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CLONING AND FUNCTIONAL EXPRESSION OF A METALLOENDOPEPTIDASE FROM HUMAN BRAIN WITH THE ABILITY TO CLEAVE A ß-APP SUBSTRATE PEPTIDE

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Using a combination of PCR and hybridization screening, we have isolated a cDNA clone for a metalloendopeptidase (h-MP78) from a human temporal cortex library. This 2.5-kb cDNA encodes a 689-amino acid protein with a predicted molecular mass of ~ 78.5 kDa. The primary structure of h-MP78 exhibits high similarity to those of porcine (94%) and rat (92%) thimet oligopeptidase. Expression of the cDNA in HEK-293 resulted in the production of an active enzyme able to cleave a chromogenic ß-APP derived substrate peptide KTEEISEVKM-P-nitro-anilide. RNA blot analysis of various human tissues revealed one major species of h-MP78 mRNA of ~ 2.55 kb. The highest level of mRNA was found in the brain.

A major pathological feature of Alzheimer's disease (AD) is the progressive accumulation of amyloid-ß-protein (Aß) in brain parenchyma and vessel walls. Aß is a 40 to 43 amino acid peptide which is derived by proteolysis from a larger membrane-spanning glycoprotein, ß-amyloid precursor protein (ß-APP) (15). Two proteolytic cleavages are necessary to release the Aß from its ß-APP, one by ß-secretase at the N-terminus, the other by γ-secretase at the C-terminus. At present, little is known about these proteases. ß-Secretase cleaves ß-APP between

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The abbreviations used are: AD, Alzheimer's disease; Aß, amyloid-ß-protein; ß-APP, ß-amyloid precursor protein; h-MP78, human metalloendopeptidase 78 kDa; EP24·15, endopeptidase 24·15; PABH, porcine angiotensin-binding protein homologue; thimet oligopeptidase, thiol-dependent and metal-dependent oligopeptidase; RT-PCR, reverse transcription-polymerase chain reaction.

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Met671 and Asp672 to produce a large secreted N-terminus-ß-APP and a membrane-associated fragment which generates Aß through further processing of the C-terminus (2). Several candidate proteases have been reported for the cleavage at the N-terminus of Aß (7,9,12-14). Recently, an 85 kDa metalloprotease isolated from AD brain has been reported to cleave ß-APP, generating a 15 kDa amyloidogenic fragment (10). The sequences of two peptides from the 85 kDa protein show high similarity to the rat testis metalloendopeptidase EP24·15 (11). For comparative studies we were interested in obtaining a human cDNA clone encoding this 85 kDa enzyme. Using nucleotide sequence information derived from rat testis metalloendopeptidase (8,11), we isolated a cDNA clone from a human temporal cortex cDNA library. Here we report the primary structure of human metalloendopeptidase h-MP78 and describe highest mRNA expression levels in brain relative to other tissues.

RESULTS AND DISCUSSION

Molecular cloning of human brain metalloendopeptidase cDNA homologuous to the rat metalloendopeptidase EP24·15. In order to isolate a human homologue of the rat metalloendopeptidase EP24·15, a cDNA probe was generated by a RT-PCR using oligonucleotide primers derived from rat EP24·15 sequences (8,11). Poly(A)$^+$RNA extracted from the human neuroblastoma cell line IMR32 was first reverse-transcribed. The resulting cDNA was then used as a template for PCR with the primer pair (nucleotides 315-338 as sense and 920-943 as antisense) derived from rat testis EP24·15 cDNA sequence (8,11). After 35 rounds of amplification, one distinct PCR fragment with the anticipated length (628 bp) was detected by agarose gel electrophoresis. The amplified DNA was excised from the gel and subcloned for sequence analysis. The sequence data confirmed that the PCR-amplified product was indeed a part of the cDNA encoding a human homologue of the rat endopeptidase 24·15 (11).

A λ Zap cDNA library of human temporal cortex was initially screened with radiolabeled PCR-amplified cDNA probe. Six positive clones were identified after screening one million plaques. The inserts from the positive clones were excised from pBluescript and analyzed on Southern blots. The largest insert (2.5 kb), isolated from clone 15-1, was sequenced (Fig. 1). The largest open reading frame in the nucleotide sequence of clone 15-1 (nucleotides -127 to 2388) started at nucleotides 1-3 and terminated at 2068-2070 with a stop codon (TGA). This 2067 bp open reading frame codes for a polypeptide of 689 amino acid residues with a predicted molecular mass of 78,500 Da. A sequence for the polyadenylation of mRNA transcripts of higher eukaryotes is found at nucleotides 2376-2381. A potential N-linked glycosylation site (ASN, Xaa, Ser/Thr) (6) was found at position 451 in the predicted amino acid sequence of h-MP78. At present,

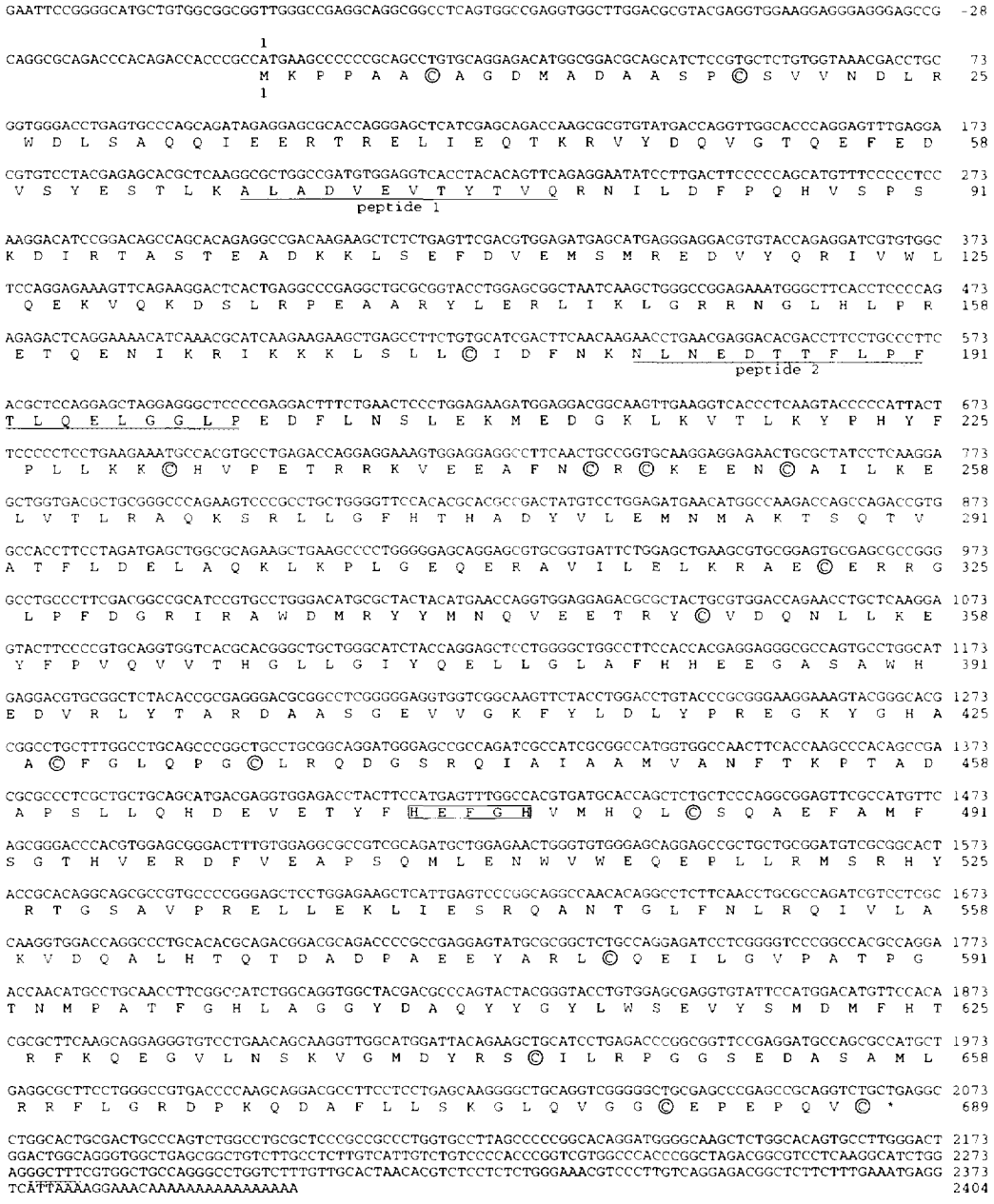

Figure 1. Nucleotide and predicted amino acid sequences of cloned human MP78. The amino acid sequence of the encoded polypeptide is shown in single-letter code below the nucleotide sequence and is numbered beginning with the initiating methionine. Nucleotides are numbered in the 5′ to 3′ direction, and positions are shown to the right. Nucleotide 1 is the A residue of the initiating methionine codon ATG. The peptides 1 and 2 that have been reported (10) for the human metalloprotease from AD brain are underlined. The putative catalytic site sequence –H–E–F–G–H– is indicated by the box. The sixteen cysteines are circled. The polyadenylation signal is overlined.

however, it is not clear if the h-MP78 is glycosylated *in vivo*. The amino acid sequences of the human 85 kDa metalloprotease peptides 1 and 2 previously reported (10) were found in the predicted protein sequence of h-MP78 (Fig. 1). This result strongly indicates that the isolated cDNA codes for the described (10) human metalloprotease purified from AD brain. The motif His-Glu-X-X-His which represents the active site of zinc-dependent metallopeptidases (16) was found at residues 473-477 of h-MP78 (Fig. 1). Interestingly, a cysteine residue (C-483) is positioned five residues away from this active site.

Comparison of the deduced amino acid sequence of h-MP78 with those of porcine thimet oligopeptidase (pig-PABH) (4) and rat testis metalloendopeptidase (rat-EP24·15) (8,11) shows high similarity. The extent of amino acid identities between h-MP78 and the porcine and rat enzymes are 91% and 88%, but increase to 94% and 92% considering conservative amino acid substitutions. These high sequence similarities clearly indicate that the h-MP78 is the human version of thimet (thiol-dependent and metal-dependent) oligopeptidase EC 3.4.24.15. In addition, sixteen cysteines are contained in h-MP78 (Fig. 1), at least 12 of which are conserved between the human, porcine and rat enzymes.

Blot hybridization of RNA. A Northern blot of Poly(A)+RNA isolated from different human tissues is shown in Fig. 2A. In all tissues tested, a single species of h-MP78 transcript of ~ 2.55 kb was detected using the nick-translated 628 bp cDNA probe. Therefore, the 2.5 kb h-MP78 clone corresponds in length to the mRNA transcript detected by Northern blotting. The strongest expression was detected in brain, with the h-MP78 transcript being abundant in all the regions investigated (Fig. 2B). Among the peripheral organs tested, the intensity of the labeling was high in skeletal muscle, placenta and heart (Fig. 2B).

Expression of h-MP78 in HEK-293 cells. To confirm that the isolated cDNA indeed encoded an enzyme with metalloendopeptidase activity, the h-MP78 cDNA was subcloned into the eukaryotic expression vector pCMV/Neo, which initiates transcription from the CMV promoter. HEK-293 cells were transfected with the resultant construct (pCMV/Neo-h-MP78) and metalloendopeptidase activity was measured in cell extracts using our ß-APP-derived chromogenic peptide substrate of the ß-secretase site (Fig. 3). Although HEK-293 cells contained some endogenous protease activity, it was not significantly decreased in the presence of the metalloprotease inhibitor phenanthroline, and this basal level did not change after transfection with the expression vector alone (not shown). In contrast, HEK-293 cells transfected with plasmid pCMV/Neo-h-MP78 showed a nearly 2-fold increase in proteolytic activity, which could be completely and dose-dependently blocked by phenanthroline. This demonstrates that the expressed h-MP78 cDNA does encode a metalloprotease, and furthermore that it has the expected proteolytic activity of a ß-secretase candidate enzyme (10).

The precise physiological role of members of the thimet oligopeptidase family with regard to substrate specificity and tissue distribution in different species is

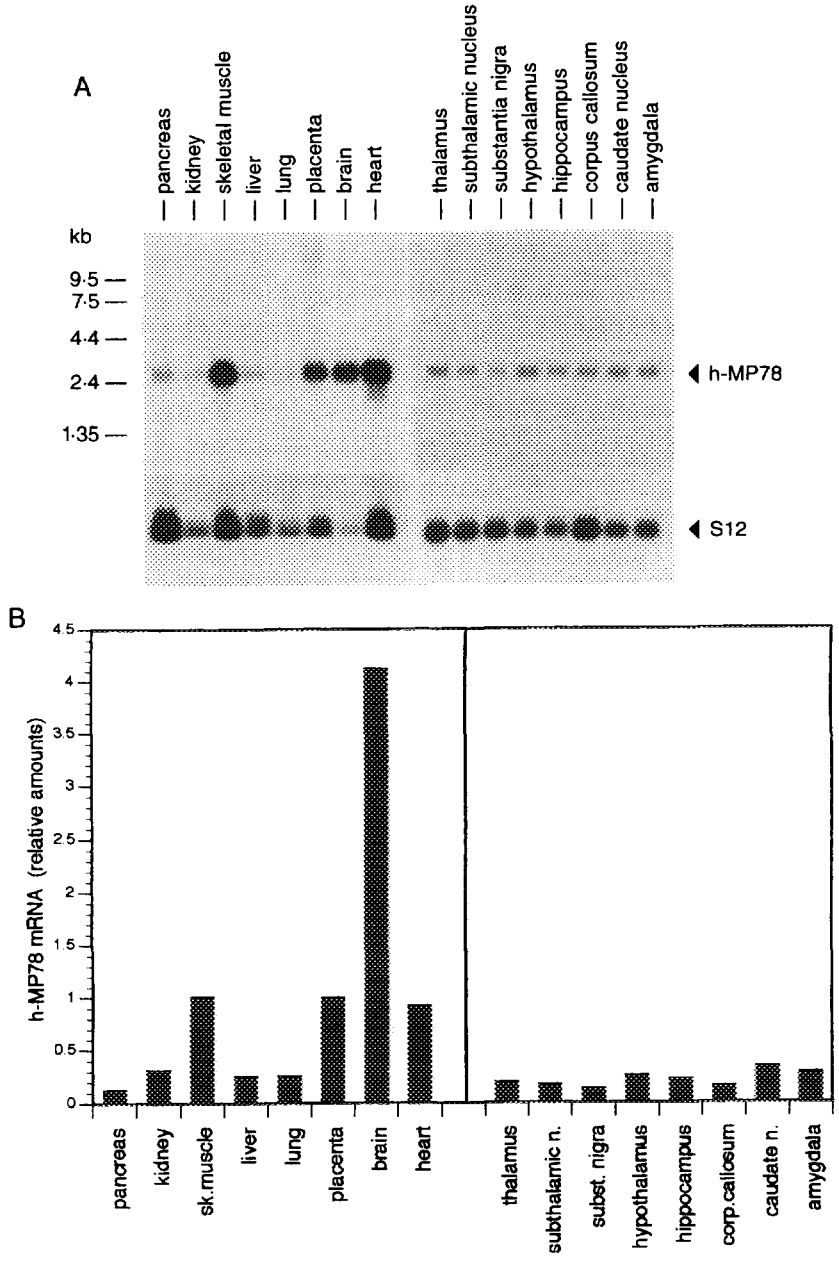

Figure 2. Expression of the h-MP78 gene in various human tissues. (A) Northern blotting shows the hybridization of ~ 2 μg of Poly(A)$^+$RNA extracted from each of the designated tissues with the [^{32}P]labeled nick-translated 625 bp h-MP78 cDNA (upper) and 368 bp ribosomal protein S12 cDNA as control (lower). Numbers on the left indicate kb as determined from RNA size markers. The arrows indicate the h-MP78 and S12 mRNAs. The blots for the peripheral organs and brain were exposed for 16 h, different region of CNS for 6 h. (B) Quantitation of h-MP78 mRNA: values are the ratio of densitometric scores for h-MP78 and S12 mRNAs.

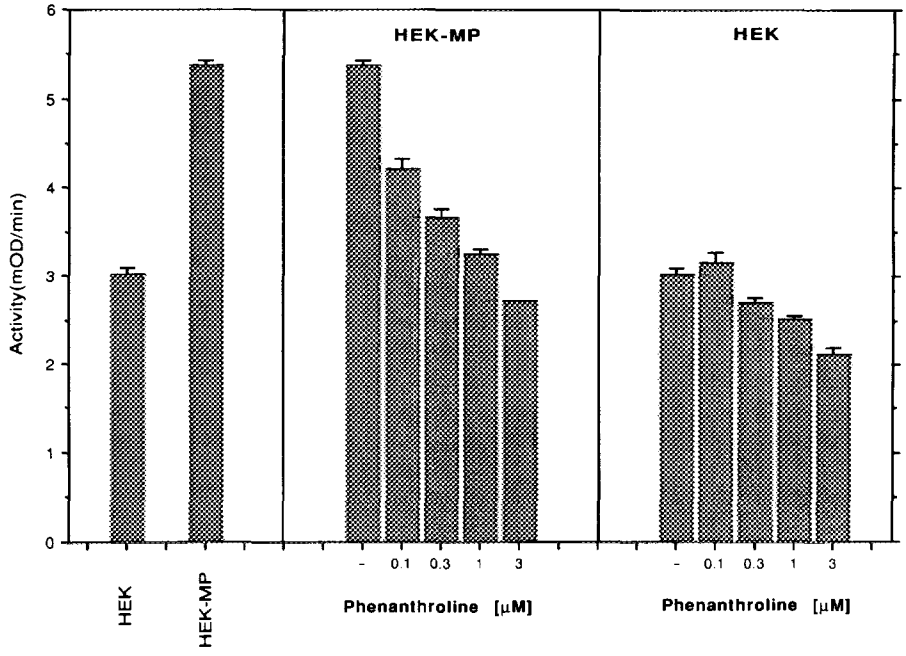

Figure 3. Metalloprotease activity of expressed h-MP78 in HEK-293 cells. Proteolytic activities in 200 µg of nontransfected control-HEK or HEK-h-MP78 cell extracts show a distinct increase in ß-APP derived chromogenic substrate proteolysis by the h-MP78 expressing cells. This h-MP78 derived enzyme activity can be dose-dependently inhibited by phenathroline.

not well established. Therefore, the availability of the recombinant human thimet oligopeptidase should provide new insights into the mechanism of action of this enzyme, its regional distribution in CNS, and its subcellular localization. These studies may shed new light on a possible role for h-MP78 in the pathology of Alzheimer's disease.

METHODS

Isolation of h-MP78 cDNA. To isolate the h-MP78 cDNA, Poly(A)$^+$RNA from human neuroblastoma cell lines IMR 32 (1 µg) was reverse-transcribed to cDNA and then amplified by means of PCR (using a gene Amp RNA-PCR kit from Perkin-Elmer). For PCR, the following oligonucleotide primers derived from the rat EP24.15 cDNA sequence (11) were used: sense (5'-CTAGGAAGGTGGCT ACTGTCTGAC-3') and antisense (5'-GCACGTGTCTCCGAACAAGGACAT-3'). The final PCR product was extracted with phenol/chloroform and then precipitated with ethanol. PCR products were electrophoresed on 1% low-melting agarose gel, and the major band of 628 bp was isolated and subcloned into the pBluescript vector for sequence analysis. A human temporal cortex cDNA library constructed in the λ zap vector (Stratagene, La Jolla, CA) was screened following

previously described procedures (3) using a [^{32}P]labeled nick-translated PCR-derived 628 bp cDNA probe

Production of recombinant human h-MP78 in HEK-293 cells and metalloendopeptidase activity measurement. The 2.5 kb insert from plasmid 15-1 was excised from the pBluescript phagemid with EcoRI and subcloned into the blunt-ended BamHI site of the expression vector pCMV/neo (1). Human embryonic kidney fibroblast cells (HEK-293 cell line, ATCC CRL1573) were transfected as described previously (5). The cell pellet was suspended in 4 volumes of cold homogenization buffer (50 mM sodium phosphate pH 7.0, 1 mM dithiothreitol (DTT)) and homogenized with a teflon pestle. After centrifugation at 20'000 x g for 30 min, 4°C, the protein concentration in the supernatant was measured (Bradford method, BioRad kit), and 200 µg of supernatant protein of transfected or nontransfected cells were assayed for protease activity as described (14). Assays were also done with increasing concentrations of the metalloprotease inhibitor phenanthroline added 10 min before the peptide substrate. All measurements were performed in duplicate. Optical density readings were done using a microplate reader (model 3550, BioRad).

Northern blot analysis. Northern blots of human Poly(A)$^+$RNA (Clontech, Palo Alto, CA) were hybridized with a [^{32}P]labeled nick-translated PCR-derived 628 bp cDNA probe as described previously (5). As a control probe, a PCR-amplified fragment of human S12 protein was used. Signal intensities on the X-ray films were quantified and analyzed using a videodensitometer (model 620, BioRad). Relative amounts of h-MP78 were calculated by determining the intensity of the h-MP78 band in relation to the internal tissue-standard band of S12.

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