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Identification of two human glutaminase loci and tissue-specific expression of the two related genes

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Abstract. Glutaminolysis is initiated by either of two isoforms, K- and L-types, of the enzyme phosphate-activated glutaminase. The chromosomal localization, genomic organization, and the tissue-specific expression of the genes have been investigated in the human by using isoform-specific cDNA probes. Results obtained from radiation hybrid mapping experiments assigned the K-glutaminase gene to human Chromosome (Chr) 2, and a second locus for L-glutaminase in Chr 12 was identified. Southern blot analysis with the L-cDNA probe showed hybridization to a single restriction fragment, while four to seven fragments were found to hybridize to the K-cDNA probe. The distribution of human glutaminase expression was also investigated: the L-cDNA probe detected a single band of 2.4 kb in liver, brain, and pancreas, whereas a single transcript of approximately 4.4 kb was detected in kidney, brain, heart, placenta, lung, and pancreas by using the K-cDNA probe. This work provides evidence that the human liver and kidney glutaminase isozymes are encoded by separate genes located on different chromosomes; furthermore, the expression pattern in human tissues revealed for both isoenzymes differs notably from the paradigm based upon the isoenzyme distribution in rats.

Introduction

Glutamine, the most abundant free amino acid in the body, plays an important role in the nitrogen and carbon-skeleton exchange among different tissues, where this amino acid fulfills many different physiological functions. In this way, glutamine is used for brain neurotransmitter synthesis, hepatic urea synthesis, renal ammoniagenesis, gluconeogenesis in both liver and kidney, and as a major respiratory fuel for many cells (Kovacevic and McGivan 1983).

In all these cases, phosphate-activated glutaminase (PAG; EC 3.5.1.2) catalyzes the first step in the transformation of glutamine. In the rat, PAG appears as two forms, denoted liver and kidney types (L-PAG and K-PAG, respectively). The L-PAG is present only in adult rat liver, whereas the K-type enzyme is found in all other rat tissues with PAG activity, including fetal liver (Smith and Watford 1990). Both enzymic forms of PAG display different kinetic, immunological, and molecular characteristics (McGivan and Bradford 1983; Curthoys and Watford 1995) that may contribute to their differential short-term regulation and tissue-specific function. The complete cDNA for these rat PAG isoforms has been cloned (Shapiro et al. 1991; Chung-Bok et al. 1997). Despite the high overall degree of identity in amino acid sequences, the scattered amino acid substitution pattern suggests that the two PAG enzymes are the products of related but separate genes (Chung-Bok et al. 1997).

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In contrast, much less is known about glutaminase expression in humans, although recent molecular cloning studies are covering this lack of information. Thus, Nagase and colleagues isolated a PAG cDNA from a human brain library; sequence analysis showed that this cDNA is homologous to the rat K-type (Nagase et al. 1998). Afterwards, Elgadi et al. (1999) isolated a cDNA from human colon cancer cells, very similar to the K-PAG obtained from brain except in the C-terminal region; the authors conclude that this new isoform derived from alternative splicing of a primary transcript originated from the same K-gene. Very recently, we have cloned a PAG cDNA from a human breast cancer cell line, and, surprisingly, it turned out to be similar to the rat L-type (Gómez-Fabre et al. 2000). Indeed, this liver-type isozyme is expressed in human brain and pancreas, apart from liver, in sharp contrast to the currently accepted model, which establishes the presence of the K isoenzyme in all nonhepatic tissues, with the L-type being expressed only in postnatal liver (Curthoys and Watford 1995). The availability of specific DNA probes for both K and L human isoforms allowed us to determine the PAG gene organization and tissue-specific expression in the human, which are detailed in this paper.

Materials and methods

Cell culture. Human breast cancer cell line ZR-75-1 (Gómez-Fabre et al. 2000) was purchased from American Type Culture Collection (A.T.C.C., Manassas, Va., USA). The cell line was grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (Engel et al. 1978) at 37°C under air/CO_2 (19:1).

Radiation hybrid mapping experiments. Chromosomal locations of the two PAG genes were determined by using human × rodent hybrid panels, GeneBridge4 (Research Genetics Inc., USA). The presence or absence of each PAG gene in the whole panel was determined by PCR. The forward primers were 5′-TACCCCATTCCCTCAGCAGAC-3′ and 5′-CAAGCCATGGAGACAGGTAGC-3′ for L- and K-PAG, respectively. The reverse primers for the L- and K-isoforms were 5′-GGGCAAGC-CATTAGGCTGTACC-3′ and 5′-TAGTCACACAAAGCGGGCTGC-3′, respectively. PCR specificity was increased by a hot-start step. Briefly, PCR reactions, in a final volume of 10 μl, containing 25 ng DNA, 230 μM each dNTP, and 0.43 μM each primer, were subjected to a denaturing step of 3 min at 95°C. One unit of *Taq* DNA polymerase (Roche Diagnostics) was then added. A further 2-min denaturing step at 95°C was followed by 30 cycles of amplification with a denaturation temperature of 95°C for 15 s; annealing and extension at 65°C for 1 min. Afterwards, PCR products were size separated by electrophoresis in large submarine gels on 2% agarose gels. The PCR product lengths were 139 bp for K-PAG and 107 bp for L-PAG.

Human isoform-specific PAG probes. A 0.95-kb K-specific PAG probe was prepared by restriction of the human cDNA clone HK03864

```
L-PAG ----------------------------------------MRSMKALQKALSRAGSHCGR 20
K-PAG MMRLRGSGMLRDLLLRSPAGVSATLRRAQPLVTLCRRPRGGGRPAAGPAAAARLHPWWGG 60
                                                *  * :*  .  *

L-PAG GGWGHPSRSPLLGGGVRHHLSEAAAQGRETPHSHQP------------------------- 56
K-PAG GGWPAEPLARGLSSSPSEILQELGKGSTHPQPGVSPPAAPAAPGPKDGPGETDAFGNSEG 120
      ***   .  :  *...  . *.* .  . ..  . .*

L-PAG ---QHQDHD5SESGMLSRLGDLLFYTIAEGQERTPIHKFTTALKATGLQTSDPRLRDCMS 113
K-PAG KELVASGENKIKQGLLPSLEDLLFYTIAEGQEKIPVHKFITALKSTGLRTSDPRLKECMD 180
         ...:. :.*:*. * ************: *:*** ****:***:******::**.

L-PAG EMHRVVQESSSGGLLDRDLFRKCVSSSIVLLTQAFRKKFVIPDFEEFTGHVDRIFEDVKE 173
K-PAG MLRLTLQTTSDGVMLDKDLFKKCVQSNIVLLTQAFRKKFVIPDFMSFTSHIDELYESAKK 240
       :: .:* :*.* :**:***:***.*.*********:******* .**.*:*.::*..*:

L-PAG LTGGKVAAYIPQLAKSNPDLWGVSLCTVDGQRHSVGHTKIPFCLQSCVKPLTYAISISTL 233
K-PAG QSGGKVADYIPQLAKFSPDLWGVSVCTVDGQRHSTGDTKVPFCLQSCVKPLKYAIAVNDL 300
       :***** ******* .*******:*********.*.**:**********.***::. *

L-PAG GTDYVHKFVCKEPSGLRYNKLSLDEECIPHNPMVNAGAIVVSSLIKMDCNKAEKFDFVLQ 293
K-PAG GTEYVHRYVGKEPSGLRFNKLFLNEDDKPHNPMVNAGAIVVTSLIKQGVNNAEKFDYVMQ 360
       **:***::*********:*** *:*:. ************:**** . *:*****:*:*

L-PAG YLNKMAGNEYMGFSNATFQSEKETGDRNYAIGYYHEEKKCFPKGVDMMAALDLYFQLCSV 353
K-PAG FLNKMAGNEYVGFSNATFQSERESGDRNFAIGYYLKEKKCFPEGTDMVGILDFYFQLCSI 420
       :*********:**********:*:****:***** :******:*.**:. **:******:

L-PAG EVTCESGSVMAATLANGGICPITGESVLSAEAVRNTLSLMHSCGMYDFSGQFAFHVGLPA 413
K-PAG EVTCESASVMAATLANGGFCPITGERVLSPEAVRNTLSLMHSCGMYDFSGQFAFHVGLPA 480
       ******.***********:****** ***.****************************

L-PAG KSAVSGAILLVVPNVMGMMCLSPPLDKLGNSHRGTSFCQKLVSLFNFHNYDNLRHCARKL 473
K-PAG KSGVAGGILLVVPNVMGMMCWSPPLDKMGNSVKGIHFCHDLVSLCNFHNYDNLRHFAKKL 540
       **.*:*.************* ******:*** :*  **:.**** ********** *:**

L-PAG DPRREGAEIRNKTVVNLLFAAYSGDVSALRRFALSAMDMEQKDYDSRTALHVAAAEGHIE 533
K-PAG DPRREGGDQRVKSVINLLFAAYTGDVSALRRFALSAMDMEQRDYDSRTALHVAAAEGHVE 600
       ******.: * *:*:*******:******************:****************:*

L-PAG VVKFLIEACKVNPFAKDRWGNIPLDDAVQFNHLEVVKLLQDYQDSYTLSETQAEAAAEAL 593
K-PAG VVKFLLEACKVNPFPKDRWNNTPMDEALHFGHHDVFKILQEYQVQYTPQGDSDNGKENQT 660
       *****:********.****.* *:*:*::*.* :*.*:**:** .** .  . :.  :

L-PAG SKENLESMV 602
K-PAG VHKNLDGLL 669
       ::**:.::
```

Fig. 1. Amino acid alignment of human K-PAG and L-PAG. The deduced amino acid sequences of human K- and L-PAG (GenBank #AB020645 and AF110330, respectively) were aligned by using the CLUSTALW program. Amino acid identities are indicated by asterisks, highly conservative substitutions by colons, and conservative by full stops. The deduced amino acid sequences corresponding to the cDNA fragments used as probes are displayed in cursive.

(Nagase et al. 1998) with the enzymes *Bgl*II and *Eco*RI (nt 841–1791). As L-specific PAG probe, the 1.1-kb 5′-RACE clone of the human breast cancer cell PAG (nt 187–1259) was used (Gómez-Fabre et al. 2000). The cDNA fragments used as probes were coding sequence in both cases (Fig. 1). Radioactive labeling of these cDNA probes was performed by the random primed method using the High Prime kit (Boehringer) and [α-^{32}P]dCTP (Amersham).

Genomic DNA Southern blot analyses. High molecular weight genomic DNA was isolated from ZR-75 cells using TriReagent (Sigma, St. Louis, Mo.) according to the manufacturer's instructions. Genomic DNA (15 μg) was digested with selected restriction enzymes, size fractionated on 0.7% agarose gel, transferred to Hybond-N$^+$ nylon membrane, and processed as described elsewhere (Gómez-Fabre et al. 2000). Briefly, the membranes were pre-hybridized at 68°C for 30 min in QuickHyb solution (Stratagene) and hybridized with ^{32}P-labeled cDNA probes in the same solution at 68°C for 1 h. Washes were carried out in 2 × SSC/0.1% SDS at room temperature for 15 min and 0.1 × SSC/0.1% SDS at 42°C for 2 × 15 min. The filters were exposed to Kodak X-OMAT AR films at −80°C in the presence of intensifying screens for 5–7 days.

Northern blot analyses. The tissue distribution of human K- and L-type PAG mRNAs was determined by the hybridization of ^{32}P-labeled cDNA probes to a commercially available Northern blot of poly(A$^+$) mRNA (Clontech). Prehybridization and hybridization were carried out as described elsewhere (Gómez-Fabre et al. 2000). Afterwards, the membranes were washed twice in 2 × SSC, 0.1% SDS at 42°C, followed by two washes in 0.5 × SSC, 0.1% SDS, and 0.1 × SSC, 0.1% SDS, both at 60°C for 30 min. The membranes were then exposed to Kodak X-OMAT AR films at −80°C for 4–5 days. The integrity of the RNAs was checked with a GAPDH cDNA probe rehybridized in the same filter.

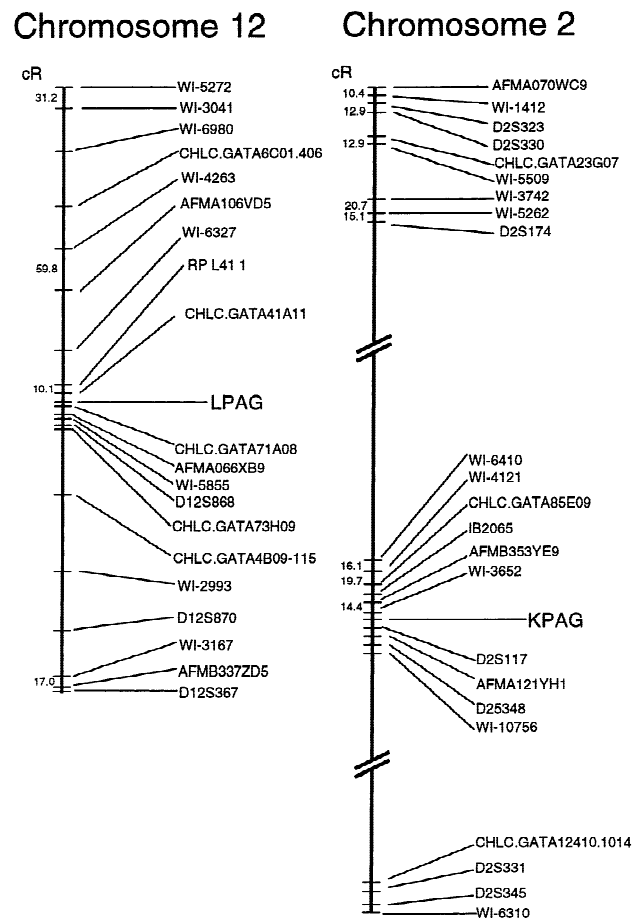

Fig. 2. Localization of the PAG loci on the human Chrs 2 and 12. The radiation hybrid map is presented as a vertical bar. Distances within the map are shown on the left, expressed as centirays (cR), where 1cR corresponds to 1% frequency of breakage between two markers after exposure to 3000 rad of X-rays.

Results and discussion

Recently, the isolation of two human PAG cDNA clones has been reported (Nagase et al. 1998; Gómez-Fabre et al. 2000). The nucleotide sequences have an overall similarity of 68%, and the deduced amino acid sequences show 72% identity (Fig. 1). The scattered amino acid substitution pattern observed is similar to that found for the rat kidney and liver isozymes (Chung-Bok et al. 1997). This pattern of similarity rules out the possibility of an alternative mRNA splicing process, suggesting that the K and L human PAG isoforms are probably encoded by different genes. Furthermore, the human PAG cDNA isolated from brain is highly similar to the rat K-isozyme, having a sequence identity of 95% in 668 amino acids (Nagase et al. 1998), whereas the human PAG cDNA isolated from breast cancer cells shares a considerable degree of identity with the rat L-isozyme (94% identity in 535 amino acids; Gómez-Fabre et al. 2000).

In spite of this information, to the best of our knowledge only one locus has been previously reported for PAG gene in mouse, rat, and human (Nagase et al. 1998; Mock et al. 1989). In this work, we have identified the chromosome on which the human L gene is located, using a comprehensive gene mapping procedure with the GeneBridge 4 radiation hybrid panel. The results indicated that the L gene is located on human Chr 12; this location is distinct from that of the K gene, located on Chr 2. The L-PAG gene is located at 6.5 cR_{3000} from the genetic marker CHL.GATA41A11 (Fig. 2). One centiray, cR_{3000}, corresponds to

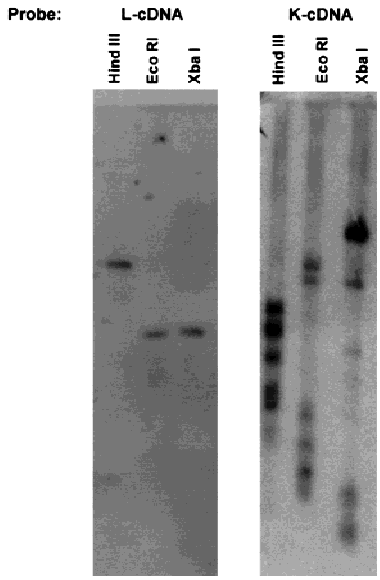

Fig. 3. Southern blot analyses of human genomic DNA. Samples containing 15 μg of ZR-75 breast cancer cell DNA were digested with indicated restriction enzymes, fractionated by gel electrophoresis, and hybridized with cDNA probes specific for each PAG isoform. Left panel taken from Gómez-Fabre et al. (2000).

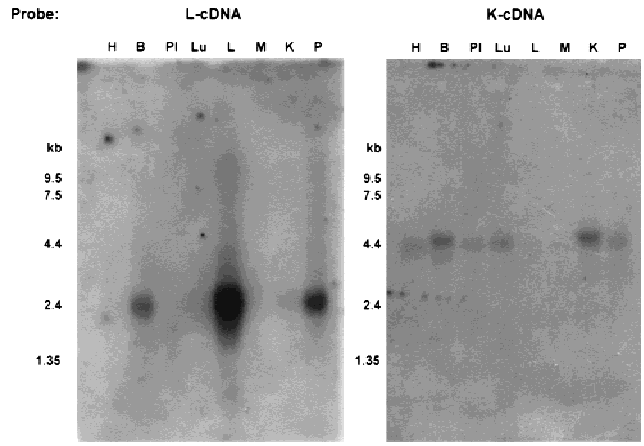

Fig. 4. Tissue-specific distribution of the human PAG isoforms. A human multiple-tissue Northern blot containing approx. 2 μg of poly(A^+) mRNA per lane was sequentially hybridized with cDNA probes for human L-PAG [left panel, taken from Gómez-Fabre et al. (2000)] and K-PAG (right panel). Lanes contained, from left to right, RNA from human heart (H), brain (B), placenta (Pl), lung (Lu), liver (L), skeletal muscle (M), kidney (K), and pancreas (P). RNA size marker bands are indicated on the left.

1% frequency of breakage between two markers after exposure to 3000 rad of X-rays (Walter et al. 1994). We have further corroborated the localization of the human K gene on Chr 2, employing radiation hybrid mapping and primers specific for the human brain K-PAG clone (Nagase et al. 1998). This last result is in good agreement with a previous work, which reported the location of human PAG on Chr 2 by using a rat brain cDNA clone as probe in Southern transfer analysis with somatic cell hybrid (Mock et al. 1989). The probe used by these authors was 100% identical to the rat kidney PAG cDNA (Shapiro et al. 1991), which further strengthens the view of the locus on Chr 2 being the K-PAG gene. Therefore, it can be concluded that there are at least two related but separate genes encoding for the K- and L-PAG, which map to distinct human chromosomes.

Total human genomic DNA was analyzed on Southern blot to further assess the complexity of the PAG genes. DNA was digested with different enzymes, electrophoresed, blotted onto a nylon membrane, and probed with a 0.95-kb and a 1.1-kb coding fragment from the K- and L-PAG cDNAs, respectively (Fig. 1). After digestion by endonucleases, a single restriction fragment hybridized to the L-cDNA probe, whereas four to seven fragments were detected when the K-cDNA probe was used (Fig. 3). The very different patterns obtained with both PAG probes are consistent with the finding of two isoloci for PAG genes. The nucleotide sequences for mammalian PAG genes have not been determined yet in any species, and, therefore, the genomic organization remains unknown. However, the very simple hybridization pattern observed with the L-PAG probe suggests that there is only one L gene. The more complex pattern obtained with the K-PAG probe may suggest that this gene is longer than the L gene, containing introns with the restriction sites used; nonetheless, the possibility of different copies for the K gene cannot be ruled out.

To determine the tissue distribution of K- and L-PAG mRNA species, we probed a human multiple-tissue Northern blot with the cDNA probes specific for each isoform (Fig. 4). The K-cDNA probe hybridized to a single transcript of approximately 4.4 kb that was highly expressed in kidney and brain. Lower levels of expression were also apparent in heart, placenta, lung, and pancreas, whereas no signal was observed in liver and skeletal muscle. This pattern of expression is identical with that previously reported by Nagase and co-workers (1998) using RT-PCR with primers specific for the cDNA clone HK03864. Furthermore, this tissue distribution of K-PAG is roughly similar to that observed by Elgadi et al. (1999) using Northern analysis with a K-PAG cDNA from human colon cancer cells, with two discrepancies: we were unable to detect a PAG transcript in skeletal muscle or the second minor band of 3.5 kb observed by these authors in kidney and pancreas.

A notably different expression pattern was revealed for L-PAG in human tissues: a cDNA probe for L-type PAG hybridized to a single band of 2.4 kb, and the most intense expression was seen in liver, although strong hybridization signals were also detected in brain and pancreas. No signal was observed in heart, placenta, lung, skeletal muscle, and kidney. It is noteworthy that neither K-type nor L-type PAG was apparent in skeletal muscle. This tissue-specific expression pattern contrasts with that reported for the rat (Curthoys and Watford 1995) and argues against the present view, which considers the K-type enzyme as the only isoform expressed in all tissues with PAG activity in mammals, with the exception of postnatal liver (Curthoys and Watford 1995).

The occurrence of different PAG transcripts in the same tissue has been reported in several species including pig (Porter et al. 1995), human (Elgadi et al. 1999), and mouse (Aledo et al. 1998). However, in all these cases, the expressed transcripts belong to the same PAG isoenzyme: the K-type or a highly related spliced form named C-type (Elgadi et al. 1999). We have presented experimental evidence that supports, for the first time, the view that in humans a single organ can simultaneously express different PAG genes, which may have profound physiological implications. For example, in brain the two PAG isoforms may play a different role in glutamate-mediated neurotransmission or may be expressed in different cellular types or subcellular compartments, allowing their regulation by separate cellular mechanisms. Interestingly, early studies have shown that PAG appears in soluble and membrane-bound forms (Nimmo and Tipton 1979), and the enzyme associates with mitochondrial and non-mitochondrial localizations (Aoki et al. 1991). Finally, it would be interesting to ascertain whether altered expression patterns of both isoforms can be ascribed to specific abnormalities or diseases.

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