Complexity and species variation of the kidney-type glutaminase gene

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Porter, L. David, Hend IbraHim, Lynn Taylor, and Norman P. Curthoys. Complexity and species variation of the kidney-type glutaminase gene. Physiol Genomics 9: 157–166, 2002. First published April 16, 2002; 10.1152/physiolgenomics.00017.2002.—Increased expression of rat kidney-type glutaminase (KGA) during metabolic acidosis results from selective mRNA stabilization. This process is mediated by an 8-base AU-sequence that functions as a pH-response element (pHRE). LLC-PK1-FBPase<sup>−</sup> cells, a pH-responsive porcine kidney cell line, express four distinct GA mRNAs. RNase H mapping indicated that three of the GA mRNAs are generated by use of alternative polyadenylation sites and are homologs of the rat KGA mRNA, while the fourth contains a different COOH-terminal coding and 3′-untranslated sequence. PCR cloning and sequencing established that the latter GA mRNA is the homolog of the human GAC mRNA. A rat GAC cDNA was also cloned from a rat kidney library. The 3′-untranslated regions of the GAC mRNAs, but not the porcine or human KGA mRNAs, contain identifiable pHREs. The human KGA gene spans 82 kb and is composed of 19 exons. The unique sequence from the hGAC cDNA is contained in a single exon. Thus in humans, alternative splicing of the initial transcript could produce two GA mRNAs, only one of which may be increased during acidosis.

pH-response element; metabolic acidosis; mRNA stabilization; LLC-PK1-FBPase<sup>−</sup> cells; COOH-terminal domains; RNase H mapping

IN RAT KIDNEY, AMMONIUM IONS are derived primarily from glutamine that is extracted from the plasma (29). Both basal and enhanced catabolism of glutamine during metabolic acidosis occur primarily within the proximal convoluted tubule (11, 26). The latter process is sustained, in part, by the increased expression of the genes that encode various transport proteins and the key enzymes of glutamine metabolism (7). For example, the level of the mitochondrial glutaminase (GA) is increased 8- to 20-fold within the rat renal proximal convoluted tubule during chronic acidosis (6, 33). This increased expression results from an increased rate of GA synthesis (31) that correlates with an increased level of the GA mRNA (17, 32). However, the apparent rate of transcription of the GA gene is not increased in acute (16) or chronic acidosis (17). These observations led to the suggestion that increased expression of the GA gene may result from the selective stabilization of the GA mRNA.

LLC-PK1-FBPase<sup>−</sup> cells, a pH-responsive porcine proximal tubule-like cell line (12), have been used to characterize the mechanism of the adaptive increase in GA activity. Through the analysis of the turnover of various chimeric β-globin (βG) constructs, it was demonstrated that the 3′-untranslated region of the rat kidney-type glutaminase (rKGA) mRNA contains a pH-response element (pHRE) (14). RNA gel shift analyses were used to characterize and map the specific binding of a rat renal protein to two adjacent 8-base AU-sequences within the 3′-untranslated region of the rKGA mRNA (20). Further studies with various βG-GA reporter constructs (18, 19) established that a single 8-base AU-sequence was both necessary and sufficient to produce a mRNA that is selectively stabilized by the transfer of LLC-PK1-FBPase<sup>−</sup> cells to an acidic media (pH 6.9, 10 mM HCO<sub>3</sub>). The protein in rat kidney cortex that binds to the pHRE was recently identified as δ-crystallin/NADPH-quinone reductase (30).

The pattern of GA mRNA expression in the porcine LLC-PK1-FBPase<sup>−</sup> cells is complex. At least four distinct GA mRNAs are differentially expressed as a function of cell growth and development (25). Treatment with cycloheximide or prolonged maintenance in the same medium can be used to substantially increase the levels of the various GA mRNAs in LLC-PK1-FBPase<sup>−</sup> cells. An initial GA cDNA that contains 528 bp of coding sequence and a 2.7-kb 3′-untranslated region was cloned from a porcine kidney cDNA library and shown by sequence comparison to encode the homolog of the rat kidney-type GA (rKGA) mRNA (25). However, probes derived from the 3′-untranslated region of the porcine KGA (pKGA) cDNA hybridized only with the 5.0- and 3.5-kb GA mRNAs (13, 25). In contrast, probes derived from the coding region of the rKGA or pKGA cDNAs hybridized to all four GA mRNAs. Furthermore, only the 4.5-kb GA mRNA, which apparently contains a unique 3′-untranslated region, is increased when confluent cultures of LLC-PK1-FBPase<sup>−</sup> cells were transferred to acidic medium (13). The two- to threefold increase in the level of the 4.5-kb GA mRNA was shown to correlate with a similar increase in the stability of the 4.5-kb GA mRNA. In contrast, the
stability of the 5.0-kb GA mRNA was not affected by treatment with acidic media. Thus it is important to determine the relationship and the structural differences among the multiple GA mRNAs that are expressed in the LLC-PK1-FBPase cells.

The human (h) homolog of the rKGA cDNA has also been cloned and characterized (8, 15). However, the 3′-untranslated region of the hKGA mRNA lacks an identifiable pHRE. An isoform of the hKGA cDNA, termed hGAC, was also cloned from a human colon carcinoma cDNA library (8). The 5′-coding region of the hGAC cDNA is identical to the corresponding sequence within the hKGA cDNA. However, the hGAC cDNA encodes a distinct COOH-terminal sequence and contains a different 3′-untranslated region, suggesting that the two mRNAs may be produced by alternative splicing. The 3′-untranslated region of the hGAC mRNA contains four separate 8-base AU sequences that are identical to either of the 8-base elements that were identified as pHREs within the rKGA mRNA. Furthermore, Northern analysis indicated that both the hKGA and hGAC mRNAs are expressed in human kidney tissue (8).

In the current study, RNase H mapping was used to further characterize the structural relationship between the multiple GA mRNAs expressed in LLC-PK1-FBPase cells. In addition, reverse transcriptase and polymerase chain reactions (RT-PCR) were used to amplify and sequence additional segments of the 5.0-kb and 4.5-kb GA mRNAs. These analyses established that the 4.5-kb GA mRNA has a COOH-terminal coding sequence that is identical to the hGAC cDNA and a 3′-untranslated region that contains three putative pHREs. Thus this mRNA is the porcine homolog of the hGAC cDNA. Finally, analysis of the current human genome database indicates that the unique sequence within the hGAC cDNA is derived from a single exon within the hGKA gene.

MATERIALS AND METHODS

Materials. [α-32P]dATP and [α-32P]dCTP (specific activity of 3,000 Ci/mmol), Hot Tub DNA polymerase and the random oligolabeling kit were obtained from Amersham Pharmacia Biotech. GeneScreen Plus and the restriction enzymes were products of New England Nuclear and Boehringer-Mannheim, respectively. The Reverse Transcription System and products of United States Biochemicals. Oligodeoxynucleotides were synthesized by Macromolecular Resources (Ft. Collins, CO). Unless designated otherwise, the oligodeoxynucleotides contained sequences that are complementary to the sequence starting with the designated 5′ base of the rat GA cDNA (27). All other biochemicals were purchased from Sigma or Fluka.

Cell culture. The LLC-PK1-FBPase cells were originally isolated by Gstraunthaler and Handler (12) and were obtained from Edward Nord (SUNY Stony Brook). The cells were cultured in a 50:50 mixture of Dulbecco’s modified Eagle’s and Ham’s F-12 media as described previously (25).

To increase the levels of the various GA mRNAs, cells were grown under standard conditions for 7 days and then maintained in the same medium for 4 days or treated with 0.5 mM cycloheximide for 16 h.

RNase H and Northern analysis. Total RNA was isolated from cultured cells using the acid guanidinium thiocyanate-phenol:chloroform protocol (4). The isolated RNA samples were dissolved in Formazol and stored at −70°C. Poly(A)+ RNA was prepared using oligo(dT)-cellulose spin columns. RNase H digests were performed as described previously (24). RNA samples were subjected to electrophoresis on a 1% agarose-formaldehyde gel. The cDNA probes used for hybridization included sequences that correspond to the coding region (r1500) of the rat GA mRNA and the 3′-untranslated region (p2400) of the porcine GA mRNA. The r1500 cDNA is an Accl restriction fragment of the rat pGA104 plasmid (27). The p2400 probe is an Nhel/NotI restriction fragment of the porcine pGA201 plasmid (25). The cDNA probes were radiolabeled with random primer labeling using either [α-32P]dATP or [α-32P]dCTP (10). The hybridization solution containing 5−10 ng/ml of the 32P-labeled cDNA probe was prepared as described (3), except that the formamide concentration was 40% and no polyethylene glycol was added. Hybridizations were carried out in glass bottles rotated overnight at 43°C in a hybridization oven. Membranes were initially washed in 300 mM NaCl, 30 mM sodium citrate, and 0.5% sodium dodecyl sulfate (SDS) at 43°C. The final wash was performed at either 56°C or 48°C using 50 mM sodium phosphate, pH 7.2, and 0.1% SDS or at 48°C using 45 mM NaCl, 4.5 mM sodium citrate, and 0.1% SDS. For low-abundance mRNAs, a stronger signal was obtained by relaxing the stringency of the final wash without changing the pattern or the relative intensities of the observed bands. The Northern blots were imaged on a PhosphorImage Analyzer (Molecular Dynamics) following a 1- to 3-day exposure or by autoradiography at −70°C for 3−21 days.

Isolation and amplification of a 5′ segment of the 5.0-kb GA mRNA. Total poly(A)− RNA isolated from LLC-PK1-FBPase cells that had been maintained in the same medium for 4 days was used as the starting material for the isolation and amplification of a 5′ segment of the porcine 5.0-kb GA cDNA. The purification protocol is diagrammed in Fig. 3A. Approximately 10 µg of poly(A)− RNA was spin dialyzed into 26 µl of water using a 30-kDa cutoff microconcentrator. Then 1 µl of 50 µM oligonucleotide 3082 and 3 µl of 10× RNase H buffer (0.5 M Tris–HCl, pH 7.4, 1.0 M NaCl, 20 mM MgCl2, 10 mM dithiothreitol) were added, and the sample was incubated at 48°C. The reaction was initiated by addition of 1.0 U of Hybridase RNase H and incubated for 15 min (24). The reaction was terminated by adding 300 µl of water and 330 µl of 2× oligo(dT) column binding buffer (1.0 M LiCl, 100 mM sodium citrate, and 0.2% SDS) and the diluted sample was heated at 60°C for 3 min. The sample was then cooled to room temperature and applied twice to an oligo(dT) column. The final flow-through fraction containing the poly(A)− RNA was collected and precipitated with an equal volume of isopropanol. The poly(A)− RNA was then resuspended in 50 µl of Formazol. The column was washed and eluted per the manufacturer’s instructions to recover the poly(A)− fraction. The fractionation was confirmed by Northern analysis.

The RT-PCR protocol employed Retrotherm, a heat-stable reverse transcriptase, to increase the priming stringency and the efficiency of polymerization through regions of possible mRNA secondary structure. The isolated poly(A)− RNA fraction was spin-dialyzed into 16.5 µl of water, and 25 pmol of
oligonucleotide 1952 was added. The sample was held at 56°C in the thermocycler while 1 μl of dNTPs (4 mM each; 1 μl each of solution A (0.2 M Tris-HCl, pH 8.3, and 1.0 M KCl) and solution B (30 mM MgCl₂ and 15 mM MnSO₄)), and 2 U of reverse transcriptase were added. The reaction temperature was then increased slowly over a 10-min period to 70°C. After 5 min at 70°C, the first-strand synthesis was considered complete. The reaction was subsequently heated to 95°C for 2 min and then held at 80°C as 20 μl from this sample was directly pipetted into the succeeding PCR reaction mix. This mix was preincubated at 80°C and consisted of 62 μl of water, 1 μl of 50 μM oligonucleotide 574-forward, 1 μl of 50 μM oligonucleotide 1689, 10 μl of 10× Hot Tub polymerase reaction buffer, and 4 μl of dNTPs (4 mM each). The Hot Tub polymerase (1.6 U) was added last. Second-strand synthesis was performed during the first amplification cycle. Amplification was carried out for 35 cycles as follows: 94°C for 30 s, 50°C for 30 s, and 70°C for 150 s. The final cycle was followed by an incubation at 70°C for 3 min and the sample was then stored at 4°C until analyzed by Southern blotting. 

Isolation and amplification of the 3′ segment of the 4.5-kb GA mRNA. The isolation and amplification of the corresponding 5′ segment of the 4.5-kb GA mRNA was accomplished using the above protocol with modifications as outlined below and in Fig. 3B. Total RNA from starved LLC-PK₁-FBPase⁺ cells was used for RNase H digestion with oligonucleotide 1952. After digestion, the sample was passed over an oligo(dT) column. The 5′ segments from the cleaved GA mRNAs eluted with the poly(A)− RNAs in the flow-through fraction, whereas the intact 4.5-kb GA mRNA eluted from the column with the poly(A)+ RNAs. This poly(A)+ fraction was used for the detailed RNase H mapping of the intact 4.5-kb GA mRNA. To remove background poly(A)− RNAs, a second RNase H digest using oligonucleotide 1689 was performed, and this sample was applied to a second oligo(dt) column. Now the 5′ segment from the 4.5-kb GA mRNA was eluted in the poly(A)− flow-through fraction, while the poly(A)+ RNAs were retained on the column. The purified 5′ segment was amplified by RT-PCR using oligonucleotides 574-forward and 1476.

Dideoxy sequencing of RT-PCR products. The two RT-PCR products were purified by agarose gel electrophoresis and sequenced without subcloning. Approximately 1 μg of extracted cDNA was transferred to 20 mM Tris-HCl, pH 9.0, by spin dialysis. An 8-μl sample containing the cDNA and 25 pmol of primer were heat denatured at 95°C for 1 min and then set on ice. DNA sequencing was performed using the Sequenase kit (United States Biochemicals).

RT-PCR amplification and cloning of the 3′ end of the 4.5-kb GA mRNA. The reverse transcription reactions were carried out using 1 μg of total RNA isolated from LLC-PK₁-FBPase⁺ cells and the Reverse Transcription System from Promega. This kit utilizes an oligo(dT)₁₂ primer and the AMV reverse transcriptase. The PCR amplification reactions utilized the Ex Taq DNA polymerase. The initial PCR reaction used the F1 and B2 primers that were described previously (8). The second PCR reaction utilized the complement of the B2 sequence as the forward primer and a reverse primer, 5′-CATAATGATTGGCATATCTG-3′, that is complementary to sequence located near the 3′ end of the hGAC cDNA. The resulting PCR fragments were cloned into the pGEM-T Easy vector. The resulting plasmids were sequenced using a Prism 377 DNA Sequencer (Applied Biosystems) and primers designed with Omega software.

Analysis of human GA genes. The human KGA (15) and rat liver-type GA (LGA) (5) cDNA sequences were used in a BLAST search of the human genome database located within the website: http://www.ensembl.org. The COOH-terminal cDNA sequence unique to the human GAC (8) sequence was used in a similar BLAST search.

RESULTS

RNase H mapping. Previous studies (13, 25) indicated that the 4.5- and 5.0-kb GA mRNAs from LLC-PK₁-FBPase⁺ cells contain divergent sequences within their 3′ ends. Thus RNase H digestions were used to confirm these results and to more precisely map the region where the two porcine GA mRNAs diverge (Fig. 1). Samples of total RNA containing elevated amounts of the two GA mRNAs were isolated from cycloheximide-treated LLC-PK₁-FBPase⁺ cells and incubated with one of three oligonucleotides (2072, 1952, and 1689) that are complementary to sequences within the COOH-terminal coding region of the rat kidney GA cDNA (27). Following RNase H digestion, the samples were probed with a cDNA (p2400) that is specific for the 3′-untranslated region of the 5.0-kb GA mRNA. Treatment with each of the oligonucleotides resulted in site-specific cleavage of the 5.0-kb GA mRNA. The sizes of the resulting bands (2.8, 2.9, and 3.1 kb, respectively) correspond closely to the expected lengths of the 3′ fragments as determined from the sequence of the pKGA cDNA (25). The blot was then stripped and reprobed with the r1400 probe that is derived from coding region of the rKGA cDNA and that hybridizes to both the 4.5- and 5.0-kb GA mRNAs. Again, the 5.0-kb GA mRNA was cleaved by all three oligonucleotides to produce the corresponding 2.2-, 2.1-, and 1.9-kb 3′ fragments, respectively. The r1400 probe also hybridized weakly to the 3.1-kb 3′ fragment that was produced following hybridization and digestion with oligonucleotide 1689. In contrast, the 4.5-kb GA mRNA was cleaved only when incubated with oligonucleotide 1689. The unexpected formation of fragments that are less than 1.9-kb in length may result from the low stringency of the oligonucleotide binding during the 37°C digestion with the E. coli RNase H. However, the resulting data establish that the sequence divergence between the two porcine GA mRNAs occurs ~150–350 nucleotides upstream of the 3′ end of the coding sequence.

A more thorough mapping analysis (Fig. 2) was performed using a total of 13 oligonucleotides that span the entire length of the 5.0-kb GA mRNA and a thermostable RNase H to achieve increased stringency (24). Each of the tested oligonucleotides cleaved at the corresponding sites in the 2.5-kb, 3.5-kb, and 5.0-kb GA mRNAs. Thus both of the smaller and less abundant GA mRNAs may be derived by use of alternative polyadenylation signals that occur at appropriate positions within the 5.0-kb GA cDNA sequence. This analysis also suggested that much of the coding sequence of the 4.5-kb GA mRNA is identical to that of the other GA mRNAs. However, this mRNA must contain unique sequences within the 3′ end of the coding region and throughout the entire 3′-untranslated region.
**Sequence analysis.** RNase H digestion and fractionation on oligo(dT)-cellulose were used to selectively purify the 5′ ends of the 5.0- and 4.5-kb GA mRNAs (Fig. 3, A and B). The oligonucleotide 3888 was used to selectively cleave the 5.0-kb GA mRNA. Following purification on oligo(dT)-cellulose, the poly(A)− fraction was shown by Northern analysis to contain only the 5′ end of the 5.0-kb GA mRNA (L. D. Porter, data not shown). This fraction was used to RT-PCR amplify and sequence the segment of the 5.0-kb GA mRNA between oligonucleotides 574 and 1476 (Fig. 4). The two amplified segments contained identical sequences, confirming the results of the RNase H mapping analysis and suggesting that the 4.5- and 5.0-kb porcine GA mRNAs may be derived from a single kidney-type GA gene.

Elgadi et al. (8) cloned a GA cDNA from a human colon carcinoma cDNA library that they termed hGAC. The 5′ end of the hGAC cDNA is identical to that of the human kidney-type GA cDNA (8, 15). However, the hGAC cDNA contains a unique COOH-terminal coding sequence and a distinct 3′-untranslated region. Since KGA cDNA coding sequences are highly conserved among species, it was probable that the unique sequence found in the hGAC cDNA would also be conserved. Thus specific primers corresponding to sequences from the hGAC cDNA were used to RT-PCR amplify and clone cDNAs from poly(A)− RNA isolated from LLC-PK1-FBPase− cells (Fig. 3C). The initial PCR fragment (464 bp) spanned the region where the 4.5- and 5.0-kb GA mRNAs diverge. The sequence of

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**Fig. 1.** RNase H mapping of the porcine 5.0-kb and 4.5-kb glutaminase (GA) mRNAs. A: the p2400 probe was obtained from the 3′-untranslated region of a porcine GA cDNA, whereas the r1400 probe was derived from the coding region of a rat GA cDNA. B: samples containing 20 μg of total RNA isolated from LLC-PK1-FBPase− cells pretreated with cycloheximide were either untreated (UNT) or incubated with the indicated oligonucleotide. RNase H treatment was performed at 37°C for 15 min. The p2400 probe hybridizes specifically with the 5.0-kb GA mRNA, whereas the r1400 probe hybridizes to both GA mRNAs.
the initial 364 bp of this fragment was 100% identical to sequence contained in the 5.0-kb GA cDNA, but only 96% identical to the corresponding segment of the hGAC cDNA (Fig. 4). All of the nucleotide substitutions in this region occur within wobble positions. Thus the two sequences encode an identical amino acid sequence. By contrast, the nucleotide sequence of the remaining 100 bp was highly divergent from the adjacent sequence in the porcine 5.0-kb GA cDNA, but was 100% identical to the corresponding sequence in the hGAC cDNA. Thus the remainder of the 3′ end of the 4.5-kb GA cDNA was amplified by RT-PCR, cloned, and sequenced. From the point of divergence to the 3′ end, the sequence of the isolated porcine cDNA is 77% identical to the 3′ end of the hGAC cDNA (Fig. 4). All of the differences in DNA sequence within the unique coding regions of the hGAC and the porcine 4.5-kb GA cDNAs occur within wobble positions. Thus the corresponding mRNAs encode identical COOH-terminal domains (Fig. 5). This level of identity clearly establishes that the porcine 4.5-kb GA mRNA is the homolog of the hGAC mRNA. Furthermore, the 3′-untranslated regions of both the 4.5-kb porcine GA and the hGAC mRNAs contain multiple pHREs (Fig. 4). In contrast, both the hKGA (8, 15) and the 5.0-kb porcine GA mRNA (25) lack the pHRE sequence identified in the rKGA mRNA. If the 2.5- and 3.5-kb porcine GA mRNAs are formed by use of alternative polyadenylation sites within pKGA transcript, then they will also lack a pHRE sequence.

RNase H experiments performed with total RNA from normal rat kidney and with poly(A)+ RNA isolated from acidic rat kidney established the presence of a 4.4-kb GA mRNA that was not cleaved when incubated with oligonucleotides 4008, 2958, 2458, or 1952, but was cleaved with oligonucleotides 1689, 1476, and 574 (L. D. Porter, data not shown). This data strongly supports the previously reported evidence (9) for the presence of a rat homolog of the hGAC mRNA. We had previously cloned a number of GA cDNAs from a rat kidney library (27). One of these clones (rat pGA105) contained a divergent sequence that was previously assumed to result from an artifact of cloning. When fully sequenced, this clone was found to diverge at the same position as the hGAC and the porcine 4.5-kb GA cDNAs. The final 147 bp of the 3′ coding sequence of the rat pGA105 cDNA is 90.5% and 91.2% identical to the unique COOH-terminal coding sequences within the human and porcine GAC cDNAs, respectively. Thus rat kidney also expresses a GAC variant of the rKGA mRNA. The rGAC mRNA encodes a COOH-terminal domain that contains five conservative amino acid substitutions compared with the corresponding human and porcine GAC proteins (Fig. 5). Thus multiple mammalian species express two distinct classes of kidney-type GA that differ in their COOH-terminal domains.

**GA genome.** Analysis of the current human genome database suggested that the two kidney-type GA mRNAs are produced by alternative splicing of an initial transcript of a single gene. The kidney-type GA gene is located on chromosome 2 (1). Sequences corresponding to the two GA cDNAs are located on four separate contigs and are distributed into 19 exons (Fig. 6). The entire gene spans 82 kb. Each of the identified junctions contains a sequence that fits to the consensus sequence for 5′ and 3′ splice sites. The hGAC mRNA is formed by joining exons 1–15, whereas the hKGA mRNA is derived from exons 1–14 and 16–19. Thus all of the sequence that is unique to the hGAC mRNA is contained in exon 15.
The rat LGA is derived from a separate but closely related gene (5). In humans, the LGA gene is located on chromosome 12 (1). The hLGA gene sequence within the human genome database is located on two nonoverlapping contigs. The missing overlap contains ~600 bp of the first intron. Thus all of the cDNA sequence was identified in 18 exons that span ~18 kb. The lengths of exons 3–17 within the hLGA and hKGA genes are identical. The nucleotide sequences of the segments of the two cDNAs derived from these exons have a 69% identity. However, the amino sequences encoded by the same exons have a 77% identity. In contrast, the COOH-terminal amino acid sequence encoded by exon 18 of the hLGA gene shares very little identity with the COOH-terminal sequence of either the hKGA (33%) or the hGAC protein (14%). This difference further suggests that the COOH-terminal domain may impart different properties and functions to the various GA proteins.

**DISCUSSION**

Multiple isoforms of the kidney-type GA mRNA are expressed in various cell lines (24) and in different...
The abundance of a particular GA mRNA variant may differ significantly depending upon the tissue type or the developmental or metabolic state of the tissue. The relative abundance of each isoform can be determined only if the structural basis for each type of GA mRNA is known. All of the KGA and GAC isoforms share a common sequence within their 5’ end but diverge significantly within the 3’-coding and 3’-untranslated regions. This variation has complicated the cloning of GA cDNAs. Given the length of the GA mRNA, it has not been possible to isolate a full-length GA cDNA to assure accurate cloning of the original mRNA. Instead, all of the reported GA cDNAs have been constructed from overlapping, but arbitrarily combined sequence segments. Thus, to obtain definitive information regarding putative isoforms of GA mRNAs, RNase H mapping was employed to characterize the relationship between the various GA mRNAs.

Fig. 5. Comparison of the COOH-terminal sequences of various kidney-type GA proteins. An underscore indicates regions where the amino acid sequence is identical to the human kidney-type glutaminase (hKGA) protein, whereas a hyphen indicates an amino acid that is identical to the divergent COOH-terminal sequence of the hGAC protein.

Fig. 6. Genomic structure of the hKGA gene. A: identified exons are shown as solid boxes and are drawn to scale. The introns are shown as lines, with lengths noted in bp. B: differential splicing of the exons to form hGAC and hKGA mRNAs. The lengths of exons are noted in bp.
that are expressed in the porcine renal LLC-PK1-FBPase+ cell line. From the results of this analysis, a protocol was developed (Fig. 2), using a modification of a previous strategy (21), to isolate similar segments from the coding regions of two specific GA mRNAs. Subsequent sequencing analysis confirmed the previously hypothesized relationship between 4.5-kb and 5.0-kb porcine GA mRNAs (25).

The KGA and GAC mRNAs are frequently expressed in the same cell line or tissue (8). The two GA mRNAs are sufficiently similar in size that they are difficult to resolve by agarose gel electrophoresis (13, 25). Thus reports in the literature that have used only a probe that hybridizes to the shared coding sequence should be interpreted with this in mind. However, the methods and probes developed in this study extend the previous techniques (8) that can be employed to detect and quantify the two GA mRNA variants.

Four GA mRNAs, that are ~2.5, 3.5, 4.5, and 5.0 kb in length, are expressed in LLC-PK1-FBPase+ cells (25). The two larger mRNAs predominate except when the LLC-PK1-FBPase+ cells are starved for nutrients by maintaining them in the same medium for 4 days. Under these conditions, very high levels of the 2.5-kb GA mRNA are selectively expressed. When poly(A)+ RNA isolated from pig kidney was probed with a porcine GA cDNA that contained the shared coding sequence, all four GA mRNAs were detected (data not shown). However, the 5.0-kb GA mRNA was predominant. The RNase H experiments reported in this study suggest that the two smaller GA mRNAs are produced from the same initial transcript by use of appropriately positioned alternative polyadenylation sites. However, both the RNase H mapping and the sequence analysis indicate that the 4.5-kb GA mRNA is the homolog of the hGAC mRNA (8). The genome analysis indicates that the GAC mRNA is produced by alternative splicing of a distinct exon within the GA gene. The finding that the unique sequence within the hGAC mRNA constitutes a single exon within the human hKGA gene provides strong evidence in support of the conclusion that the hKGA and hGAC mRNAs are formed by alternative splicing of the initial transcript of the same gene. In many well-conserved gene families, the number and lengths of the component exons are also conserved between species. Given the high percent identity between the hGAC cDNA and the porcine and rat homologs, it is highly likely that the exon arrangement present in the hKGA gene is conserved in the porcine and rat KGA genes.

An alternative splicing reaction could generate two distinct isoforms of the GA protein. Currently, the only evidence that the GAC mRNA encodes an active GA protein is the high GA activity measured in TSE cells that express only the hGAC mRNA (8). Translation of the rKGA mRNA yields a 74-kDa precursor protein that is translocated into the mitochondrial matrix and processed to yield the 68- and 66-kDa peptides that constitute the mature rat renal GA (23, 26). Assuming that the precursor translated from the GAC mRNA is similarly processed, the mature GAC protein would contain 60-kDa and/or 58-kDa peptides. In addition, the two proteins would contain distinct COOH-terminal domains. The sequence of the LGA is very homologous to the KGA protein except within the NH2- and COOH-terminal regions (5). The COOH terminus of the hLGA protein mediates its association with two PDZ domain-containing proteins, α1-syntrophin, and a glutaminase interacting protein termed GIP (22). The recombinant GIP protein inhibits GA activity when added to crude rat liver extracts (2). These observations suggest that the COOH-terminal domains of the various GA isoenzymes may constitute sites for selective protein-protein interactions. Thus alternative splicing may result in the formation of two KGA isoforms that contain distinct COOH-terminal protein interactive domains. Selective interactions of the GA protein with a membrane anchoring protein could account for its loose association with the inner surface of the mitochondrial inner membrane (28). Alternatively, selective association of either KGA protein with the mitochondrial glutamine transporter or the subsequent enzymes of glutamine metabolism could facilitate the acute activation of renal glutamine catabolism that precedes increased expression of the GA protein during the rapid onset of an acute acidosis (7).

Increased expression of rat renal GA during chronic acidosis results from selective stabilization of the rKGA mRNA (14, 20). This process is mediated by 8-base AU sequences that function as pHREs (18, 19). In the rat, both the KGA and GAC mRNAs contain multiple pHRE sequences. Thus both mRNAs may be stabilized and increased during chronic acidosis. However, in the pig and human, only the GAC variant of the KGA mRNA contains multiple pHRE sequences. Based upon the rat model, one would hypothesize that only the pGAC and hGAC mRNAs would be increased within the proximal tubule of their respective species during chronic acidosis. Such an induction could be mediated by cell-specific alternative splicing such that only the GAC mRNA is produced within the proximal tubule cells. Alternatively, only the KGA mRNA may be constitutively expressed in multiple segments of the nephron during normal acid-base balance, and the onset of acidosis could lead to a cell-specific change in splicing that results in formation of the pH-responsive GAC variant solely within the proximal tubule. Either mechanism would result in the cell-specific induction of GA activity.

Based upon nucleotide and protein sequence homology, it was previously concluded that the rat LGA and KGA proteins were derived from separate, but structurally related genes (5). A comparison of the composition of the two genes established that the extensive region of high homology within both genes is encoded by 15 exons. Furthermore, the lengths of the 15 corresponding exons within the two genes are identical. These observations support the previous conclusion. They also suggest that the separate genes probably arose from gene duplication followed by divergent evolution. During evolution, significant differences in the composition and lengths of the introns arose. In addi-
tion, different 5’- and 3’-exons were added to the two genes to encode different NH2-terminal mitochondrial targeting signals and COOH-terminal protein interacting domains, respectively. Finally, an alternative 3’-exon was inserted within the KGA gene to allow for the formation of the GAC isoform of the KGA protein.

The nucleotide sequences for the pGAC and rGAC cDNAs have been deposited in the GenBank under accession numbers AF490841 and AY063459, respectively. This work was supported, in part, by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-37124.

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