The Kidney Is a Major Site of \( \alpha_2 \) -Antiplasmin Production

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Abstract

The serpin \( \alpha_2 \) -antiplasmin (\( \alpha_2 \)-AP) is the major circulating inhibitor of plasmin; it plays a determining role in the regulation of intravascular fibrinolysis. In addition to blood plasma, plasmin formation occurs in various organs where it is thought to fulfill a spectrum of functions not restricted to clot lysis. \( \alpha_2 \)-AP is synthesized by hepatocytes, but other possible sites of production have not been investigated. To explore the potential extravascular contribution of \( \alpha_2 \)-AP in the regulation of proteolysis, we have isolated the murine \( \alpha_2 \)-AP cDNA and determined its mRNA distribution in adult tissues.

In addition to liver, kidneys are major sites of \( \alpha_2 \)-AP mRNA accumulation in the mouse. The transcript is present in epithelial cells lining the convoluted portion of proximal tubules, and its accumulation is under androgen control. Human kidneys also contain high levels of \( \alpha_2 \)-AP mRNA. Moderate amounts of \( \alpha_2 \)-AP mRNA are detected in other murine tissues such as muscle, intestine, central nervous system, and placenta. Our observations indicate that \( \alpha_2 \)-AP can be synthesized in a number of tissues, where it could function as a distal regulator of plasmin-mediated extracellular proteolysis. (J. Clin. Invest. 1996. 97:2478–2484.)

Key words: fibrinolysis • plasmin • plasminogen • proteolysis • serpins

Introduction

\( \alpha_2 \) -Antiplasmin (\( \alpha_2 \)-AP) is a serine-protease inhibitor (serpin) that is regarded as the primary physiological inhibitor of plasmin. Structural and kinetic studies have shown that \( \alpha_2 \)-AP has three functional sites: a plasminogen/plasmin binding site, a reactive site that binds covalently the catalytic serine residue of plasmin, and a cross-linking site to the fibrin \( \alpha \) chain (1–4). \( \alpha_2 \)-AP is abundant in plasma, where it exerts its antifibrinolytic properties by competing with fibrin for plasminogen binding and through plasmin inhibition. The functional importance of \( \alpha_2 \)-AP is illustrated by the rare reported cases of congenital \( \alpha_2 \)-AP deficiency who exhibit severe lifelong hemorrhagic tendency. Hepatocytes are considered to be the site of production of circulating \( \alpha_2 \)-AP. To our knowledge no other cell type has been demonstrated to synthesize the protein; \( \alpha_2 \)-AP released by glioblastoma organ cultures (5) and cultured monocytes (6) may originate from the circulating pool of the protein, and \( \alpha_2 \)-AP in platelet \( \alpha \)-granules (7), like other constituents of these organelles, could have been taken up from plasma.

A wide body of evidence suggests that the role of plasmin may not be limited to intravascular fibrinolysis. Plasmin is a serine protease generated by an enzymatic cascade that is controlled by the combined actions of proteases, plasminogen activators (PAs), and antiproteases, plasminogen activator inhibitors (PAIs) (8). In contrast to PAs, plasmin is a proteolytic enzyme of broad specificity that is capable of catalyzing the degradation not only of fibrin but also, directly or indirectly, of most extracellular proteins. Therefore, plasmin is thought to fulfill a large spectrum of biological functions (8). For instance, plasmin-mediated proteolytic activity is postulated to participate in tissue repair processes (9, 10), to help maintain tubular patency in renal tubules (11, 12), and to be involved in extracellular metabolism in the central nervous system (13, 14).

Therefore, in addition to preventing excessive intravascular fibrin dissolution, plasmin inhibition could influence biological phenomena in multiple tissues. In this view, a more complete understanding of the sites and circumstances of \( \alpha_2 \)-AP production in vivo is mandatory to assess the potential role of this antiprotease in modulating physiological and pathological processes associated with expression of the PA/plasmin system.

We report here the cloning of the murine \( \alpha_2 \)-AP cDNA and show that its RNA transcription is not restricted to hepatocytes. In particular, we identify the kidney as a major source of \( \alpha_2 \)-AP production and demonstrate that testosterone modulates \( \alpha_2 \)-AP mRNA accumulation in epithelial cells lining the convoluted segments of proximal tubules. Similarly, we provide evidence that \( \alpha_2 \)-AP is produced by the human kidney.

Methods

Murine and human tissues. Murine tissues were obtained from NMRI mice killed by cervical dislocation and processed as described elsewhere (12). Normal human kidney samples were obtained from nephrectomy specimens performed for carcinomas and normal liver tissue was obtained from hepatomec- tomy. Macroscopically uninvolved areas were carefully dissected, frozen in liquid nitrogen, and stored at −70°C until analyzed. Tissue integrity was verified by conventional histological evaluation. Serum-free culture supernatant and RNA from human kidney tumor–derived cells, UOK and Ge1, were kindly provided by Dr. P.-Y. Dietrich (Hôpital Universitaire de Genève, Geneva, Switzerland).

Cloning of \( \alpha_2 \)-AP cDNA. Oligonucleotides of degenerate sequences (5’-ATGTAYTNCAARGGNTT-3’ and 5’-TCAAYTRTTNCKCCARA-3’), based on the human \( \alpha_2 \)-AP cDNA sequence (amino acids 166–172 and 240–246) (4), were prepared. We used 1 µg of total murine liver RNA to synthesize cDNA with the oligonucleotide spanning the 3’ region as a primer (15). The mouse \( \alpha_2 \)-AP cDNA was then amplified by PCR, yielding a fragment of 242 bp which was subcloned into pBluescript KS+ vector (Stratagene, La Jolla, CA) and sequenced. This fragment was labeled by random

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1. Abbreviations used in this paper: \( \alpha_2 \)-AP, \( \alpha_2 \)-antiplasmin; PA, plasminogen activator; PAI, plasminogen activator inhibitor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

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priming (16) and further used to screen a lambda-ZAP murine liver cDNA library (Stratagene). Inserts from three positive phage clones were excised from the lambda-ZAP vector according to the instruction manual and sequenced on both strands by the chain termination method using successive primers or exonuclease III/mung bean nuclease unidirectional deleted recombinant plasmids (17). The full-length cDNA-containing plasmid was called pmu $\alpha_2$-AP fl. Nucleic acid and deduced amino acid sequences were compared with EMBL/GenBank and Swissprot databases using the FASTA program (18). The full-length cDNA-containing plasmid was called pmu $\alpha_2$-AP fl. Nucleic acid and deduced amino acid sequences have been deposited in the EMBL data library under accession number Z36774.

Probe preparation and RNA analysis. Complementary and sense RNA probes were synthesized with T3 or T7 RNA polymerases using the HindIII- and BamHI-digested pmu $\alpha_2$-AP plasmid, respectively. This plasmid is derived from one clone of the lambda-ZAP liver cDNA containing a 704-bp cDNA fragment of the murine $\alpha_2$-AP corresponding to bases 199–902 of pmu $\alpha_2$-AP fl. For in situ hybridization the probes were either labeled with $^{33}$PUTP or $^{3}H$UTP and $^{3}H$CTP. Plasmids containing murine urokinase-type PA (uPA) (19) or tissue-type PA (tPA) (20) cDNA sequences were linearized and used as templates for in vitro transcription as described (12). The human $\alpha_2$-AP cDNA was obtained by reverse transcription–PCR of total RNA extracted from normal liver, using the same degenerate oligonucleotides as mentioned above.

Total RNAs were extracted from murine and human tissues according to the guanidium/acid phenol method (21). For Northern blot analyses, 5 μg of total RNA was separated by formaldehyde gel electrophoresis and blotted onto nylon membranes as described (22). RNase protection assays were performed following standard methods (17) with slight modifications. Total RNA, 0.5–2 μg, isolated from murine tissues (21), was hybridized at 45°C to 300,000 cpm (10⁸ cpm/μg) of a 360-base $^{33}$P-labeled murine $\alpha_2$-AP cRNA probe (XmnI-digested $\alpha_2$-AP cDNA fragment subcloned in pBluescriptKS ). The hybridization was carried out in 20 μl of buffer containing 80% formamide, 0.4 M NaCl, 40 mM Pipes, pH 7.4, 2 mM EDTA, and yeast tRNA to make up a final RNA concentration of 0.5 μg/μl. After 4 h of hybridization, 200 μl of 0.3 M NaCl, 20 mM Tris, pH 7.4, 4 mM EDTA, 50 ng/μl RINase A were added to the hybridization mixture and the incubation was continued for 30 min at 37°C. Then, protected fragments were precipitated for 30–60 min at −20°C after addition of 50 μl of a denaturing solution (4 M guanidine-thiocyanate, 2% Na-laurylsarcosinate, 25 mM Na-citrate, pH 7.0, 0.1% β-mercaptoethanol) and 250 μl isopropanol. After centrifugation at 14,000 g for 15 min at 4°C, the pellets were resuspended in 8 μl of RNA-loading buffer (80% formamide, 1 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene-cyanol) and heated at 70°C for 2 min. Samples were loaded on a 16 × 20 cm 8 M urea/6% polyacrylamide gel and electrophoresed at 25 V/cm for 120 min in 1× TBE. After electrophoresis, the gel was fixed with acetic acid/ethanol/water (1:2:7), dried, and exposed to autoradiographic film or phospho-imager screen for counting.

Figure 1. Amino acid sequences of murine, human, and bovine $\alpha_2$-AP. Amino acid sequences deduced from the cDNA sequences of mouse, human, and bovine were aligned. Shading indicates residues conserved in mouse and human and/or bovine $\alpha_2$-AP.

α₂-Antiplasmin in the Kidney 2479
Degenerate primers were prepared on the basis of the human
was labeled with \(\text{[}^{32}\text{P}]dCTP\) and used to screen 0.5-
242-bp fragment
Northern blots of RNAs from murine tissues and recognized
vitro transcription of this 242-bp cDNA was used to probe
strands were sequenced. The nucleic acid sequence showed
analyzed. 5-
4 h in 2 ml of serum-free defined medium. Protease-inhibitor binding
Inhibitor-containing fluids were incubated with
purified human plasmin for 10 min at 37
125
10
32
I-labeled
3
2
C. Plasmin–inhibitor com-
three independent experiments.

In situ hybridizations. Kidneys were obtained from 3–6 mo-old
NMRI male mice, frozen in methyl butane, and kept at \(-80^\circ\text{C}\) until analyzed. 5-\(\mu\text{m}\) cryostat tissue sections were fixed in 1% glutaraldehyde in PBS. The hybridization and posthybridization washes were carried out as described previously (12). Macroscopic mRNA localizations were revealed by film autoradiography (Kodak-SB) whereas microscopic detection was performed by emulsion autoradiography (Kodak-NTB2).

Castration and testosterone injection. Males were anesthetized and castrated as described by Vassalli et al. (23). After 2 wk of recovery, mice were daily injected subcutaneously with 0.5 ml of corn oil alone or containing 100 \(\mu\text{g}\) of testosterone-propionate per gram of body weight. After 5 d, the animals were killed and kidneys, liver, and seminal vesicles were dissected and their RNA was extracted as mentioned above.

Protein analysis. Kidneys from wild-type (C57/B6) and \(\text{uPA-}\text{deficient mice (24) were flushed with 15 ml of serum-free defined medium (25, 26). After dissection, kidneys were minced and cultured for 4 h in 2 ml of serum-free defined medium. Protease-inhibitor binding assays were performed with either diluted human or murine plasma or supernatant of primary kidney cultures. Kidney tissue was also homogenized in PBS buffer containing 0.1% Triton X-100, centrifuged at 800 \(g\) for 5 min, and the supernatant was collected for further processing. Inhibitor-containing fluids were incubated with \(\text{[}^{125}\text{I}]\)labeled purified human plasmin for 10 min at 37\(^{\circ}\)C. Plasmin–inhibitor complexes were immunoprecipitated with either rabbit anti-human \(\alpha_{\text{2}}\)-AP or rabbit anti–human plasminogen, according to standard procedures (17). Samples were subjected to SDS-PAGE in 8% polyacrylamide gels and the gels were analyzed by autoradiography.

Results

Isolation and characterization of murine \(\alpha_{\text{2}}\)-AP cDNA clones. Degenerate primers were prepared on the basis of the human \(\alpha_{\text{2}}\)-AP cDNA sequence, in a region devoid of strong amino acid homology with other serpins; they corresponded to the sequence coding for amino acid residues 166–172 and 240–246 (4, 27). PCR amplification of reverse-transcribed mouse liver RNA yielded the expected 242-bp DNA fragment. This fragment was subcloned into pBluescriptKS\(^+\) plasmid and both strands were sequenced. The nucleic acid sequence showed 80% identity with its human counterpart. The product of in vitro transcription of this 242-bp cDNA was used to probe Northern blots of RNAs from murine tissues and recognized single-size transcripts (data not shown). This 242-bp fragment was labeled with \(\text{[}^{32}\text{P}]dCTP\) and used to screen 0.5 \(\times\) 10\(^6\) pfu of

![Figure 2. Tissue distribution of murine \(\alpha_{\text{2}}\)-AP mRNA. A 1 \(\mu\text{g}\) (liver and kidney) or 10 \(\mu\text{g}\) of total RNA from various murine tissues was analyzed by RNase protection using a murine 360-base \(\alpha_{\text{2}}\)-AP \(\text{[}^{32}\text{P}]\)labeled cRNA probe. C, yeast RNA; Li, liver; Ki, kidney; St, stomach; Pa, pancreas; In, intestine; Du, duodenum; Sp, spleen; Lu, lung; He, heart; Th, thymus; Mu, striated muscle; Ov, ovary; U, uterus; Te, testis; Vd, vas deferens; Vs, seminal vesicle; Pr, prostate; Co, brain cortex; Hi, hippocampus; Cc, cerebellum; Bo, bone; Sk, skin; Pl, placenta.](image)

![Figure 3. Quantification of \(\alpha_{\text{2}}\)-AP mRNA in adult murine tissues. Results were obtained by phospho-imager quantification of RNase protection assays performed as in Fig. 2. Bars represent the mean \(\pm\) SD, expressed as percentage of the liver signal, from at least three independent experiments.](image)
The presence of \( \alpha_2\)-AP mRNA in adult murine tissues was assessed by RNase protection. \( \alpha_2\)-AP mRNA was detected in liver, kidney, intestine, spleen, lung, muscle, ovary, testis, cerebral cortex, hippocampus, cerebellum, bone, skin, and placenta (Fig. 2). The highest levels of \( \alpha_2\)-AP mRNA were observed in liver and kidney, while the abundance of \( \alpha_2\)-AP mRNA in the other tissues ranged between 0.3 and 2% of that in the liver (Fig. 3).

The renal sites of \( \alpha_2\)-AP mRNA accumulation were identified by hybridization of cRNA probes to kidney cryostat tissue sections. Macroscopic localizations performed with a \( ^{32}\)P-labeled probe revealed circumscribed zones of \( \alpha_2\)-AP mRNA accumulation in the cortex (Fig. 4). The specificity of in situ hybridization was confirmed by the lack of signal when hybridizing consecutive sections to an \( \alpha_2\)-AP sense probe and by the nonoverlapping distributions of uPA and tPA mRNAs (Fig. 4) (12). Microscopic localization studies performed with a \( ^{3}H\)-labeled probe showed that hepatocytes are responsible for \( \alpha_2\)-AP synthesis in the liver (not shown). In the kidney, they confirmed the restricted distribution observed macroscopically and ascribed \( \alpha_2\)-AP mRNA accumulation to epithelial cells present in the cortex. The labeled epithelial cells displayed a brush border and were contained within a single type of tubule that, on transverse sections, had a large diameter without a visible lumen and that predominated around glomeruli. Therefore, \( \alpha_2\)-AP mRNA-containing cells were identified as epithelial cells lining the convoluted portion of proximal tubules (Fig. 5).

Incubation of supernatants from murine renal tissue short-term cultures with \( ^{125}\)I-labeled plasmin and analysis of the samples by SDS-PAGE after immunoprecipitation with human antiplasminogen revealed the presence of a 140-kD radiolabeled plasmin–inhibitor complex (Fig. 6, lane 3); this complex comigrated with the \( \alpha_2\)-AP–plasmin complex formed in murine plasma (Fig. 6, lane 2). When renal tissue was obtained from uPA-deficient mice (24), the amount of 140-kD \( \alpha_2\)-AP–plasmin complex was higher (Fig. 6, lane 4); uPA may interact with \( \alpha_2\)-AP (34, 35), and in samples from uPA-deficient mice the amount of free \( \alpha_2\)-AP available for reaction with \( ^{125}\)I-plasmin may be higher. These results suggest that \( \alpha_2\)-AP is pro-
Figure 6. α2-AP in murine kidney. 1 ml of serum-free supernatant of murine kidney short-term cultures was incubated with 125I-labeled plasmin and the α2-AP/plasmin complex was immunoprecipitated using rabbit anti–human plasminogen polyclonal antibodies. Lane 1 shows immunoprecipitate of 125I-labeled plasmin. Lane 2 shows immunoprecipitate of 1 μl of murine plasma (dilution 1:50). Lane 3 shows immunoprecipitate of wild-type kidney culture supernatant. Lane 4 shows immunoprecipitate of uPA-deficient murine kidney culture supernatant.

**Discussion**

Most studies on the cellular origin and regulation of expression of the components of the PA/plasmin system have been focused on PAs and PAIs. Much less is known regarding plasminogen and α2-AP, which are produced by hepatocytes and circulate at relatively high concentrations in blood plasma and other extracellular fluids. In particular, possible extrahepatic sources of these distal components of a proteolytic system implicated in a large number of physiological and pathological circumstances have not been systematically explored. To address this issue, we have cloned and sequenced murine α2-AP cDNA. The deduced amino acid sequence of murine α2-AP shows a high degree of identity with its human and bovine counterparts. The putative signal peptide cleavage site appears to be similar between the murine and human and bovine proteins, although the mature forms of the inhibitor would have a different amino-terminal residue. The three functional sites of the inhibitor (i.e., the fibrin-binding site, the reactive site, and the plasminogen-binding site) are also very well conserved.

We have found that the α2-AP gene is expressed in several adult murine tissues in addition to the liver. The kidney is a major site of α2-AP mRNA accumulation, and in situ hybridization localizes the transcript exclusively in epithelial cells lining the convoluted segments of proximal tubules. Since the contribution of these cells to total kidney RNA is certainly much less than that of hepatocytes to total liver RNA, the relative abundance of α2-AP mRNA is most likely higher in these tubular cells than in hepatocytes.

The human kidney, in particular its cortical region, also contains high levels of α2-AP mRNA. The variable amounts of α2-AP mRNA detected in tumors and normal tissues analyzed...
suggest that α₂-AP expression may be modulated depending on physiological or pathological parameters. Although direct evidence for the translation of kidney α₂-AP mRNA is lacking, the detection of free α₂-AP (as revealed by the formation of ²¹²⁴l-plasmin-α₂-AP complexes) in supernatants of short-term mouse kidney cultures and of Ge1 cells (derived from human kidney tumor) strongly suggests that α₂-AP can be synthesized by renal tissues. Interestingly, this observation corroborates the postulated histogenesis of renal cancers which are thought to arise from proximal tubules (36).

Whether kidney-derived α₂-AP may also contribute to the circulating pool of the protein cannot be decided at this time. The identification of the kidney as a site of high level α₂-AP production should be considered in the context of our present understanding of the PA/plasmin system’s involvement in renal biology. Kidneys have long been recognized as a major source of PA production; more recently, uPA and tPA have been shown to be produced by epithelial cells lining distinct portions of renal tubules and to be secreted in urine (12). The present study documents the accumulation of α₂-AP mRNA in cells lining portions of tubules located upstream of the segments that produce uPA. Though the functional relevance of plasmin formation in the tubular compartment remains to be elucidated, these findings suggest that α₂-AP could exert a control over plasmin-mediated proteolysis in renal tubules. As illustrated by experiments performed with tissues of uPA-deficient mice, α₂-AP may interact not only with plasmin but also uPA, thereby providing powerful means to regulate plasmin formation and plasmin activity within tubules. Exploration of experimental pathologies indicates that plasmin-catalyzed proteolysis may prevent intratubular protein deposition and thereby may help maintain tubular patency (11). It will be of interest to investigate a possible implication of α₂-AP in tubular dysfunctions.

Male mouse kidneys were found to contain larger amounts of α₂-AP mRNA than female kidneys, and this sexual dimorphism was ascribed to the effect of testosterone. Other androgen-responsive genes are known to be expressed by the same epithelial cells within proximal tubules, such as the kidney androgen-regulated protein (37), β-glucuronidase, ornithine-decarboxylase (38, 39), and P450 cytochromes (40, 41). Testosterone is considered to control the expression of the β-glucuronidase and ornithine-decarboxylase genes at a post-transcriptional level, whereas it increases the transcription rates of the kidney androgen-regulated protein and P450 cytochrome genes. Since the rates of α₂-AP gene transcription estimated by nuclear run-on experiments are equivalent in both sexes, testosterone appears to regulate α₂-AP gene expression at the level of transcript processing and/or stability. Of interest, mouse liver α₂-AP mRNA accumulation was not influenced by androgens, demonstrating a tissue-specific endocrine regulation of α₂-AP production. Another serpin, protease nexin I (PN-I), is also under androgen control in a subset of the tissues in which it is expressed (23). Further studies are required to determine if the accumulation of α₂-AP mRNA in the human kidney is also sexually dimorphic. In any event, our results indicate that the production of α₂-AP, like that of many other serpins, can be modulated as a function of the hormonal status of the animal.

In addition to liver and kidney, a number of other murine tissues also contain α₂-AP mRNA, albeit at much lower levels; however, without knowledge of the cells that contain the transcript in these other tissues, it is not possible to determine whether the α₂-AP gene is expressed at low levels in many cells or at high levels in a fraction of the cells. It is noteworthy that these other tissues, for instance the placenta and the central nervous system, are recognized sites of PA synthesis. The presence of α₂-AP mRNA in the central nervous system is of particular interest since, while most neurons produce tPA, plasmin-mediated proteolysis is limited to defined cerebral areas (13, 14). This apparent discrepancy is not attributable to the three known PAIs, raising the possibility that other inhibitors, including α₂-AP, are involved in the local control of plasmin activity. In conclusion, our observations provide novel evidence to suggest that a distal regulation of the activity of the PA/plasmin system may operate to prevent excessive proteolysis in discrete extracellular compartments, through the local production of α₂-AP.

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References


