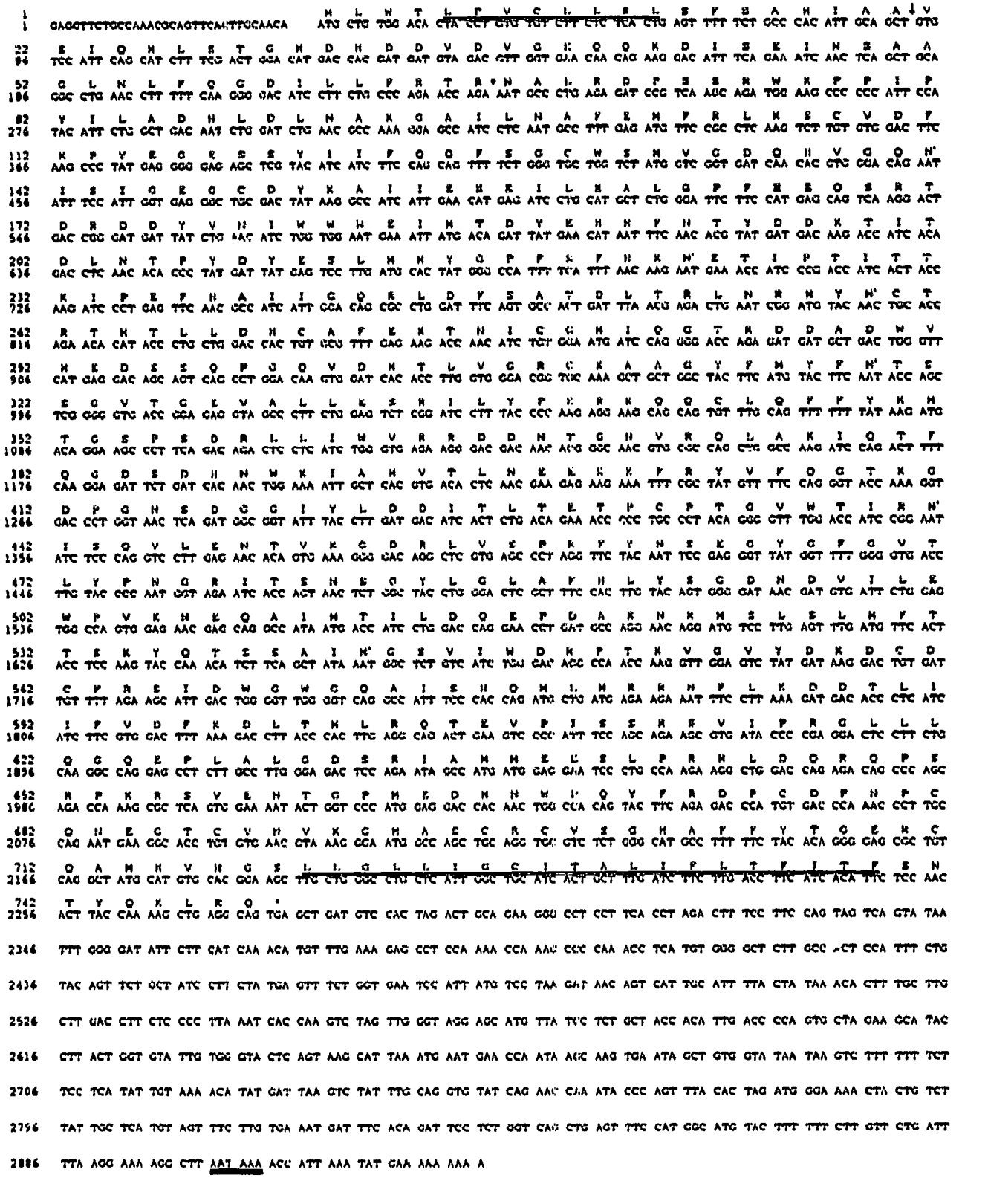

Fig. 2. Combined nucleotide sequence of clones 6.2, 4 and the PCR product, with the deduced amino acid sequence of the α-subunit of rat E-24.18. The hydrophobic domains are underlined. The arrow indicates the possible cleavage site of a signal peptide. The solid triangle indicates the N-terminus of the papain-released form of the protein. Asterisks mark the potential *N*-glycosylation sites. The putative extended zinc-binding site appears in bold. In the 3′ untranslated region the putative polyadenylation signal is indicated by a double line.

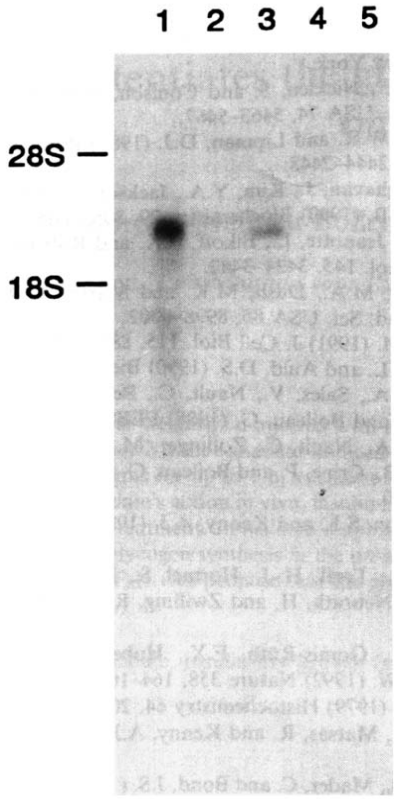

Fig. 3. Northern blot analysis of rat RNA. RNA samples were electrophoresed on a 1% agarose-formaldehyde gel, transferred to nitrocellulose membranes and hybridized with ^{32}P-labelled cDNA (clone 6.2), as described in section 2. 10 μg of total RNA isolated from kidney (lane 1), 1 μg of poly(A)$^+$ RNA from skeletal muscle (lane 2), 1 μg of poly(A)$^+$ RNA from kidney (lane 3), 1 and 2 μg of poly(A)$^+$ RNA from brain (lanes 4 and 5, respectively). The blot was exposed for 10 days. Positions of 28 S and 18 S rRNA are indicated on the left margin.

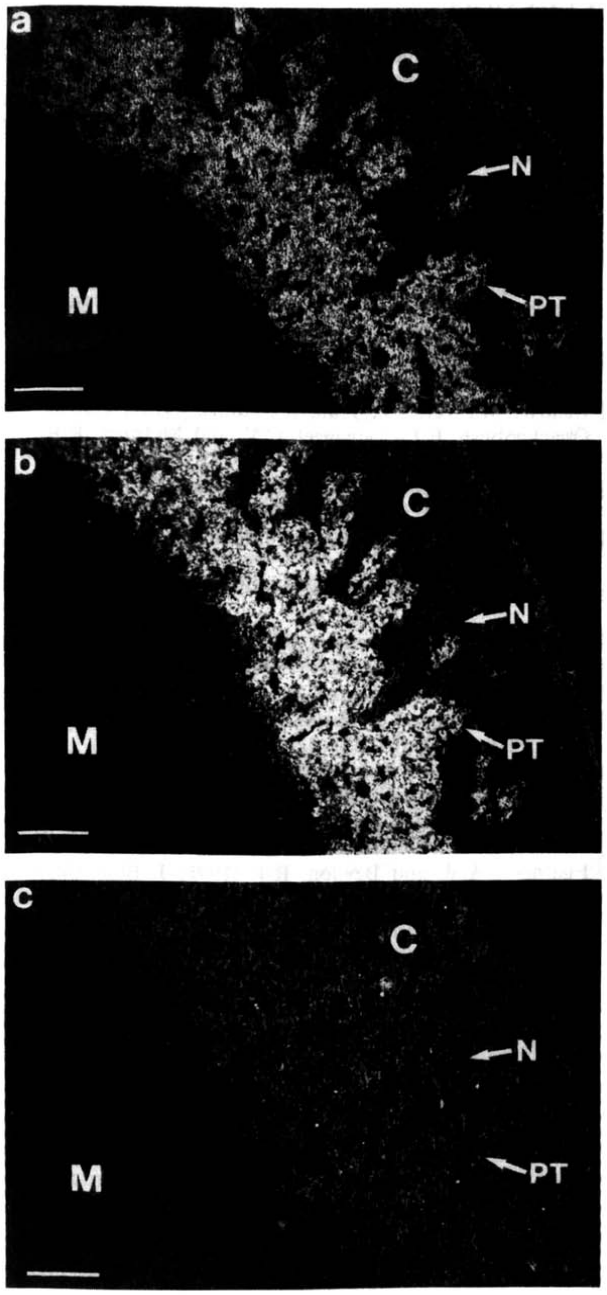

Fig. 4. In situ hybridization of adjacent longitudinal sections of rat kidney. Sections (5 μm thick) were hybridized with ^{35}S-labelled antisense cRNA for E-24.18 (a), E-24.11 (b) and sense cRNA for E-24.18 (c). C, cortex; M, medulla; PT, proximal tubules; N, nephron. Bars = 500 μm.

nephrons in some other species [31,32]. No labelling was observed with E-24.18 sense RNA (Fig. 4c), nor with E-24.11 sense RNA (not shown). Consistent with the Northern and Western blots [3], no E-24.18-specific staining was observed in the rat brain by in situ hybridization after 3 weeks of exposure (not shown). The distribution of E-24.18 in rat kidney closely resembles that of meprin A in mouse kidney [3,33]. The co-localization of mRNAs for both E-24.18 and E-24.11 in the proximal tubules of the juxtamedullary nephrons suggest that these enzymes may have similar or complementary roles in the degradation of peptides and proteins filtered at the glomerulus. The preference of E-24.18 is for longer peptides and even some proteins [2,15], while E-24.11 is more efficient with a wide range of small biologically-active peptides [13,34].

Understanding the primary structure of E-24.18, especially details of its active site, should facilitate the design of highly potent and specific inhibitors. Such compounds would be valuable in elucidating the physiological role of this metallo-endopeptidase.

Acknowledgements: We are grateful to Dr. J.N. Keen (Department of Biochemistry and Molecular Biology, Leeds) and Dr. N.G. Seidah (Clinical Research Institute of Montreal) for sequencing the protein and to C. Landry for preparing total rat kidney RNA. This work was supported by a grant from the Medical Research Council of Canada (MRC-C) to G.B. and P.C. G.B. was the recipient of a Chercheur-Boursier Award from Le Fonds de la recherche en santé du Québec. S.W. was the recipient of a fellowship from MRC-C and D.C. was the recipient of a studentship from FCAR.

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