Cloning of human agmatinase. An alternate path for polyamine synthesis induced in liver by hepatitis B virus

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Mistry, Sanjay K., Tim J. Burwell, Rebecca M. Chambers, Laura Rudolph-Owen, Frank Spaltmann, W. Jim Cook, and Sidney M. Morris, Jr. Cloning of human agmatinase. An alternate path for polyamine synthesis induced in liver by hepatitis B virus. Am J Physiol Gastrointest Liver Physiol 282: G375–G381, 2002; 10.1152/ajpgi.00386.2001.—Agmatinase, which hydrolyzes agmatine to putrescine and urea, not only represents a potentially important mechanism for regulating the biological effects of agmatine in mammalian cells but also represents an alternative to ornithine decarboxylase for polyamine biosynthesis. We have isolated a full-length cDNA encoding human agmatinase whose function was confirmed by complementation in yeast. The single-copy human agmatinase gene located on chromosome 1 encodes a 352-residue protein with a putative mitochondrial targeting sequence at the NH3-terminus. Human agmatinase has about 30% identity to bacterial agmatinasises and <20% identity to mammalian arginases. Residues required for binding of Mn2+ at the active site in bacterial agmatinase and other members of the arginine superfamily are fully conserved in human agmatinase. Agmatinase mRNA is most abundant in human liver and kidney but also is expressed in several other tissues, including skeletal muscle and brain. Its expression in human liver is induced during hepatitis B virus infection, suggesting that agmatinase may play a role in the pathophysiology of this disease.

brain; kidney; putrescine; arginase superfamily

THE PATHWAY OF AGMATINE METABOLISM has long been recognized in bacteria, plants and invertebrates (40) where agmatine [4-(aminobutyl)guanidine] is synthesized from L-arginine by L-arginine decarboxylase (ADC). Agmatine, in turn, is hydrolyzed by agmatinase (agmatine ureohydrolase; EC 3.5.3.11) to form urea and putrescine, thus providing a route for polyamine biosynthesis that is an alternative to the exclusive route for polyamine synthesis in mammals.

Because agmatine itself is biologically active, agmatinase may not only provide a route for putrescine synthesis but may represent a mechanism for regulating cell signaling via changes in agmatine levels. Examples of agmatine effects include inhibition of cell proliferation (29, 31, 36), stimulation of glomerular filtration rate (18), activation of constitutive nitric oxide synthase (23, 37), irreversible inactivation of neuronal nitric oxide synthase (10), inhibition of inducible nitric oxide synthase (1, 5, 12), and inhibition of monoamine oxidase (27). Moreover, the relative distributions of ADC and agmatinase activities in mammalian tissues are not identical (32), which is also consistent with the possibility that these enzymes do not simply comprise a pathway for putrescine biosynthesis. Accordingly, changes in activity or expression of agmatinase could play an important role in regulating the physiological actions of agmatine.

The physiological and pathophysiological roles of agmatinase in mammals have not yet been defined, in part, because no vertebrate agmatinase has been purified or cloned nor has agmatinase expression been determined for a broad range of mammalian tissues. Moreover, it has not previously been established that agmatinase is expressed in humans. To begin evaluating potential functions of agmatinase, we isolated a full-length cDNA for human agmatinase, demonstrated tissue-specific expression, and showed that its expression is altered in human disease. Cloning of human agmatinase provides opportunities to elucidate the function of endogenous agmatine via development of specific agmatinase inhibitors and the ability to manipulate agmatinase expression by recombinant DNA techniques.
MATERIALS AND METHODS

Materials. Materials not described previously (21) were obtained from Sigma or were of the highest quality commercially available.

Isolation of cDNA clones. A human kidney cDNA library in phage lambda (Stratagene, La Jolla, CA) was screened with [*32P]*cDNA, using procedures described previously (20). Phage clones were converted to plasmids as recommended by Stratagene. Plasmids were sequenced using an ABI PRISM 377 sequencer at the University of Pittsburgh DNA Sequencing Facility.

Plasmid construction. With the use of appropriate restriction sites and standard procedures for subcloning, the entire 1.9-kb human agmatinase cDNA was inserted into the Pst I site downstream of the alcohol dehydrogenase promoter in the yeast expression plasmid pXZ134, which contains the *ura3* selectable marker (42). The plasmid containing the agmatinase cDNA in the correct orientation was designated pXZ134-agmatinase and used for complementation.

Yeast strains and complementation. A yeast strain containing a disruption of the *spe1* gene encoding ODC (strain yASG1–8), as well as strains complemented with the *Escherichia coli speA* gene encoding ADC (strain yASG1–8/pbADC), the *E. coli speB* gene encoding agmatinase (strain yASG1–8/pbAUH), or with both *E. coli* genes (strain yASG1–8/pbADC + pbAUH), were generously provided by Dr. Guan Zhu (Texas A&M University) and used for complementation studies essentially as described (15). Yeast strains were maintained on enriched medium or on yeast minimal medium (YMM) supplemented with the appropriate amino acids, nucleotides, or spermidine (10 μg/ml) as described (15).

The specific yeast strains designated in RESULTS were transformed with pXZ134-agmatinase by the lithium acetate procedure (14), and transformants were selected on medium lacking uracil. To test for complementation, each yeast strain transformed with pXZ134-agmatinase by the lithium acetate procedure (14), and transformants were selected on medium lacking uracil. To test for complementation, each yeast strain was incubated overnight in 10-ml liquid cultures of YMM supplemented with the appropriate amino acids, nucleotides, and spermidine (10 μg/ml). Each culture was then diluted to an optical density at 600 nm of 0.2, and 5 μl of serial 10-fold dilutions were spotted onto YMM plates containing the appropriate amino acids and nucleotides, with and without 10 μg/ml spermidine. Complementation of the ODC deficiency was identified by growth on plates lacking spermidine after incubation at 30°C.

RNA analysis by RT-PCR and Northern blot test. Normal human tissues were collected by the Millennium Pathology Department from various clinical sources. Hepatitis B virus (HBV)-infected liver needle biopsies and purified HBV-infected liver RNA were the kind gifts from Dr. Peter R. Galle (Johannes Gutenberg University, Mainz, Germany) and Dr. Fabio Marra (University of Florence, Florence, Italy). HepG2 and HepG2.2.15 cells were obtained from Dr. Erwin Graef (Bayer-AG, Wuppertal, Germany).

For real-time quantitative RT-PCR purposes, total RNA was purified from tissues and cells using RNA STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. After DNase-I treatment (Qiagen), total RNA went through a secondary purification using RNeasy columns (Qiagen). cDNA was synthesized using the Omniscript Reverse Transcriptase Kit (Qiagen) after the manufacturer’s suggested protocol. Design of the PCR primers and Taqman probe for human agmatinase were done using Primer Express (Applied Biosystems) software. The forward primer was 5’-ACATAGCATCTCACAACAGCAGGTGTAG, the reverse primer was 5’-AAGTTTACACCCGTATGATCCTTC, and the Taqman probe was 5’-FAM-CGCCAGCACGGCTGTTCCTCC-3’ TAMRA. Primers and the VIC-labeled Taqman probe for the housekeeping control gene human β2 microglobulin (hu β2M) were purchased from Applied Biosystems. Real-time quantitative RT-PCR analyses were performed with reagents recommended by the manufacturer (Applied Biosystems) using an ABI PRISM 7700 Sequence Detection System instrument. Briefly, ~300 pg cDNA was added to 250-μl reactions containing 12.5 μl of PCR Universal Mix (Applied Biosystems), 600 nM agmatinase F primer, 600 nM agmatinase R primer, 200 nM agmatinase Taqman probe, 200 nM hu β2M F primer, 200 nM hu β2M R primer, and 100 nM hu β2M Taqman probe. The number of PCR cycles needed for FAM and VIC fluorescence to cross a threshold where a statistically significant increase in change in fluorescence (Ct = threshold cycle) was measured using Applied Biosystems software according to their recommendations. Relative agmatinase RNA expression was determined using the formula Rel Exp = 2ΔΔCt, where ΔΔCt = (Ct agmatinase–Ct hu β2M in experimental sample)–(Ct agmatinase–Ct hu β2M in a no-template control sample). Relative expression was plotted after setting the sample with lowest measurable expression equal to 1. Taqman RT-PCR experiments were performed in duplicate and were independently repeated three to four times.

Total RNA samples were subjected to Northern blot analysis using [*32P]*cDNA (a 1.25-kb EcoRI fragment of human agmatinase) containing the entire open reading frame (ORF). Procedures for gel electrophoresis and hybridization were as previously described (22).

In situ hybridization and immunohistochemistry. For immunohistochemistry, frozen sections of infected or human liver were cut at 6 μm thickness, mounted on charged slides, fixed in cold acetone (Sigma), and immersed in 0.3% H2O2 in methanol for 10 min to block endogenous peroxidase. A 1:2,000 dilution of anti-HBV surface antigen (DAKO) was applied in 5% BSA/PBS for 30 min at room temperature. Slides were then incubated with anti-goat secondary antibody, followed by an alkaline phosphatase-labeled polymer (Dako) for detection. After multiple PBS washes, fast red substrate-chromogen solution was applied for 10 min and the slides were then counterstained with hematoxylin.

For in situ hybridization, frozen 6-μm sections of infected or normal human liver were fixed in 4% paraformaldehyde, washed in 1× PBS, acetylated in 0.1M TEA-HCL and 0.25% acetic anhydride (Sigma) for 10 min, washed in 2× SSC, dehydrated with ethanol, and air dried. Slides were hybridized overnight at 55°C with [*35S]*labeled human agmatinase riboprobes at 2.5 × 106 cpm/slide. Slides were subsequently washed and dipped in photographic emulsion (Kodak) and exposed for 2 to 4 wk at 4°C, developed, and counterstained with hematoxylin.

RESULTS

Isolation of full-length human agmatinase cDNA. Based on the homology between arginases and bacterial agmatinases (26), we initially identified several potential human agmatinase sequences by using the rat arginase I sequence as the query in a basic local alignment search tool (BLAST) search (2) of the expressed sequence tag (EST) database at the National Center for Biotechnology Information. Several candidate human EST clones were obtained from the IMAGE Consortium, but none contained a complete ORF. Based on preliminary Northern blot analyses of human RNAs with a partial human agmatinase cDNA, we used one of the candidate EST clones (GenBank accession no. A1653589) to screen a human kidney cDNA library. We isolated a 1884-bp cDNA

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containing a short 5’ untranslated region of 43 bp, a coding region of 1059 bp, and a longer 3’ untranslated region of 757 bp, plus a 25-bp polyA tail. The predicted ORF corresponds to a protein of 352 residues with a calculated molecular weight of 37,731 daltons. Alignment of the cDNA sequence with sequences in the human genome database identified a single-copy gene composed of eight exons spanning 12.6 kb on chromosome 1 (Fig. 1A). As shown in Fig. 1B, the predicted ORF exhibits ~30% identity to two bacterial agmatinases whose activity has been established (6, 38), but it has <20% identity to either human arginase I or arginase II (not shown). However, human agmatinase does contain all amino acid residues that are absolutely conserved in the arginase superfamily (26, 38). These residues include the six aspartic acids and histidines (Fig. 1B) that bind divalent manganese ions required for activity of the arginases and bacterial agmatinases (4, 8). An additional histidine required for maximal activity of E. coli agmatinase (9) also is conserved in the human enzyme.

Identification of human agmatinase by complementation in yeast. Because it is generally very difficult to obtain enzymatically active bacterial preparations of recombinant mammalian proteins that normally undergo proteolytic processing into the final active form, we chose to confirm the identity of the candidate human agmatinase by functional complementation in yeast. Fortunately, polyclone-requiring yeast strains suitable for such complementation studies have recently been developed (15). Yeast strain yASG1–8 contains a disruption in the spe1 gene encoding ODC and thus requires exogenous polyamines for growth. Transformants of yASG1–8 expressing either E. coli ADC (speA) or E. coli agmatinase (speB) also require exogenous polyamines, but a yASG1–8 strain expressing both speA and speB genes can grow on minimal medium without polyamines (15). The complementation scheme for yeast deficiency in ODC is illustrated in Fig. 2A. After transformation with plasmid pXZ134-agmatinase, the ability to grow on minimal medium lacking polyamines was restored in the yASG1–8 strain complemented with speA, whereas there was little or no growth in the yASG1–8 strain complemented only with speA or human agmatinase (Fig. 2B), thus confirming the identity of our cloned DNA as an agmatinase cDNA. Transformation with the parental plasmid pXZ134 alone failed to restore growth in the yASG1–8 strain complemented with speA (not shown).
Expression of agmatinase in human tissues. Because the expression of agmatinase in human tissues has not been reported previously, agmatinase mRNA levels were determined for 30 tissue types (Fig. 3). Although there is individual variation, agmatinase mRNA is most abundant in adult human liver and kidney, with lesser amounts in skeletal muscle, fetal liver, brain, testis, skin, and the gastrointestinal tract (Fig. 3). Human tissue contains two hybridizing RNA species, one of about the same size as the 1.9-kb human agmatinase cDNA and a larger molecular weight species of 3.9 kb (Fig. 4). In contrast, only the smaller mRNA is found in rat and mouse. As shown in the preceding sections, the smaller mRNA is of sufficient size to encode a full-length agmatinase.

Changes in agmatinase activity or expression may be informative for elucidating the physiological or pathophysiological roles of this enzyme. Because agmatinase is strongly expressed in liver, we investigated whether its expression could be altered in this organ. We found that agmatinase mRNA is induced two- to threefold in HBV-infected liver (Fig. 5). This induction is cell autonomous, because agmatinase mRNA levels also are elevated in the human hepatoma cell line HepG2.2.15, which expresses infectious HBV (Fig. 5) (39). Immunohistochemical analysis and in situ hybridization revealed that agmatinase and HBV antigen were coexpressed within virtually all cells in the field, primarily parenchymal cells (Fig. 6).

DISCUSSION

The existence of an alternate pathway for polyamine synthesis via ADC and agmatinase in mammals was discovered only recently (16, 17, 35). However, it has not been previously established that humans express either of these enzymes nor have these enzymes been characterized for any vertebrate species. As a first step in analyzing agmatine metabolism in humans, we set out to clone human agmatinase, identify the enzyme family to which it belongs, and characterize its expression in human tissues. A full-length human agmatinase cDNA was isolated, and an initial BLAST search of the EST databases with the human agmatinase sequence identified homologous sequences in rats, mice, pigs, and chickens (GenBank accession no. AF401291), indicating that this gene is expressed in a wide range of vertebrate species. Sequence alignments showed human agmatinase has significant similarity to bacterial agmatinases and thus is a member of the arginase superfamily (26, 38). The presence of key conserved residues indicates that human agmatinase likely requires two divalent manganese ions for full activity, as do the arginases and E. coli agmatinase (4, 8). More detailed studies of agmatinase structure and
enzymatic properties will be useful in designing specific inhibitors that can be used in elucidating its physiological functions. On the basis of its general similarity to the NH$_3$-termini of the precursor forms of mitochondrial type II arginases, the NH$_3$-terminus of the human agmatinase ORF appears to represent a mitochondrial import/processing sequence. This also is consistent with the mitochondrial localization of agmatinase activity (35).

Although agmatinase is present in rodent tissues, including brain (35), liver (7), and a murine macro-

phage line (34), there is no published information on agmatinase expression in human tissue. The present study shows that expression of agmatinase in humans is tissue specific, with the highest abundance of agmatinase mRNA in liver and kidney. The lower level of agmatinase mRNA in human brain likely represents high-level expression only in selected subpopulations of brain cells, consistent with reports of inhomogenous distribution of agmatinase activity in rat brain (35). The highest levels of agmatinase expression are not found in tissues with the highest rates of cell proliferation and turnover, suggesting that this enzyme does not serve the same role as ornithine decarboxylase. Instead, the primary function of agmatinase in organs with high expression may be to regulate levels of agmatine, which can act as a cell-signaling molecule. For example, agmatinase expression in liver may serve to limit the extent and duration of any increases in circulating levels of agmatine from endogenous or dietary sources. Because agmatine stimulates glomerular filtration rates in the kidney (18), renal expression of agmatinase may serve to modulate its effects in this organ. The presence of agmatinase in skeletal muscle may prevent inhibition of neuronal nitric oxide synthase, which can be irreversibly inactivated by agmatine (10). Similarly, the agmatinase in the brain likely modulates its activity as a cell-signaling molecule in that organ (33). Availability of a mammalian agmatinase cDNA will provide opportunities to begin testing these hypotheses.

Because the identification of circumstances and mechanisms involved in regulation of agmatinase expression will be important in establishing the physiological role(s) of this enzyme, we sought to identify cell culture models for studying regulation of agmatinase expression. Using primary cultures of rat hepatocytes readily responsive to cAMP and glucocorticoids (24), we found that levels of agmatinase mRNA were completely unaffected by these agents (results not shown), indicating that hepatic agmatinase expression is not regulated by hormonal signals commonly involved in responses to changes in diet or metabolic status. Because it expresses agmatinase and has increased agmatinase expression after infection with HBV, we identified the HepG2 hepatoma cell line as a potentially useful model for studying the regulation and function of hepatic agmatinase.

Regarding the increase in agmatinase expression after HBV infection, we note that HBV infection also upregulates another enzyme of arginine metabolism in human liver and in HepG2 cells, namely the inducible isoform of nitric oxide synthase (3, 19). Whether the elevated agmatinase expression is a direct effect of viral factors on agmatinase gene expression or is a secondary effect of the induced nitric oxide production remains to be determined. In any case, the elevated agmatinase expression may provide a source of polyamines for efficient viral replication (13, 28) and thus exacerbate the disease. If so, inhibition of hepatic agmatinase activity or expression could represent a new treatment strategy for patients with viral hepatitis.

Fig. 6. Localization of agmatinase mRNA in human liver by in situ hybridization. Serial sections are from HBV-infected (A, C, and E) and control liver (B, D, and F). Shown are dark-field views of liver sections hybridized with antisense (A and B) or sense (C and D) probes for human agmatinase. Hybridization is indicated by bright white spots. E and F: sections of liver after immunohistochemical staining (in red) for HBV surface antigen are shown.
The possibilities described here are based on two assumptions: first, that increased agmatinase mRNA levels result in increased agmatinase activity. Although we believe this to be likely, it remains to be determined. The second assumption is that increases in agmatinase activity result in physiologically significant increases in polyamine production. Further experimentation is required also to test this assumption.

Physiological and pathophysiological roles of endogenous agmatinase in mammals are poorly understood, in part, because relatively little is known regarding the location, regulation, and properties of the enzymes involved in synthesis and degradation of agmatine. Although most speculation as to the physiological roles of agmatinase in mammals has focused on its potential involvement in the tryptophan degradation, and functional effects in the kidney of the rat. Evidence for a virus-induced gene upregulation. J Clin Invest 101: 1343–1352, 1999.


