Aminopeptidase-A. I. cDNA cloning and expression and localization in rat tissues

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Troyanovskaya, Marta, Gomathi J ayaraman, Lijun Song, and Dennis P. Healy. Aminopeptidase-A. I. cDNA cloning and expression and localization in rat tissues. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R413–R424, 2000.—Aminopeptidase-A (APA) is an ectoenzyme that selectively hydrolyzes acidic residues from the amino terminus of oligopeptides, including biologically active [Asp1]ANG II and [Asp1]CCK-8. We sought to characterize rat APA by cDNA cloning and expression and to determine its tissue distribution by in situ hybridization and immunohistochemistry. Sequence analysis of overlapping cDNA clones isolated from rat kidney cDNA libraries indicated that the full-length cDNA encoded a 945-amino acid protein with a predicted molecular mass of 108 kDa; the size was confirmed by in vitro translation of a full-length cDNA construct. Transient transfection of the full-length cDNA construct in mammalian cells yielded a protein ~140 kDa in size, a size that agrees with the immunoblots of APA from rat tissue and is consistent with APA being known as a glycosylated protein. Tissue APA activity and mRNA expression were highest in the kidney and ileum. Localization of APA by in situ hybridization and immunohistochemistry indicated that, with the exception of the kidney and ileum, where APA was localized to the luminal brush border of proximal tubules and enterocytes, respectively, APA was associated with either capillaries or the lining of sinusoids. Areas known to be physiological targets for ANG II, including glomeruli, the zona glomerulosa, and anterior pituitary, had high levels of APA. The localization pattern suggests that APA may subserve multiple functions, i.e., a generalized role in peptide scavenging and perhaps a more specific role in metabolism of circulating or locally produced ANG II or CCK-8.

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phage was obtained. The cDNA inserts were then rescued from the Uni-Zap phage according to the manufacturer’s protocol using the R408 helper phage. The sizes and sequence of the inserts were determined by digestion of the individual plasmids with EcoR I and Xho I followed by gel electrophoresis and sequence analysis, respectively.

Initial screening resulted in isolation of several large clones (Fig. 1A, clones K1 and K2) whose sequence terminated in an open reading frame. A second rat kidney cDNA library in lambda gt10 that had been primed with random primers was kindly provided by Dr. Kevin Lynch (Charlottesville, VA) and screened as described above. cDNA inserts were digested directly from the lambda gt10 phage and subcloned into the EcoR I site of pBluescript.

5’-RACE. Rapid amplification of cDNA ends (5’-RACE) was performed according to the manufacturer’s recommendations (GIBCO-BRL, Grand Island, NY) to obtain sequence information on the 5’portion of the APA cDNA. First-strand cDNA synthesis of total rat kidney RNA was performed using an antisense primer (RACE-2, 5’-TGTTCAAGGGATACACACT-3’) based on sequence from the partial cDNA clone K5 (Fig. 1A) and Superscript II RT. An anchor sequence was then added to the 3’end of the single-stranded cDNA using TdT and dCTP. PCR was then done using an anchor primer (GIBCO-BRL) and a nested antisense primer (RACE-3, 5’-AGCTCCGGCAGCTTGGTGATC-3’ and Taq polymerase (Perkin-Elmer, Norwalk, CT). The PCR product consisted of a single major band of ~600 bp (data not shown). The PCR product was subcloned into a TA cloning vector (Invitrogen) and used to transform bacteria. Individual colonies were selected, and the largest inserts were analyzed.

APA expression vector. A full-length cDNA expression vector for APA was constructed from partially overlapping cDNA clones (Fig. 1B). Plasmids (pBluescript, Stratagene) containing two clones isolated from the rat kidney cDNA library (pBlueK1 and pBlueK5) and the 5’-RACE product in the TA cloning vector (pCR-5’) were digested with appropriate restriction enzymes (Fig. 1B) and ligated into the EcoR I and Xho I sites of the pcDNA3 (Invitrogen, Carlsbad, CA) expression vector. The full-length clone in pcDNA3 (pcDNA-APA) was fully sequenced to confirm that proper ligation had been obtained.

Transfection. Transient transfection of pcDNA-APA into mammalian cells was performed essentially as reported previously (8). Briefly, HEK-293 or CHO cells were grown to 50% confluence and transfected with the pcDNA3-APA expression vector using the Lipofectamine reagent (GIBCO-BRL) method. Control transfections consisted of the pcDNA-3 vector alone. Transfections were done in serum-free and antibiotic-free medium according to the manufacturer’s protocol. One day before transfection, confluent cells grown in 15-cm petri dishes were split 1:3 and plated 4–6 × 106 cells per 15-cm dish and grown overnight. For each transfection, 20 µg of DNA diluted in 1.8 ml of DMEM and 180 µl of Lipofectamine diluted in 1.8 ml of DMEM were combined, mixed gently, and incubated in RT for 10–15 min. After the incubation, 14.5 ml of DMEM was added and the mixture was overlayed onto the cells prewashed with DMEM. Cells were incubated overnight with DNA-Lipofectamine mixture. The next day the DNA-containing medium was replaced with normal growth medium containing FBS and antibiotics. The cells were either harvested 2 days later for immunoblotting or APA activity measured in situ within the wells.

In vitro translation. In vitro translation of the APA was performed using a coupled transcription/translation system (TNT Lysate System, Promega, Madison, WI). Briefly, the pcDNA3-APA vector (1 µg) was added to rabbit reticulocyte lysate containing T7 RNA polymerase, an amino acid mixture (1 mM) minus methionine, [35S]methionine (sp act 10 mCi/ml, NEN), and RNAsin (RNase inhibitor, 40 U/µl, Boehringer Mannheim, Indianapolis, IN) and incubated for 2 h at 30°C. An aliquot of reaction mix was diluted with sample buffer (2 ml glycerol, 2 ml 10% SDS, 0.25 mg bromophenol blue, 2.5 ml stacking gel buffer, 0.5 ml β-mercaptoethanol) and separated on a 7% SDS-PAGE gel at 180 V for 2.5 h. The gel was then dried and exposed to X-ray film.

Immunoblots. Immunoblots of HEK cells transfected with the APA expression vector (pcDNA-APA) were conducted essentially as previously reported for immunoblots of rat tissues using the enhanced chemiluminescence (ECL) method (Amerham Pharmacia Biotech, Piscataway, NJ) (22). Briefly, after transient transfection, cells were harvested with lysis buffer, samples were mixed with loading buffer, and 10–25 µg protein were separated on a 10% SDS-PAGE gel. The protein was then transferred to nylon membranes at 70 V for 50 min. The membranes were washed with transfer buffer and incubated with primary antibody at a dilution of 1:1,000 in PBS buffer containing 2% BSA. The next day, the membranes were washed 3 × 5 min with PBS buffer at room temperature and then incubated with secondary antibody (1:3,000) in PBS buffer containing 3% BSA for 60 min. The membranes were then washed 3 × 5 min in PBS buffer, incubated with ECL solution, and exposed to film for 5 min.

Fig. 1. Schematic drawing of rat kidney aminopeptidase-A (APA) cDNA and construction of APA expression vector. A: at top is a schematic of APA cDNA with coding region shown as a thick line and 5’- and 3’-untranslated regions shown as thin lines. Also shown are locations of major restriction sites. Bottom shows partial cDNAs that were isolated by library screening and rapid amplification of cDNA ends (5’-RACE) product. B: strategy for construction of APA expression vector. E, EcoR I; K, Kpn I; N, Nar I; S, SacI; X, Xho I.
Enzyme activity. Because APA is an ectoenzyme, APA activity can be measured in cultured cells within the culture dish without disruption of the cells. Similarly, for cells either transiently or stably transfected with APA, APA expression can be measured within the well. APA was measured in situ as described previously for membrane preparations (25) using a-glutamyl-2-naphthylamide (Bachem Bioscience, Philadelphia, PA) as substrate. Enzyme specific activity was expressed as nanomoles per milligram per hour per well.

Northern blot analysis. Northern blot analysis of total RNA from various tissues with a partial APA cDNA probe was conducted as previously described for rat kidney RNA (25). Briefly, tissue total RNA was isolated using the method of Chomczynski and Sacchi (4). Ten micrograms of total RNA was run on a denaturing 1% formaldehyde-agarose gel and transferred to nitrocellulose membranes (Nitropure, MSI Transfer Membrane, Fisher Scientific, Pittsburgh, PA). The membranes were then baked for 2 h at 80°C. The blots were hybridized overnight in hybridization buffer (25 mM KPO4, pH 7.4, 5x SSC, 5x Denhardt’s solution, 50 µg/ml salmon sperm DNA, 50% formamide, and 10% dextran sulfate). The next day, the membranes were washed two times for 15 min in 1x SSC plus 0.1% SDS at room temperature followed by two 15-min washes in 0.25x SSC plus 0.1% SDS at room temperature. The membranes were then wrapped in plastic wrap and exposed to X-ray film.

In situ hybridization. In situ hybridization of rat APA was performed identically as reported previously (25). Briefly, male Sprague-Dawley rats (175–200 g) were decapitated, and tissues were removed and frozen immediately on dry ice. Cryostat sections (10 µm) were placed on microscope slides and fixed with 3% paraformaldehyde in phosphate buffer for 5 min, dehydrated, and vacuum dried. An 35S-labeled riboprobe, in either the antisense or sense orientation (control) to the APA mRNA, was transcribed in vitro using a partial APA probe, in either the antisense or sense orientation (control) to the APA mRNA, was transcribed in vitro using a partial APA cDNA as template. Sections were prehybridized for 2 h at 50°C in a 1:1 mixture of formamide and prehybridization buffer, containing 0.6 M NaCl, 20 mM PIPES buffer (pH 6.7), 1x Denhardt’s solution, 1 mM EDTA, 500 µg/ml yeast total RNA, 50 µg/ml yeast tRNA, 1 mg/ml salmon sperm DNA, and 500 µg/ml sodium pyrophosphate. Hybridization buffer was identical to prehybridization buffer, except that it contained 2.5 M α-thiododecylphosphates and 200 mM diithiothreitol (Sigma). Slides were incubated overnight at 50°C. The next day, the sections were washed in 10 mM Tris·HCl (pH 8), 0.3 M NaCl, 0.1% sodium pyrophosphate, and 10 mM dithiothreitol and then treated with RNase (30 µg/ml) for 30 min at room temperature in 0.3 M NaCl, 10 mM Tris·HCl, and 1 mM EDTA. The sections were then washed for 10 min at room temperature in 0.3 M NaCl, 2 mM Tris·HCl, 1 mM EDTA, 0.05% sodium pyrophosphate, and 1 mM dithiothreitol followed by a 3-h wash at 50°C in 0.075 M NaCl, 2 mM Tris·HCl, 1 mM EDTA, and 1 mM dithiothreitol and then overnight with fresh buffer at room temperature. The next day, the sections were vacuum dried and exposed to Kodak XAR film for 1–20 days.

Immunocytochemistry. Immunocytochemical localization of APA was conducted in a similar manner as reported previously (25). Male Sprague-Dawley rats (175–200 g) were anesthetized with pentobarbital sodium (50 mg/kg ip) and perfused transcardially with ~100 ml of PBS (pH 7.4) followed by 250 ml of Zamboni’s fixative (2% paraformaldehyde, 15% picric acid, 83% 0.12 M phosphate buffer, pH 7.4). Tissues were removed and postfixed for 1 wk in fixative. Slide-mounted cryostat sections were incubated overnight at 4°C with diluted immune sera (1:5,000 to 1:10,000) in PBS containing 0.1% Triton X-100 and 0.1% BSA. The sections were then rinsed with PBS and incubated 45 min with biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:222 with PBS-Triton X-100-BSA and then 45 min with streptavidin-horseradish peroxidase conjugate. The peroxidase reaction was developed by treating the sections with a 0.05% solution of 3,3’-diaminobenzidine (Sigma Chemical) containing 0.003% hydrogen peroxide in 50 mM phosphate buffer, pH 7.4, for 5–10 min. Some sections were lightly counterstained with 1% cresyl violet.

RESULTS

A partial cDNA for rat kidney APA (25) was used to screen rat kidney cDNA libraries. A series of overlapping clones (Fig. 1) was isolated, but because the 5’ terminus of each clone was still within an open reading frame it was apparent that the clones did not extend to the translation start site. Antisense primers (RACE-2 and RACE-3; Fig. 2) were constructed from the 5’-most clone and 5’-RACE performed with rat kidney poly(A)+RNA to obtain the 5’ end of the cDNA. A product of ~600 bp was characterized (5’-RACE; Fig. 1A). Sequence analysis indicated that the product extended 200 bp upstream of the translation start site. The 5’-RACE product was then used to rescreen the lambda gt10 rat kidney cDNA library. An additional partial cDNA clone was isolated and found to extend 38 bp beyond the RACE product (5’-clone; Fig. 1A).

When overlapping cDNAs were combined, the full-length cDNA sequence was ~3.6 kb in length and contained an open reading frame that would encode a protein of 945 amino acids with a predicted molecular mass of 108 kDa. Human APA (i.e., BP-1/6C3) has been shown to be 957 amino acids in length and mouse APA 945 amino acids (12, 20, 31). Sequence comparisons indicated that rat APA was 85% identical with human APA and 93% identical with mouse APA (Fig. 3). Hydropathy analysis of rat APA indicated that there was a single hydrophobic domain from position +19 to +40. This is consistent with the wild-type protein known to be a type II membrane-associated protein with the amino terminus inside the cell and the catalytic domain contained within the large extracellular portion of the protein. The 5‘-untranslated region (UTR) contains an ATG at position 209 followed by an in-frame TGA stop codon at position 221. The ATG at position 239 initiated the longest open reading frame of 945 amino acids and is the presumptive start codon. APA has been characterized as being a metalloenzyme with Zn2+ in the catalytic site (29). The signature Zn-binding motif HEV/LHx5E was shown previously to be essential for catalytic activity of human APA (28), and the amino acid sequence surrounding this region was conserved in all three species (Fig. 3). There were 12 possible N-linked glycosylation sites within the extracellular domain, of which six were conserved in rat, mouse, and human sequences. There were nine Cys residues within the rat APA sequence and seven within the extracellular domain, and, of these, all nine were conserved in all three species (Fig. 3).
Fig. 2. Complete cDNA sequence of rat kidney APA. A: nucleotide sequence 1 to 1768. B: nucleotide sequence 1769 to 3639. Shown is cDNA sequence with first nucleotide numbered +1. Below the nucleotide sequence is the predicted amino acid sequence of the protein. Boxed amino acid sequence from 19–40 represents single hydrophobic transmembrane-spanning domain. Shaded box represents Zn$^{2+}$ binding domain with core sequence HELVHX$_n$E. Irregular clear box represents sequence of previously characterized partial cDNA (25). Areas of sequence from which antisense primers were made for 5$'$-RACE are noted (RACE-2 and RACE-3) as is 5$'$ end of 5$'$-RACE product (position relative to the isolated cDNA clone beginning at +1). The 5$'$-untranslated region start codon at position 209 is shown by thick underlining (A), and 3$'$-untranslated region RNA instability element (AUUUA) is underlined with a dashed line (B).
Fig. 2—Continued
conserved across the three species. APA exists as a homodimer, and its enzyme activity is inhibited by reducing agents, presumably reflecting an importance of intramolecular or intermolecular disulfide bonds. It is not clear which, if any, of these Cys residues are essential for APA activity. Finally, there are two conserved putative phosphorylation sites within the cytoplasmic domain. The Ser-9 residue is a protein kinase C consensus site, and Tyr-12 is a possible tyrosine kinase substrate site (Fig. 3). Although the BP-1/6C3 differentiation antigen is known to be phosphorylated (31), it is not known which residue(s) is phosphorylated.

The 3'-UTR was ~563 bp in length. There was one RNA destabilization element (ATTTA) at position 3256 but no detectable polyadenylation signals (AATAAA) were seen, although the terminal region was AT rich (63%). Three clones were spliced together and inserted into a mammalian expression vector to form pcDNA-APA (Fig. 1B). The size of the protein encoded by the APA cDNA was determined experimentally by in vitro translation using a rabbit reticulocyte lysate-coupled transcription/translation system. A major product slightly less than 110 kDa was obtained (Fig. 4A). This size is in agreement with the predicted size based on sequence analysis and represents the nonglycosylated form of the enzyme. There was some indication that the in vitro translation product was a doublet (Fig. 4A, inset). To determine the size of the protein expressed in mammalian cells, the expression vector was transiently transfected into HEK-293 cells and immunoblots were performed. There was a single major immunoreactive band of ~140 kDa (Fig. 4B), in close agreement with the size of the APA protein seen in immunoblots of rat...
HEK-293 cell protein was loaded. It cannot be seen in nontransfected cells, even when 2-fold more HEK-293 cells. Analysis of APA after transient transfection of pcDNA-APA into lane 2, lysate plus pcDNA-APA. Lane 3, same as lane 2 but shorter exposure. Protein size markers are shown on left (in kDa). Inset: magnification of predominantly labeled band from lane 3. Note that major product is slightly smaller than 110-kDa markers and with less exposure appears as a doublet. B: Immunoblot analysis of APA after transient transfection of pcDNA-APA into HEK-293 cells. Lane 1, 10 g of HEK-293 cell lysate 48 h after transfection of pcDNA-APA. Lane 2, 20 µg of control HEK-293 cell lysate. Note that single major positive band of ~140 kDa (arrow) cannot be seen in nontransfected cells, even when 2-fold more HEK-293 cell protein was loaded.

APA mRNA was also localized by in situ hybridization. In the kidney, APA mRNA was localized to the outer stripe of the outer medulla that appeared to follow the medullary rays and extend into the cortex (Fig. 7A). In the cortex, labeling appeared punctate. Previous studies using higher resolution autoradiography indicated that this pattern was due to labeling of proximal tubules and glomeruli (25). In the brain (Fig. 7B), APA mRNA was concentrated within the choroid plexus and the ependymal lining. Although less intense, a clearly recognizable pattern of labeling was seen adjacent to the dorsal third ventricle, consistent with labeling of the hypothalamic paraventricular nucleus. The labeling was not seen with tissue hybridized with sense strand probes (Fig. 7B'). The adrenal gland exhibited positive hybridization within the capsular region (Fig. 7C), corresponding to the zona glomerulosa. In the ileum, APA mRNA was very high in the basal portion of villi (Fig. 7D). In the pituitary, APA mRNA was primarily localized to the anterior lobe (Fig. 7E).

The in situ hybridization pattern was then compared with the immunohistochemical staining pattern using antiserum against rat kidney APA. We previously reported that APA in the brain was primarily associated with microvessels and was not associated with neuronal or glial elements (10). This again appeared to be the pattern, but the Zamboni's fixative used here gave better localization to the vascular elements than the previous fixative that was used. As reported previously, heavy staining of vascular elements was seen within the hypothalamic paraventricular nucleus (Fig. 8A). This pattern exactly matches the pattern seen in the in situ hybridized sections (Fig. 7B). High resolution staining of the pattern of microvessel staining that was seen throughout the brain indicated clearly that the cellular elements that were stained were adventitial pericytes (Fig. 8, B and C). At high power, the pericyte cell body and thin processes were positively stained, whereas the endothelial cells lining the vessel wall were devoid of staining. In the heart, the in situ hybridization was nondescript (data not shown), and immunohistochemical staining indicated a fairly uni-
form distribution of staining within the ventricle, with staining restricted to the thin vascular elements, presumably capillaries, intermingled within the microvilli (Fig. 8D). The liver, another organ with only a diffuse signal by in situ hybridization (data not shown), had positive staining within the central vein and the liver sinusoids (Fig. 8E). In the ileum, APA immunoreactivity was intensely rich within the enterocytes lining the lumen, with some impression that immunoreactivity was higher within the proximal portions of the microvilli (Fig. 8F). Within the adrenal gland, heavy APA immunohistochemical staining could be seen within the adrenal capsule (Fig. 8, G and H) and less intense staining within the vascular elements in the adrenal cortex and medulla. Higher resolution indicated that the cortical staining was localized to the lining of the sinusoids. The staining pattern within the pituitary (not shown) was very similar to that seen in the brain, with again, the staining being restricted to small vessels. Whereas the intermediate and posterior lobes were not enriched in RNA and thus appeared negative relative to the anterior pituitary in sections treated for in situ hybridization (Fig. 7E), immunocytochemical staining indicated that vessels were positive for APA immunoreactivity in a nearly identical pattern as seen in the brain. In the anterior lobe, the staining was even more intense but again only seen within the small vascular elements, presumably reflecting staining within capillaries.

**Table 1. APA activity in rat tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Activity</th>
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<tr>
<td>Kidney</td>
<td>684 ± 72</td>
</tr>
<tr>
<td>Ileum</td>
<td>230 ± 19</td>
</tr>
<tr>
<td>Lung</td>
<td>71 ± 3</td>
</tr>
<tr>
<td>Thymus</td>
<td>56 ± 1</td>
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<tr>
<td>Pituitary</td>
<td>47 ± 1</td>
</tr>
<tr>
<td>Liver</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Ventricle</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Adrenal</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Atrium</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>Aorta</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Pancreas</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Brain</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Spleen</td>
<td>4 ± 1</td>
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Data are expressed as means ± SE. Tissue from 4 animals was used for each measurement. Aminopeptidase-A (APA) activity was measured using γ-glutamyl-2-naphthylamide as substrate and is expressed as nmol·mg protein⁻¹·h⁻¹.

**DISCUSSION**

Rat APA is highly similar to both mouse (93%) and human APA (85%). Areas functionally important for APA enzymatic activity were found to be highly conserved in all three species. In particular, APA is a zinc metalloenzyme (29) and the zinc binding domain and the regions immediately surrounding it are completely conserved. Interestingly, all nine Cys residues are conserved across all three species. Although APA is a homodimer and loses activity under reducing conditions, it is not clear whether the disulfide bonds are intramolecular or intermolecular. Mild treatment of the membrane-bound enzyme with papain can solubilize the enzyme activity, presumably by clipping the enzyme near the membrane surface (23). Under nonreducing conditions, the solubilized enzyme complex is smaller but retains activity, indicating that the dimerization does not require the transmembrane domains but not revealing whether the disulfide bonds are intramolecular or intermolecular.

The 5'-UTR contains an ATG at position 209 followed by an in-frame TGA stop codon at position 221 and, if translated, would encode a 4-amino acid peptide. Interestingly, the upstream ATG is contained within a more conserved Kozak consensus site GCCA/GCCATGG than is the downstream start codon for the major open reading frame; compare

**Fig. 6. Northern blot analysis of APA mRNA from rat tissues.** Ten micrograms of total RNA was separated on a formaldehyde-agarose gel and transferred to nitrocellulose membranes and probed with a partial APA cDNA. Single major hybridized band was ~4.1 kb in size based on markers run in parallel (not shown). Kidney RNA was included in most blots for comparative purposes.
Fig. 7. In situ hybridization of APA mRNA. Sections were hybridized with $^{35}$S-labeled antisense riboprobes synthesized in vitro from a partial APA cDNA. Sections were exposed to X-ray film for 2–4 wk, and reverse film images were examined. A: transverse section from rat kidney hybridized with an antisense APA riboprobe. Heavy labeling was seen in outer stripe (os) of outer medulla, and punctate labeling was seen in cortex (c). Inset: lower magnification of an adjacent section incubated with a radiolabeled sense strand APA riboprobe. B: coronal section from rat brain hybridized with an antisense APA riboprobe. Labeling was seen within ependymal (e) lining of brain, choroid plexus (cp), and hypothalamic paraventricular nucleus (pvn). B': same as B except incubated with a radiolabeled sense strand APA riboprobe. Ependyma, choroid plexus, and paraventricular nucleus are not labeled (arrows). C: section of rat adrenal hybridized with an antisense APA riboprobe. Detectable labeling was restricted to capsular region (arrows). C': same as section in C except incubated with a sense strand APA riboprobe. Capsular region is not labeled (arrows). D: section of rat ileum hybridized with an antisense APA riboprobe. Inset: lower magnification of an adjacent section incubated with a sense strand APA riboprobe. E: section of rat pituitary hybridized with an antisense APA riboprobe. Heavy labeling was seen within anterior lobe (a) but not intermediate (i) or posterior (p) lobes. Inset: lower magnification of an adjacent section incubated with a sense strand APA riboprobe.
was no evidence of a doublet and, because APA is glycosylated, there was no indication from the size as to whether the protein contained the additional NH2-terminal amino acids.

Ectoenzymes such as APA are generally considered to be housekeeping enzymes. Lending support to this notion are early studies from renal hypertensive patients (18). It was argued that because ANG II is a substrate for APA, renin-dependent renal hypertensive patients might be expected to have altered levels of APA. However, plasma levels of APA were unchanged, arguing that APA expression was insensitive to changes in ANG II. We reported recently, however, that plasma levels of APA do not accurately reflect what is going on within tissues (9, 22) and because ANG II is synthesized in extrarenal tissues (5), regulation of the half-life of ANG II might play a more important role in regulating ANG II levels than previously appreciated. Specifically, we found that within two-kidney, one-clip hypertensive rats, glomerular APA was increased in both clipped and nonclipped kidneys, whereas tubular APA was diminished (22). Likewise, ANG II infusion increases glomerular APA expression and angiotensin-converting enzyme inhibition decreases APA activity in spontaneously hypertensive rats (9). Thus it seems as if APA can indeed be regulated by ANG II in a cell-specific manner.

As our previous studies on the localization of APA have been conducted primarily within the kidney (25) and brain (10, 23), we extended the study here to localize APA to additional tissues. First of all, Northern blot analysis and direct measurement of APA activity were generally in agreement, with the exception of the lung, where APA activity was measurable but mRNA was not detected. APA mRNA and APA activity were highest within the kidney and ileum. Previously, we
reported that APA within the kidney is heavily localized to the brush border of proximal tubule cells, with lesser staining detected within glomeruli. Both the immunohistochemical staining pattern and the in situ hybridization labeling pattern within glomeruli were consistent with expression within mesangial cells (25), and, indeed, cultured mesangial cells have APA enzyme activity and detectable APA mRNA (27). In the ileum, APA immunohistochemical staining was restricted to the brush border of enterocytes lining the intestinal microvilli. The localization pattern by low resolution in situ hybridization was consistent with this and suggested that the mRNA levels were higher in the proximal portion of the villi than in the distal tips. In general, the high levels of expression of APA in the brush border of both proximal tubule cells and ileal enterocytes are consistent with the role of these tissues in the degradation of proteins and peptides and the reabsorption of essential amino acids for nutritive purposes. These areas are enriched with a battery of proteolytic enzymes, and APA is thought to participate in the degradation of peptides generated from such degradation. Interestingly, APA would not appear to be critical in this function because BP-1/6C3 (i.e., APA)-deficient mice develop normally (14).

We previously reported that in the brain APA was localized primarily to small vascular elements, the choroid plexus, and the ependymal lining of the brain (10). The in situ hybridization was consistent with this pattern of expression. Because APA is so widely distributed, the in situ hybridization pattern was diffuse and only concentrated in structures expressing higher levels, such as the choroid plexus, or in structures more heavily vascularized, such as the paraventricular nucleus of the hypothalamus (compare Figs. 7B and 8A). Whereas in previous studies based on the pattern of staining we argued that the distribution of APA within microvessels within the brain was consistent with localization to adventitial pericytes, here, using an alternative fixation approach, the staining evidence is more convincing in this regard. Quite clearly, the positive-stained cells are on the outer surface of the vessels and can be distinguished from the endothelial cells lining the lumen (see Fig. 8C). Interestingly, pericytes and renal mesangial cells are thought to have similar origins (21) and it is thus consistent that both cell types express APA. Moreover, as we demonstrated that pericytes readily take up large molecules such as horseradish peroxidase (10), the localization of APA to the adventitial lining of cerebral capillaries would suggest that APA plays a protective role as part of an enzymatic component of the blood-brain barrier. Interestingly, Alliot et al. (2) recently reported using electron microscopy that immunoreactive APA is localized on the abluminal surface (i.e., facing the interstitial space) of brain pericytes. Thus APA may be preferentially positioned to metabolize peptides arising from the brain parenchyma rather than from the circulation. This may explain why immunostaining of microvessels for APA is enriched in brain areas that also contain ANG II immunoreactive neurons, areas such as the hypothalamic paraventricular nucleus (10). We have also shown that an APA inhibitor or APA antiserum with anticytotoxic activity administered within the cerebroventricular space inhibits the actions of intracebroventricularly administered ANG II (24). Together these results suggest that expression of APA on the abluminal surface of brain microvesSEL pericytes may play a functional role in modulating activity of the brain ANG system.

We further localized APA to other tissues. In the heart, ANG II is known to have positive inotropic effects on cardiac function (5). Northern blot analysis indicated cardiac APA mRNA was higher within the ventricular muscle than within atrial muscle. Immunohistochemical localization indicates that this simply might reflect differences in microvessel density between these two areas, because in both atrium (not shown) and ventricle (Fig. 8D), APA was restricted to capillaries or very small caliber arterioles or veins. APA might thus influence the levels of circulating or locally produced ANG II within the heart. In the adrenal gland and liver, APA was associated with the lining of sinusoids. Again, one could speculate that this localization is consistent with either processing or degradation. In addition to sinusoids within the adrenal cortex, APA was very high within the capsule directly adjacent to (or overlapping) the zona glomerulosa. Because blood flow within the adrenal gland is from the superficial cortex to the inner medulla, ANG II reaching the adrenal gland via the circulation would be accessible to high levels of APA. Indeed, this localization pattern is consistent with physiological studies that indicate ANG III is equieffective as ANG II at stimulating aldosterone from the adrenal cortex (7) and may reflect the metabolism of ANG II to ANG III. Finally, the anterior pituitary had high levels of APA mRNA and APA immunoreactivity. ANG II is known to stimulate release of ACTH and prolactin from the anterior pituitary (6). Thus APA may play an important role in regulating ANG II levels within the anterior pituitary, either locally synthesized or from the general circulation or, possibly, the portal circulation, because ANG II immunoreactivity is present in the externa lamina of the median eminence (10).

Although our focus has been primarily on the role of APA in metabolism of ANG II, APA is also the principal enzyme involved in the degradation of CCK-8 (16), the other known biologically active peptide that is a substrate for APA. CCK-8 is a gastrointestinal hormone that stimulates pancreatic secretion, gallbladder contraction, gastric emptying, and induction of satiety (13). Although APA activity or mRNA levels are not high in the pancreas, APA levels are very high in the small intestine, suggesting that APA might be important in regulating CCK-8 levels locally.

Perspectives

APA levels are highest in the brush border of the small intestine and kidney proximal tubules, areas enriched in proteases and peptidases involved in protein/peptide catabolism. However, the widespread distribu-
tion of APA within capillaries and sinusoids suggests a possible role in metabolism of biologically active circulating peptides. In this regard, it is interesting to consider that acidic amino acids are rarely found at the amino terminus of bioactive peptides. The only exceptions that we are aware of are ANG II and CCK-B, both of which contain an Asp residue at their amino termini. It is further interesting to note that the two tissues with the highest levels of APA (i.e., kidney and small intestine) are the principal tissues for ANG II and CCK-B synthesis. Recently we showed that kidney APA levels can be regulated by variations in ANG II levels in a cell-specific manner (9, 22). Given that APA is expressed in tissues that are targets for circulating or locally produced ANG II (i.e., proximal tubules, glomeruli, zona glomerulosa, circumventricular organs, anterior pituitary, etc.), it is reasonable to speculate that ANG II may regulate expression of APA by a receptor-mediated mechanism. Therefore, it is possible to further speculate that modulation of peptidase activity may be yet another way by which biologically active peptides are regulated.

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REFERENCES