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Identification of Human Aminopeptidase O, a Novel Metalloprotease with Structural Similarity to Aminopeptidase B and Leukotriene A_4 Hydrolase*

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We have cloned and characterized a human brain cDNA encoding a new metalloprotease that has been called aminopeptidase O (AP-O). AP-O exhibits a series of structural features characteristic of aminopeptidases, including a conserved catalytic domain with a zinc-binding site (HE*XX*H*X*$_{18}$E) that allows its classification in the M1 family of metallopeptidases or gluzincins. The structural complexity of AP-O is further increased by the presence of an additional C-terminal domain 170 residues long, which is predicted to have an ARM repeat fold originally identified in the *Drosophila* segment polarity gene product Armadillo. This ARM repeat domain is also present in aminopeptidase B, aminopeptidase B-like, and leukotriene A_4 hydrolase and defines a novel subfamily of aminopeptidases that we have called ARM aminopeptidases. Northern blot analysis revealed that AP-O is mainly expressed in the pancreas, placenta, liver, testis, and heart. Human AP-O was produced in *Escherichia coli*, and the purified recombinant protein hydrolyzed synthetic substrates used for assaying aminopeptidase activity. This activity was abolished by general inhibitors of metalloproteases and specific inhibitors of aminopeptidases. Recombinant AP-O also cleaved angiotensin III to generate angiotensin IV, a bioactive peptide of the renin-angiotensin pathway with multiple actions on diverse tissues, including brain, testis, and heart. On the basis of these results we suggest that AP-O could play a role in the proteolytic processing of bioactive peptides in those tissues where it is expressed.

Aminopeptidases are exopeptidases that have the ability to catalyze the hydrolysis of amino acid residues from the N terminus of peptide or protein substrates. These enzymes are widely distributed in multiple organisms from bacteria to human and play essential roles in protein maturation and regulation of the metabolism of bioactive peptides (1, 2). Furthermore, alterations in the function and regulation of aminopeptidases underlie several human diseases, including cancer and cardiovascular disorders (3–5). Among the different families of human aminopeptidases, there has been a growing interest in the analysis of the biological and pathological relevance of members of the M1 family of zinc metallopeptidases or gluzincins (6, 7). To date, 12 members of this family have been identified in human tissues (www.uniovi.es/degradome) (8). Aminopeptidase A (APA or ENPEP) is a type II membrane-bound protease that cleaves N-terminal amino acid residues from peptides such as cholecystokinin-8 and angiotensin II (9, 10) and contributes to the control of blood pressure and angiogenesis (11–13). Aminopeptidase N (APN,[1] ANPEP, or CD13) is also a membrane-bound exopeptidase associated with angiogenesis regulation (14–16) that acts as a receptor for the coronavirus and tumor-homing peptides (17, 18). Aminopeptidase Q (APQ or laeverin) is structurally related to APN and participates in regulation of the extravillous trophoblast invasion of maternal decidual tissues (8, 19). The thyrotropin-releasing hormone degrading ectoenzyme (TRHDE) is found in serum as a soluble enzyme or anchored to the cell surface of neuronal cells and selectively inactivates TRH, a peptide that stimulates hormone secretion from adenohypophyseal cells (20–22). Placental leucine aminopeptidase (PLAP, LNPEP, or IRAP), puromycin-insensitive, leucyl-specific aminopeptidase (PILS-AP, ERAAP, ERAP1, or ARTS1), and leukocyte-derived arginine aminopeptidase (L-RAP, AMPEP, or LPEP) belong to the subfamily of oxytocinases. Placental leucine aminopeptidase is regulated by insulin and degrades peptide hormones such as oxytocin and vasopressin, maintaining homeostasis during pregnancy (23–28). The puromycin-insensitive, leucyl-specific aminopeptidase and the leukocyte-derived arginine aminopeptidase are involved in the N-terminal trimming of major histocompatibility complex class I-presented peptide precursors (29–31) and the shedding of cytokine receptors (32, 33). The puromycin-sensitive aminopeptidase (PSA or NPEPPS) has a broad substrate specificity and is involved in multiple processes including protein turnover, neuropeptide metabolism, cell cycle control, reproduction, and regulation of anxiety and pain (34–38). Leukotriene A_4 hydrolase (LTA4H) and aminopeptidase B (APB or RNPEP) are structurally related and have been linked to inflammatory processes and tumor progression (39–46). Finally, aminopeptidase B-like (APB-L or RNPEPL1) has been identified in the course of global projects of human genome analysis (8, 47), but to date no functional characterization of this enzyme hasbeen provided.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ560639, AJ810420, and AJ810421.

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[1] The abbreviations used are: APN, aminopeptidase N; APB, aminopeptidase B; APB-L, APB-like; AP-O, aminopeptidase O; AMC, 7-amino-4-methylcoumarin; GST, glutathione *S*-transferase; LTA4H, leukotriene A_4 hydrolase; PAP, porcine leucine aminopeptidase; SH3, Src homology 3.

This paper is available on line at http://www.jbc.org

Recently, as part of our studies aimed at trying to get a complete view of the human degradome, the complete set of proteases produced by human tissues (48), we detected a genomic sequence encoding a putative protein with structural similarity to aminopeptidases of the M1 family of metalloenzymes. In this work we report the cloning and characterization of a cDNA coding for this new aminopeptidase that has been tentatively called aminopeptidase O (AP-O). We also examine the distribution of this enzyme in human tissues and perform an analysis of its enzymatic activity on synthetic and endogenous substrates. Finally, we discuss the putative physiological role of this novel enzyme in the processing of bioactive peptides in heart and reproductive tissues.

EXPERIMENTAL PROCEDURES

Materials—Human cDNA libraries and Northern blots containing polyadenylated RNAs from different tissues were from Clontech. Restriction endonucleases and other reagents used for molecular cloning were from Roche Diagnostics. Double-stranded DNA probes were radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) from Amersham Biosciences, using a commercial random-priming kit purchased from the same company. Antibodies against GST were developed in our laboratory as described previously (49). The aminopeptidases APN and porcine leucine aminopeptidase (PAP) were obtained from Calbiochem. The fluorogenic aminopeptidase substrates, as well as angiotensins I, II, and III, were purchased from Bachem, and protease inhibitors and AMC were from Sigma.

Bioinformatic Screening of the Human Genome and cDNA Cloning—The BLAST program was used to search public (www.ncbi.nlm.nih.gov) and private (www.celera.com) human genome databases to look for regions with sequence similarity to previously described M1 aminopeptidases. After identification in human chromosome 9q22 of DNA contigs encoding regions similar to the catalytic domain of these enzymes, we designed specific oligonucleotides to PCR amplify a cDNA for this protein using a brain cDNA library. All PCR amplifications were performed in a GeneAmp 2400 PCR system from PerkinElmer Life Sciences. After cloning the amplified PCR products in pUC19, the identity of the products was confirmed by nucleotide sequencing.

Nucleotide Sequence Analysis—Cloned cDNAs were sequenced by the dideoxy chain termination method using the DR Terminator Taq FS kit and an ABI-Prism 310 DNA sequencer (Applied Biosystems). Computer analysis of DNA and protein sequences was performed with the GCG software package of the University or Wisconsin Genetics Computer Group.

RNA Extraction—Samples were homogenized in guanidinium thiocyanate buffer with a blender. Then, a one-tenth volume of sodium acetate 3 M pH 4.0, 1 volume of phenol/water, and 1 volume of chloroform/isoamylic alcohol (1:25) were added. The resulting mixture was centrifuged at 13,000 rpm for 20 min at 4 °C. The aqueous phase was recovered, and 1 volume of cold 2-propanol was added. After centrifugation, the pellet was washed with 70% ethanol.

Northern Blot Analysis—Nylon filters containing 2 μg of poly(A$^+$) RNA from human tissues or 20 μg of total RNA from mouse tissues were prehybridized at 42 °C for 3 h in 50% formamide, 5× SSPE (1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4), 10× Denhardt's solution, 2% SDS, and 100 μg/ml denatured herring sperm DNA. Then, filters were hybridized with a radiolabeled human AP-O-specific probe (377 bp) containing nucleotides 2101–2477 of the isolated cDNA. Hybridization was performed for 20 h under the same conditions used for prehybridization. Finally, blots were washed once with 2× SSC, 0.05% SDS for 30 min and three times in 0.1× SSC, 0.1% SDS for 30 min at 50 °C and exposed to autoradiography. RNA integrity and loading was assessed by hybridization with probes specific for β-actin or glyceraldehyde-3-phosphate dehydrogenase.

Reverse Transcription PCR—About 1 μg of total RNA extracted from the testes of mice of different ages was used to carry out reverse-transcriptase reactions using the GeneAmp kit from PerkinElmer Life Sciences. A PCR reaction was then performed with the primers 5′-GAAGAGGTGTTTGAAAAGCTTC-3′ and 5′-GGGAGAGAATCAC-CTCG-3′ for 25 cycles of denaturation (94 °C for 20 s), annealing (62 °C for 20 s), and extension (72 °C for 30 s). As a control, β-actin was PCR-amplified from all samples under the same conditions.

Production, Refolding, and Purification of Recombinant Proteins—A cDNA containing the catalytic domain of AP-O was obtained by PCR amplification using two oligonucleotides containing BamHI and EcoRI sites, respectively, namely 5′-CAT<u>GGATCC</u>AATCTGTGTTAAAAGTC-GAGGAGG-3′ and 5′-CAG<u>GAATTC</u>CCCTAGCCCTTTAAATAAT-GCACCTGC-3′ (where the BamHI and EcoRI sites are underlined). The PCR amplifications were performed with 25 cycles of denaturation (95 °C for 15 s), annealing (58 °C for 10 s), and extension (68 °C for 2 min) using the Expand™ Long High Fidelity PCR system. The PCR product was digested with BamHI and EcoRI and cloned between the corresponding sites of the pGEX-5x-2 expression vector (Amersham Biosciences). The resulting construct was transformed into BL21(DE3)-pLysE-competent *Escherichia coli* cells, and expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration 1 mM), followed by 5 h of incubation at 28 °C. The cells were then harvested by centrifugation, washed with phosphate-buffered saline, and lysed by incubation in phosphate-buffered saline with 100 μg/ml lysozyme, 10 μg/ml DNase, and 0.1% Triton X-100 overnight at 4 °C. The extract was centrifuged, and the pellet was washed three times with phosphate-buffered saline and dissolved in a buffer containing 20 mM Tris, pH 7.4, and 6 M guanidinium chloride. The recombinant protein was refolded at 4 °C in two dialysis steps, first against a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, and 2 M urea for 48 h and twice against 50 mM Tris, pH 7.5, and 150 mM NaCl for 24 h. Refolded protein was purified by affinity chromatography using a glutathione-Sepharose column. The identity of the recombinant proteins was verified by Western blot and in-gel digestion followed by mass spectrometry analysis.

Western Blot Analysis—The supernatant and the pellet of the bacterial extracts were separated in 10% SDS-PAGE. After electrophoresis, gels were electrotransferred onto nitrocellulose filters, and then the filters were blocked with 5% nonfat dried milk in PBT (phophate-buffered saline with 0.1% Tween 20) and incubated for 1 h with rabbit anti-GST serum diluted 1:1000 in 3% nonfat dried milk in PBT. After three washes with PBT, filters were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at a 1:20000 dilution in 1.5% milk in PBT and developed with a West Pico enhanced chemiluminescence kit (Pierce).

In-gel Digestion—Gel bands were manually excised and placed into 0.5-ml tubes. Gel pieces were washed twice with 180 μl of 25 mM ammonium bicarbonate/acetonitrile (70:30), dried at 90 °C, and incubated with 12 μg/ml trypsin (Promega) in 25 mM ammonium bicarbonate. The digestion was allowed to proceed for 4–12 h at 37 °C. The peptide mixture was then desalted by C_{18} reverse phase chromatography (ZipTip, Millipore) and eluted with α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.05% trifluoroacetic acid. In a typical experiment, 1 μl of this solution was analyzed by mass spectrometry.

Mass Spectrometry Analysis—Matrix-assisted laser desorption ionization was performed on a time-of-flight mass spectrometer equipped with a nitrogen laser source (Voyager-DE STR, Applied Biosystems). Data from 50–200 laser shots were collected to produce a mass spectrum.

Enzyme Assays—Enzymatic activity of the purified recombinant human AP-O was assayed using AMC-coupled amino acids (Asp-AMC, Thr-AMC, Leu-AMC, Glu-AMC, His-AMC, Val-AMC, Asn-AMC, Ser-AMC, Ile-AMC, Trp-AMC, Phe-AMC, Ala-AMC, Gln-AMC, Gly-AMC, Lys-AMC, Tyr-AMC, Pro-AMC, Met-AMC, and Arg-AMC). Assays were carried out at 37 °C at a substrate concentration of 5 μM in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 0.05% Brij-35, pH 7.5. The fluorometric measurements were made in an LS55 spectrofluorometer from PerkinElmer Life Sciences (λ_{ex} = 360 nm and λ_{em} = 460 nm). The fluorescent signal was calibrated using known concentrations of AMC. For inhibition experiments, the reaction mixture was preincubated for 30 min at 37 °C with *o*-phenanthroline, E-64, 4-(2-aminoethyl)-benzenesulfonyl fluoride, or arphamenine A (20 μM), and the hydrolyzing activity against Arg-AMC was then determined by fluorometric measurements as described above. Kinetic studies were performed using different concentrations of the fluorogenic compounds (0.5–500 μM) in 100 μl of assay buffer containing 5 nM recombinant AP-O or the commercially available aminopeptidases APN and PAP (5 pM), and peptide hydrolysis was measured as the increase in fluorescence at 37 °C over time. Initial velocities were calculated using the analysis package FL WinLab 2.01 (PerkinElmer Life Sciences), and data were fitted to the Michaelis-Menten equation (50) using GraFit v4.0 (Erithacus). Assays with angiotensin peptides were performed by incubation of 2.5 nM refolded AP-O or 2.5 pM APN and PAP with 10 μM angiotensins I, II, or III. Reactions were carried out at 37 °C in a buffer containing 50 mM Tris-HCl and 150 mM NaCl pH 7.5 for 2, 6, and 24 h. Samples were purified using ZipTip and analyzed by mass spectrometry.

A

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1
ATGgacatacagctggaccctgccagagatgacctgcctctcatggccaacaccagccac
M  D  I  Q  L  D  P  A  R  D  D  L  P  L  M  A  N  T  S  H
61
atacttgtgaagcactatgtactggatttggatgtggattttgaaagtcaagtcattgag
I  L  V  K  H  Y  V  L  D  L  D  V  D  F  E  S  Q  V  I  E
121
gggaccatagtgcttttcctcgaggatggaaacagattcaagaaacagaatagctctatt
G  T  I  V  L  F  L  E  D  G  N  R  F  K  K  Q  N  S  S  I
181
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E  E  A  C  Q  S  E  S  N  K  A  C  K  F  G  M  P  E  P  C
241
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H  I  P  V  T  N  A  R  T  F  S  S  E  M  E  Y  N  D  F  A
301
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I  C  S  K  G  E  K  D  T  S  D  K  D  G  N  H  D  N  Q  E
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601
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661
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721
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P  H  A  I  R  I  W  Y  K  T  K  P  E  G  R  S  V  T  W  T
781
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S  D  Q  S  G  R  P  C  V  Y  T  V  G  S  P  I  N  N  R  A
841
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901
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1141
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1501
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1561
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I  L  S  Q  D  F  L  Q  M  L  L  E  N  I  P  E  E  K  R  L
1921
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E  L  S  V  E  N  I  Y  Q  D  W  L  E  S  S  G  I  P  K  P
1981
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V  T  K  W  I  G  V  N  R  R  P  R  K  R  K  R  R  E  K  E
2101
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2401
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R  T  K  E  Q  M  D  R  S  S  A  Q  V  V  A  E  M  L  F  *
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B

FIG. 1. **Sequence and domain structure of human AP-O.** *A*, nucleotide and amino acid sequences of AP-O. The zinc-binding site characteristic of aminopeptidases is highlighted by a *black background*. The complete catalytic domain is shown in *bold*. The GMEN motif and YXKG box are highlighted by *gray backgrounds*. The SH3-like motif is in *italics* and is *underlined*. The C-terminal ARM repeat domain is *underlined*. *B*, graphic representation of the AP-O domain structure showing the LTA4H-like N-terminal domain, the gluzincin catalytic domain, the SH3-like motif, and the ARM domain.

RESULTS

Identification and Characterization of a New Human Aminopeptidase—An extensive search for DNA genomic sequences encoding novel aminopeptidases allowed us to identify a contig in human chromosome 9q22 containing coding information for the putative catalytic domain of a yet uncharacterized metalloprotease. The full-length cDNA for this enzyme was predicted by comparison with the structure of other aminopeptidases and then amplified by PCR experiments. We used specific oligonucleotides to amplify, from a brain cDNA library, a 2.5-kb fragment containing in frame initiator and stop codons. After cloning and sequencing of the PCR-amplified products, we confirmed by conceptual translation that the generated sequence encoded a new protein (Fig. 1) that we tentatively called aminopeptidase O or AP-O.

Computer analysis of the identified amino acid sequence revealed that it contains a large catalytic domain typical of M1 aminopeptidases flanked by N- and C-terminal extensions, which are 150 and 170 residues long, respectively (Fig. 1). Moreover, the C-terminal extension is connected to the catalytic domain through a hydrophobic loop that resembles SH3 domain recognition sequences (51, 52). The N-terminal extension of AP-O is recognized by the InterPro program (www.ebi.ac.uk/interpro) as being similar to the N-terminal domain of LTA4H despite the low percentage of identities between both regions (about 15%). The C-terminal region is recognized by the

A

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AP-O     : RVFAPVCLTGACQETLLRLIPPCLS--AAHSVLGAHPFSRLDVLIVPANFPSLGMASPHIMFLSQSILTGG--------NHLCGTRLCHEIAHAWFGLAIGARDWTEEWLSEGFATHLE
APB      : RVWAEPCLIDAAK-EYNGVIEEFLA--TGEKLFGPYVWGRYDLLFMPPSFPFGGMENPCLTFVTPCLLAGD---------RSLADVIIHEISHSWFGNLVTNANWGEFWLNEGFTMYAQ
APB-L    : RVWAEPCLLPTATSKLSGAVEQWLS--AAERLYGPYMWGRYDIVFLPPSFPIVAMENPCLTFIISSILESD---------EFLVIDVIHEVAHSWFGNAVTNATWEEMWLSEGLATYAQ
LTA4H    : LVWSEKEQVEKSAYEFS-ETESMLK--IAEDLGGPYVWGQYDLLVLPPSFPYGGMENPCLTFVTPTLLAGD---------KSLSNVIAHEISHSWTGNLVTNKTWDHFWLNEGHTVYLE
NPEPPS   : RVYT-PVGKAE-QGKFALEVAAKTLPFYKDYFNVPYPLPKIDLIAIA-DFAAGAMENWGLVTYRETALLIDPKNSCSSSRQWVALVVGHELAHQWFGNLVTMEWWTHLWLNEGFASWIE
APN      : RIWARPSAIAAGHGDYALNVTGPILNFFAGHYDTPYPLPKSDQIGLP-DFNAGAMENWGLVTYRENSLLFDPLSSSSSNKERVVTVIAHELAHQWFGNLVTIEWWNDLWLNEGFASYVE
AMPEP    : SIYASPDKRNQ--THYALQASLKLLDFYEKYFDIYYPLSKLDLIAIP-DFAPGAMENWGLITYRETSLLFDPKTSSASDKLWVTRVIAHELAHQWFGNLVTMEWWNDIWLKEGFAKYME
PPILS    : SVYAVPDKINQ--ADYALDAAVTLLEFYEDYFSIPYPLPKQDLAAIP-DFQSGAMENWGLTTYRESALLFDAEKSSASSKLGITVTVAHELAHQWFGNLVTMEWWNDLWLNEGFAKFME
LNPEP    : SIYAVPENIGQ--VHYALETTVKLLEFFQNYFEIQYPLKKLDLVAIP-DFEAGAMENWGLLTFREETLLYDSNTSSMADRKLVTKIIAHELAHQWFGNLVTMKWWNDLWLNEGFATFME
APA      : TIYVQPEQKHT--AEYAANITKSVFDYFEEYFAMNYSLPKLDKIAIP-DFGTGAMENWGLITYRETNLLYDPKESASSNQQRVATVVAHELVHQWFGNIVTMDWWEDLWLNEGFASFFE
APQ      : RIWARKDAIANGSADFALNITGPIFSFLEDLFNISYSLPKTDIIALP-SFDNHAMENWGLMIFDESGLLLEPKDQLTEKKTLISYVVSHEIGHQWFGNLVTMNWWNNIWLNEGFASYFE
TRHDE    : RLYARPDAIRRGSGDYALHITKRLIEFYEDYFKVPYSLPKLDLLAVP-KHPYAAMENWGLSIFVEQRILLDPSVSSISYLLDVTMVIVHEICHQWFGDLVTPVWWEDVWLKEGFAHYFE
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B

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AP-O     : LLPDQLVLLLEHLLEQKTLSPRTLQSLQRTYH--LQDQDAEVRHRWCELIVKHKFTKAYKSVERFLQED--QAMGVYLYGELMVSEDARQQQLARRCFERTKEQMDRSSAQVVAEML---
APB      : WKTYQLVYFLDKILQKSPLPPGNVKKLGDTYPSISNARNAELRLRWGQIVLKNDHQEDFWKVKEFLHNQGKQKYTLPLYHAMMGGSE-VAQTLAKETFASTASQLHSNVVNYVQQI----
APB-L    : WRTFQTALFLDRLLDGSPLPQEVVMSLSKCYSSLLDSMNAEIRIRWLQIEVRNDYYPDLHRVRRFLESQMSRMYTIPLYEDLCTG---ALKSFALEVFYQTQGRLHPNLRRAIQQIL---
LTA4H    : LSSHQLNEFLAQTLQRAPLPLGHIKRMQEVYN-FNAINNSEIRFRWLRLCIQSKWEDAIPLALKMATEQGRMKFTRPLFKDLAAFD--KSHDQAVRTYQEHKASMHPVTAMLVGKDLKVD
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FIG. 2. **Amino acid sequence alignment of catalytic and ARM repeat domains of AP-O and related enzymes.** *A*, alignment of amino acid sequences around the zinc-binding site of the catalytic domains of human aminopeptidases. Designations not defined in the abbreviations footnote are as follows: *AMPEP*, leukocyte-derived arginine aminopeptidase; *APA*, aminopeptidase A; *APQ*, aminopeptidase Q; *LNPEP*, placental leucine aminopeptidase; *NPEPPS*, puromycin-sensitive aminopeptidase; *PPILS*, puromycin-insensitive, leucyl-specific aminopeptidase; *TRHDE*, thyrotropin-releasing, hormone-degrading ectoenzyme. *B*, alignment of amino acid sequences of the C-terminal domain of aminopeptidases AP-O, APB, APB-like, and LTA4H. Residues common to all of these aminopeptidases are *shaded gray*. The alignment was performed using ClustalX (version 1.81).

SMART program (smart.embl-heidelberg.de) as an ARM repeat domain. This domain was first recognized in the *Drosophila* segment polarity protein known as Armadillo, but it is also present in other proteins such as β-catenin and the tumor suppressor APC (adenomatous polyposis coli) (53–57). Interestingly, detailed structural analysis of all members of the M1 family of aminopeptidases demonstrated that a similar ARM repeat fold is also predicted in the equivalent regions of APB, APB-L, and LTA4H (Fig. 2). In this latter case, the occurrence of this fold has been confirmed by analysis of the three-dimensional structure of the enzyme (52).

Further comparative analysis of the catalytic domain of AP-O revealed a low overall similarity with other members of the family, the highest percentage of identities being with APB and LTA4H (25 and 21%, respectively) (Fig. 2). Nevertheless, and despite this low level of similarity with other gluzincins, the AP-O catalytic domain contains a series of structural features characteristic of this family of enzymes. Thus, it contains the archetypal metalloprotease zinc-binding site ending in a family-specific Glu residue (HEXXHX_{18}E) that acts as the third zinc ligand (Figs. 1 and 2). A series of residues around this zinc-binding site are also conserved in AP-O with respect to those of other aminopeptidases. These conserved amino acids include a Glu residue seven residues downstream of the third zinc ligand that is proposed as contributing to subsite interactions of M1 aminopeptidases with basic substrates (58) (Fig. 2). By contrast, the G(G/A)MENP motif present in the catalytic domain of aminopeptidases (59) is not well conserved in AP-O. The equivalent sequence in AP-O is LGMASP, which lacks the conserved glutamic acid residue reported to be important for the activity of these enzymes (59–62). On the other hand, the catalytic domain of AP-O contains a conserved Tyr at the position 595, included in a YXKG box, which has been implicated in transition state stabilization (58, 60, 63, 64). It is also remarkable that all of the structural features of aminopeptidases found in human AP-O are also present in the sequences deduced for its mouse and rat orthologues, which exhibit 80 and 76% identities with the human enzyme (EMBL accession numbers AJ810420 and AJ810421). Interestingly, no AP-O orthologous sequences were found in *Caenorhabditis elegans* or *Drosophila melanogaster*, suggesting that AP-O may be a protease specific to vertebrates.

It is noteworthy that AP-O lacks any recognizable signal sequence or type II transmembrane domain characteristic of other enzymes of the M1 family of aminopeptidases such as oxytocinases (PLAP, the puromycin-insensitive, leucyl-specific aminopeptidase, and the leukocyte-derived arginine aminopeptidase), the thyrotropin-releasing, hormone-degrading ectoenzyme, the puromycin-sensitive aminopeptidase, aminopeptidase A, and APN. Therefore, we suggest that this novel enzyme is a protease with a domain organization similar to that of other family members such as APB, APB-L, and LTA4H. To further explore the structural and evolutionary relationships between AP-O and other members of the M1 aminopeptidase family, we performed a computational phylogenetic tree analysis (Fig. 3). According to this phylogenetic analysis, together with the exclusive presence of a C-terminal ARM repeat fold in these aminopeptidases, we suggest that APB, APB-L, LTA4H, and AP-O form a defined subfamily of M1 metalloproteases that we propose to call ARM aminopeptidases.

Analysis of Aminopeptidase O Distribution in Human Tissues—To investigate the presence of AP-O mRNA transcripts in human tissues, Northern blots containing poly(A^+) RNAs prepared from a variety of human tissues (leukocytes, colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, liver, lung, placenta, brain, and heart) were hybridized with a specific probe for human AP-O. As can be seen in Fig. 4*A*, AP-O mRNA transcripts are present at low levels in several tissues, being predominantly detected in the pancreas, placenta, liver, testis, and heart. Nevertheless, reverse transcription PCR analysis also revealed the expression of AP-O in a number of additional tissues such as brain, lung, and kidney (data not shown). The two mRNA transcripts of 7.5 and 5 kb observed in some tissues are likely derived from alternative splicing events reported previously to occur in other aminopeptidases (28, 29, 33, 65). The detection of AP-O expression in the heart suggests a potential role for this enzyme in the processing of bioactive peptides that regulate cardiac muscle physiology. Likewise, the expression in testis also suggests that this aminopeptidase could be implicated in reproductive functions as described previously for other family members (42). To further study the putative implication of AP-O in testis development, we analyzed the expression of this gene in samples

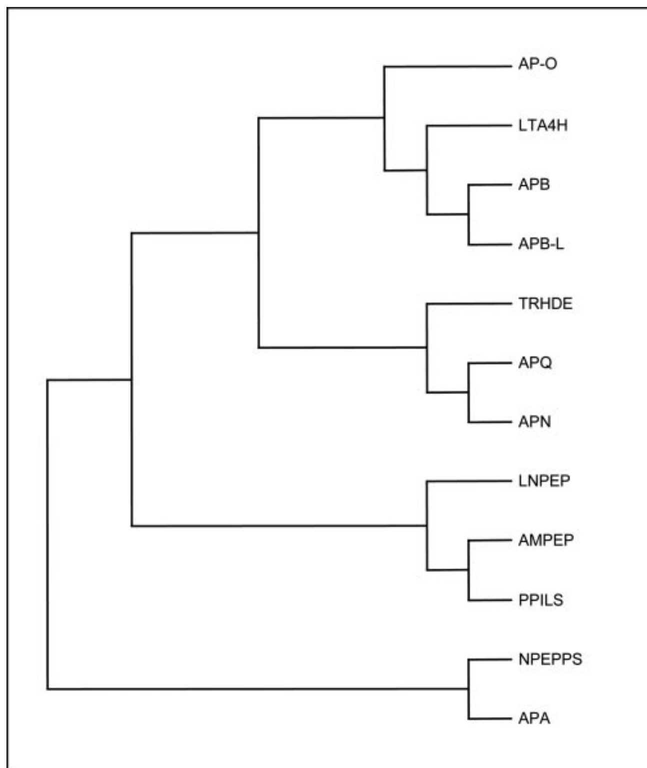

FIG. 3. **Phylogenetic relationships of AP-O and other M1 aminopeptidases.** The sequence of AP-O was aligned to the sequences of the other members of the aminopeptidase family to deduce their phylogenetic relationship. The tree was calculated with the Protpars program of the Phylip program package (version 3.6). *APA*, aminopeptidase A; *APQ*, aminopeptidase Q; *LNPEP*, placental leucine aminopeptidase; *NPEPPS*, puromycin-sensitive aminopeptidase; *PPILS*, puromycin-insensitive, leucyl-specific aminopeptidase; *TRHDE*, thyrotropin-releasing, hormone-degrading ectoenzyme.

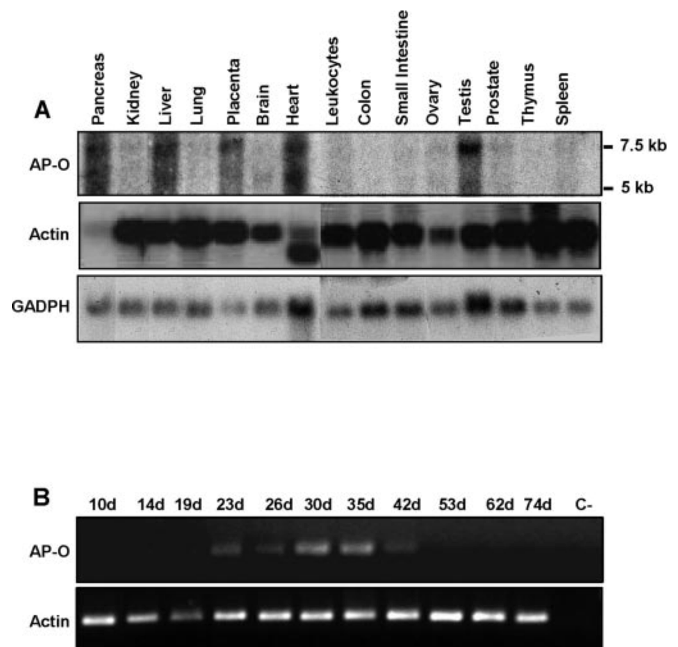

FIG. 4. **Analysis of AP-O expression.** *A*, filters containing ~2 μg of polyadenylated RNAs from human adult tissues were hybridized with a human AP-O-specific probe. Probes for β-actin and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) were used to assess RNA integrity and equal loading. RNA sizes are indicated in kilobase values. *B*, reverse transcription PCR analysis of testis samples from mice of different ages using specific AP-O primers (*d*, day).

from mice of different ages (from 8 to 74 days). As shown in Fig. 4*B*, AP-O expression was detected in samples from day 23 to day 42, reaching a peak in testis from 30–35-day old mice. This peak of AP-O expression overlaps with the period when mice reach sexual maturity.

Enzymatic Activity of the Recombinant Human Aminopeptidase O—To investigate the enzymatic activity of human AP-O, we produced in *E. coli* a fusion protein containing the putative catalytic domain of the enzyme linked to GST at the N terminus. After isopropyl-1-thio-β-D-galactopyranoside induction of bacterial cells transformed with the plasmid encoding the fusion protein, a band of the expected size (70 kDa) was detected by Western blot analysis of protein extracts with antibodies against GST (Fig. 5*A*). This recombinant GST-aminopeptidase protein was contained in insoluble inclusion bodies, which were solubilized, refolded, and purified by affinity chromatography as described under "Experimental Procedures." As can be seen in Fig. 5*A*, two bands of ~70 and ~30 kDa were detected with anti-GST antibodies in both bacterial extracts and purified recombinant AP-O. To assess the identity of the proteins in these bands, they were digested with trypsin and analyzed by mass spectrometry. The obtained spectra confirmed that the 70-kDa band corresponded to the GST-AP-O fusion protein, whereas the 30-kDa band corresponded to a processed GST-AP-O protein lacking the catalytic site of this enzyme (data not shown).

The recombinant human AP-O was then used in enzymatic assays with fluorescent peptides commonly employed for assaying other aminopeptidases. These assays showed that recombinant AP-O exhibits a significant proteolytic activity against Arg-AMC and, to a lesser extent, against Asn-AMC (Fig. 5*B*). To further characterize this activity, we performed a kinetic analysis of the Arg-AMC proteolysis catalyzed by AP-O (Fig. 5*C*). The fitting of the resulting data to the Michaelis-Menten equation yielded k_{cat} and K_m values of 4×10^{-4} s^{-1} and 6 μM, respectively. Similar experiments with purified APN and PAP showed higher k_{cat} (9 and 7 s^{-1}, respectively) and K_m (140 and 210 μM) values. We then tested the ability of different protease inhibitors to block the enzymatic activity of AP-O. This activity was strongly inhibited by *o*-phenanthroline, a metalloprotease inhibitor, but not by 4-(2-aminoethyl)-benzenesulfonyl fluoride and E-64 (Fig. 5*D*), which are common inhibitors of serine- and cysteine proteases, respectively. Furthermore, AP-O activity was significantly inhibited by arphamenine A, which is a potent inhibitor of aminopeptidases such as APB and LTA4H (66–68), thereby reinforcing the proposal that this new enzyme belongs to this family of metalloproteases.

The expression of the AP-O gene in heart and testis prompted us to evaluate the possibility that this aminopeptidase could participate in the proteolytic processing of peptides involved in the regulation of cardiac and male reproductive physiology (5, 69). To this purpose, we incubated angiotensins I, II, and III with 2.5 nM recombinant AP-O or the commercially available aminopeptidases APN and PAP (2.5 pM), and the resulting fragments were analyzed by mass spectrometry. As shown in Fig. 6*A*, angiotensin III was detected by this method as a 932-Da peak, corresponding to the sequence RVYIHPF. By contrast, incubation with AP-O or APN produced an additional 775-Da peak corresponding to angiotensin IV (VYIHPF) (Fig. 6, *B* and *C*, respectively), although the AP-O-catalyzed reaction was significantly slower. However, PAP failed to hydrolyze angiotensin III (data not shown). Similar experiments using angiotensins I and II yielded no additional peaks when incubated with AP-O (data not shown).

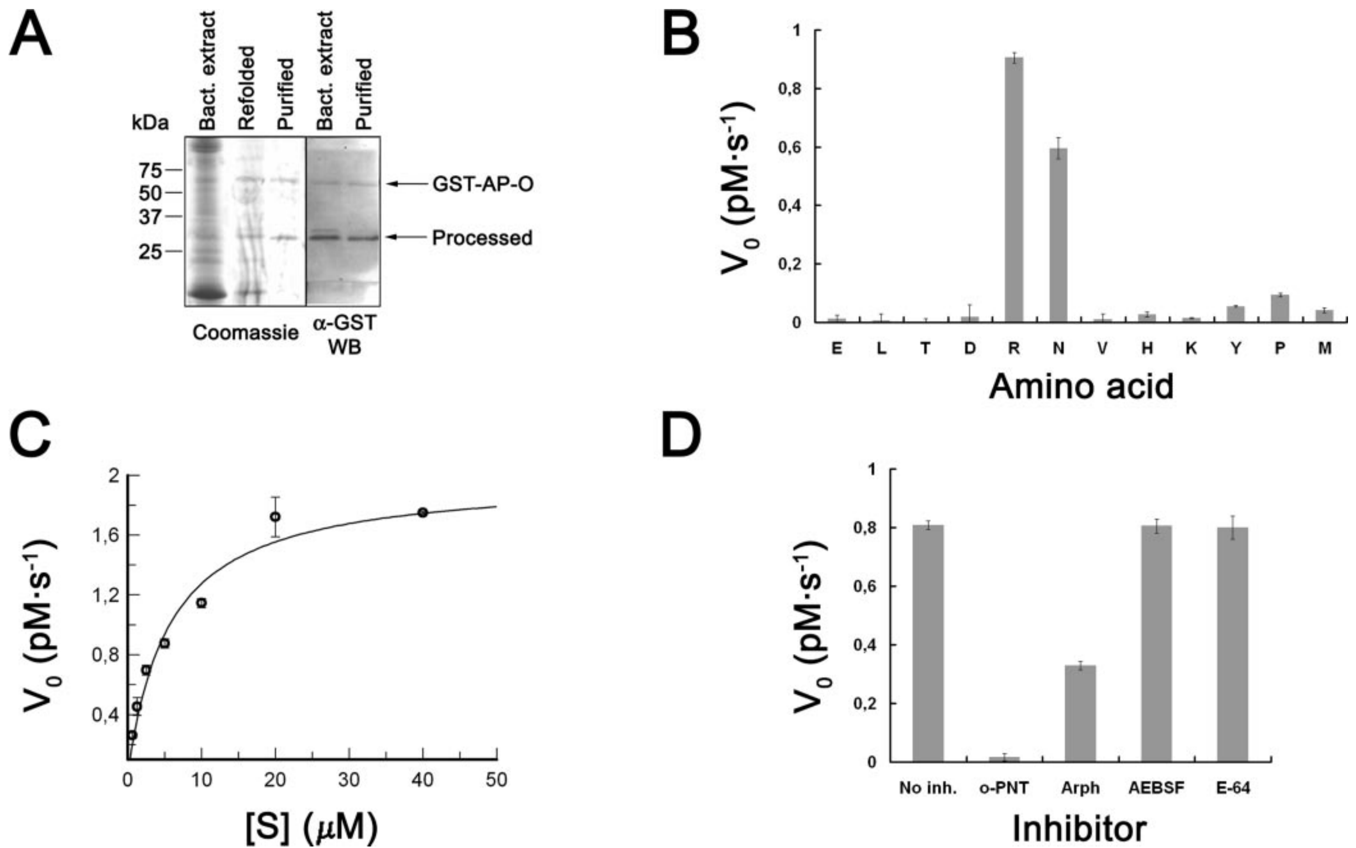

FIG. 5. **Production and enzymatic analysis of recombinant human AP-O.** *A*, a Coomassie Blue-stained SDS-PAGE and a Western blot show the results of the expression, refolding, and purification of the recombinant catalytic domain of human AP-O. 5-μl aliquots of the insoluble fraction of the lysate (*Bact. extract*), the refolded supernatant (*Refolded*), and the refolded purified AP-O (*Purified*) were analyzed in each *lane*. The two *far right lanes* correspond to the Western blot analysis of bacterial pellet and purified AP-O using anti-GST antibodies. The sizes of the molecular size markers are indicated on the *left*. The *upper arrow* on the *right* indicates the position of the fusion protein GST-AP-O. *B,* analysis of the hydrolyzing activity of AP-O using different AMC-bound amino acids. The initial velocity of each reaction (V_0) is represented. *C*, kinetic analysis of the AP-O-catalyzed Arg-AMC proteolysis. Different concentrations of Arg-AMC were incubated with refolded AP-O, and the resulting data were fitted to the Michaelis-Menten equation. *D*, AP-O sensitivity to different inhibitors. Recombinant GST-AP-O was incubated alone (*No inh.*) or in the presence of *o*-phenanthroline (*o-PNT*), arphamenine A (*Arph*), 4-(2-aminoethyl)-benzenesulfonyl fluoride (*AEBSF*), and E-64, and the remaining activity was measured using Arg-AMC as the substrate.

DISCUSSION

In this work we describe the finding in human tissues of a new member of the M1 zinc aminopeptidase family that we have called aminopeptidase O or AP-O. The strategy followed to identify human AP-O was first based on a genomic search of sequences with similarity to those encoding the catalytic domain of known aminopeptidases such as aminopeptidase A, APN, APB, or LTA4H. After the identification of candidate sequences in the human genome and a series of PCR amplification experiments using a cDNA brain library as template, a full-length cDNA coding for human AP-O was finally isolated, cloned, and characterized. Detailed structural analysis of the identified sequence revealed that this protein shows a domain organization characterized by an N-terminal region similar to that present in LTA4H, namely a large catalytic domain, a short SH3 recognition sequence domain, and a C-terminal extension possessing an ARM repeat fold. According to the three-dimensional structural analysis of LTA4H, the N-terminal domain of this enzyme contains a large concave surface exposed to the solvent that could participate in the recognition of specific substrates (52). On the basis of the observed sequence similarity between LTA4H and AP-O at this region, we suggest that the equivalent domain in AP-O plays a similar role. This domain is followed by a typical catalytic domain of the M1-family of zinc-dependent metalloprotease or gluzincins as assessed by the glutamic acid residue located 18 residues C-terminal to the archetypal zinc-binding motif (HE*XX*HX$_{18}$E). This catalytic domain also contains most of the conserved residues in aminopeptidases that have been linked to substrate recognition and catalysis but lacks specific amino acids such as the Glu residue in the G(G/A)MENP motif, proposed to be important for the catalytic activity of these enzymes (59–62). The catalytic domain of AP-O is followed by a hydrophobic loop that resembles an SH3 domain recognition sequence found in a variety of intracellular or membrane-associated proteins (51). This domain could mediate the putative binding of AP-O with the Pro-rich motifs present in other proteins. Finally, the C-terminal domain of AP-O has been predicted to have an ARM repeat fold. This fold is characterized by a three-dimensional structure containing two layers of parallel α-helices arranged in an antiparallel manner with perpendicular loops containing short helical segments on top (56, 57). This unusual coil of helices seems to be suitable for protein-protein interactions, although it is smaller in aminopeptidases than in other ARM repeat-containing proteins such as Armadillo or HEAT repeats (52). The presence of this ARM domain in AP-O as well as in other aminopeptidases such as LTA4H, APB, and APB-like have prompted us to group these proteins in a novel subfamily of M1-proteases that we have called ARM aminopeptidases.

In this work we have also analyzed the distribution of AP-O in human and mouse tissues. These studies allowed us to detect the expression of the *AP-O* gene in several tissues, mainly in the pancreas, placenta, liver, heart, and testis. This pattern of expression suggests that AP-O is implicated in the develop-

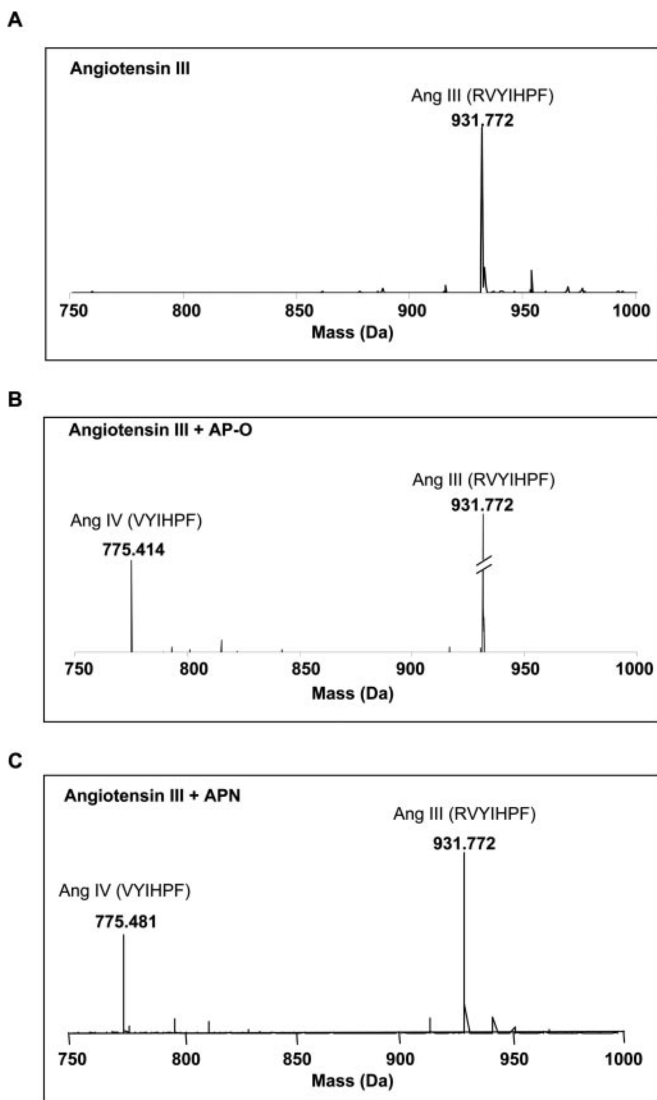

FIG. 6. **Mass spectrometry analysis of the angiotensin III proteolysis catalyzed by AP-O.** Human angiotensin III was incubated alone (*A*) or in the presence of 2.5 nM recombinant GST-AP-O (*B*) or 2.5 pM APN (*C*) for 24 h. The resulting peptide mix was analyzed by mass spectrometry. The peaks corresponding to angiotensin III (*Ang III*) (931.8 Da) and angiotensin IV (*Ang IV*) (775.4 Da) are shown.

ment or physiology of these organs. Interestingly, expression analysis of AP-O in testes from mice of different ages showed a peak of expression in samples from 30–35-day old mice during the sexual maturation stage. This result supports the putative implication of this novel enzyme in testis development as described for APB (42, 70, 71).

To further explore the physiological implications of AP-O, we performed a functional analysis of the recombinant enzyme produced in *E. coli*. This analysis revealed that this protein is a catalytically active aminopeptidase and that this activity is abolished by both general and specific aminopeptidase inhibitors. Remarkably, the preferred substrate of AP-O in these studies was Arg-AMC. This preference for N-terminal Arg residues is similar to that of other aminopeptidases such as APB (39, 46, 68). To further characterize the specificity of AP-O for Arg-AMC, we performed parallel studies with other commercially available aminopeptidases. These studies showed that APN and PAP can also cleave Arg-AMC, although, unlike AP-O, they also cleave Lys-AMC to a similar extent (Fig. 5*B* and data not shown). Interestingly, the K_m value was ~100 times lower in the AP-O-catalyzed Arg-AMC cleavage than in the APN- or PAP-catalyzed reactions. This result would support the possibility that the cleavage of N-terminal Arg residues catalyzed by AP-O could also be of *in vivo* relevance. This possibility, together with the expression of the AP-O gene in the heart and testis, tissues in which the renin-angiotensin system plays important roles (5, 69), prompted us to investigate the putative involvement of AP-O in the processing of peptides of this system. The renin-angiotensin system regulates the homeostasis, blood pressure, endocrine processes, and behavior at the systemic level as well as at the local tissue level (5, 69). Angiotensin peptides derive from a precursor, called angiotensinogen, which is converted to angiotensin I and II by the successive actions of the endopeptidases renin and ACE (angiotensin-converting enzyme). Angiotensin II is an octapeptide involved in homeostasis and hemodynamic processes (72). An aminopeptidase activity, possibly aminopeptidase A (5, 73), converts angiotensin II to angiotensin III, a heptapeptide important in brain and cardiovascular physiology (74). Finally, angiotensin III is converted to angiotensin IV by another aminopeptidase activity (73, 74). Consistent with the specificity of AP-O, this enzyme cleaved the N-terminal residue of angiotensin III *in vitro*, but not that of angiotensin I or II. This finding opens the possibility that AP-O belongs to the group of angiogenic enzymes that play an important role in the biological processes depending on the generation of angiotensin IV in tissues such as brain, testis, and heart (73, 75). However, it must be noticed that AP-O assays were performed with the recombinant catalytic domain of this enzyme, whereas APN and PAP were purified from a natural source. This difference could explain the low k_{cat} value obtained in the AP-O-catalyzed Arg-AMC cleavage, as well as its slow angiotensin III hydrolyzing activity. Thus, further experiments with purified AP-O are required to confirm the putative activity of this enzyme under physiological conditions.

Additionally, AP-O might also process angiogenic peptides as reported previously for other M1 aminopeptidases and have a role in tumor progression (11, 12, 76–78). In this regard, the gene encoding AP-O maps at chromosome 9q22, a region associated with loss of heterozygosity in different malignant tumors including ovarian, bladder, basal cell, and esophageal carcinomas (79–82). Furthermore, 9q22 amplification is associated with cisplatin resistance of human male germ cell tumors (83), whereas a subset of familial colorectal neoplasia kindreds has been linked to 9q22 (84). Unidentified loci located at the region containing AP-O have also been linked to cases of Alzheimer disease (85), schizophrenia (86), and diabetes (87). Finally, duplication of this region leads to learning disability and pyloric stenosis (88). Further studies will be required to ascertain if AP-O could be a direct target of any of these genetic abnormalities resulting in cancer or other pathological conditions.

In summary, we have cloned and characterized AP-O, a novel M1 aminopeptidase that shows a structural organization similar to other members of this group but also shows some structural peculiarities. Notably, we have identified orthologous sequences in mouse and rat, but not in *C. elegans* or *D. melanogaster*, which contain orthologous genes for the remaining members of this aminopeptidase family. We have also shown that AP-O is a proteolytically active enzyme with a profile of activity and sensitivity to inhibitors characteristic of metallopeptidases. The expression of the *AP-O* gene is detected at significant levels in diverse tissues including pancreas, placenta, liver, testis, and heart. Further experimental work, including the generation of mutant mice deficient in this protease, will be necessary to clarify the role of AP-O in physiological processes.

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