Nuclear Localization of L-type Glutaminase in Mammalian Brain*

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In mammals, there are two different genes encoding for glutaminase isoforms, named liver (LGA) and kidney (KGA) types. LGA has long been believed to be present only in liver mitochondria from adult animals. However, we have recently reported the presence of LGA mRNA in human brain. We now describe the expression of LGA mRNA in the brain of other mammals (cow, mouse, rabbit, and rat) and in different areas of human brain as assessed by Northern blot analysis. The presence of mRNA encoding for this isoform in rat brain was further confirmed by reverse transcriptase-PCR cloning and sequencing. Although it has been well accepted that glutaminase is a mitochondrial enzyme, using newly generated isoform-specific antibodies, we have found a differential intracellular immunolocalization of both glutaminase isoforms in rat and monkey brain. In both species, KGA protein was present in mitochondria, whereas LGA protein was localized in nuclei. Furthermore, subcellular fractionation and Western blot analysis revealed that brain LGA was enriched in nuclei where it was catalytically active. Nuclear glutaminase exhibited a kinetic behavior that resembles that of the liver-type enzyme with regard to the low phosphate concentration requirement; however, nuclear glutaminase was susceptible to glutamate inhibition, a property that is absent in the rat liver enzyme.

Glutamate is the major excitatory neurotransmitter in mammalian brain and has been implicated in a plethora of physiological and pathological processes. Not surprisingly glutamate receptors are among the most studied and best understood molecules of the nervous system (1). Nevertheless, understanding the mecanism by which glutamate plays its role in diverse processes requires not only the detailed knowledge of the implicated postsynaptic receptors but also of the enzymes involved in the production of glutamate in the presynaptic neurons. Phosphate-activated glutaminase (GA, EC 3.5.1.2), an enzyme that catalyzes the conversion of glutamine to glutamate, has been considered as a major enzyme for the production of neurotransmitter glutamate (2). In mammals, there are two different genes encoding for GA, known as liver (LGA) and kidney (KGA) types (3). Both enzymatic forms of GA display different kinetic and molecular characteristics (4). In rat, LGA is believed to be present only in liver mitochondria from adult animals, whereas K-type enzymes are found in all other rat tissues with GA activity, including brain (5). In contrast, we have previously reported that human brain expresses transcripts for both isoenzymes KGA (3) and LGA (6). Now we have generated isoform-specific antibodies and cDNA probes to study the expression of both glutaminase isoforms in the brain of diverse mammalian species. Our results suggest a nuclear localization of the LGA protein, which is catalytically active and displays kinetic properties different from both the liver and kidney glutaminases.

MATERIALS AND METHODS

Northern Blot Analyses—KGA (nt 841–1791) and LGA-specific (nt 1118–1896) probes were prepared by restriction of the human cDNA clones HK03864 (7) and hGAZRT75 (6), respectively. The regional expression of K- and L-type mRNAs in human brain was determined by hybridization of *P-labeled probes to commercially available Northern blots of poly(A)+ mRNAs (Clontech). Poly(A)+ mRNA from whole cow, chicken, mouse, rabbit, and rat brain were purchased from Sigma or Clontech, fractionated, and processed as described previously (8). As an internal standard, the mRNA for β-actin was detected using a mouse cDNA (Ambion).

Reverse Transcriptase-PCR (RT-PCR)—Single-stranded cDNA was synthesized by reverse transcription of poly(A)† from rat brain by using SuperScript II RNase H reverse transcriptase (Invitrogen) and the antisense primer 5′-TCCAGAGGCCACACTGCTGGAC-3′ (nt 316–293, GenBank™ accession no. NM_138904). The cDNA was subsequently subjected to PCR for 30 cycles with primers deduced from the rat LGA cDNA: forward (nt 67–89), 5′-GAGGGGCATCTCTAGGCTCCTGC-3′; and reverse (nt 276–257), 5′-GCATCTGGCTACAGTCC-3′.

Generation of Antibodies against Recombinant KGA and LGA—A 596-bp fragment, encoding the last 119 amino acids of the KGA protein, was obtained by PCR using the human clone HK03864 (7) as template and the primers 5′-GAAGGTGGGATCCAGGGTAAATT- C-3′ (forward) and 5′-GTTAATATGCTAAGCTTGAGGAC-3′ (reverse). This PCR product, containing BamHI and HindIII restriction sites, was cloned into the pQE-31 vector to express in Escherichia coli a His-tagged recombinant protein (KGA (551–669)), which was purified by immobilized metal ion affinity chromatography. The whole coding sequence of KGA was also expressed in E. coli as a fusion protein (GST-KGA) using the pGEX-6P-1 vector. The whole coding sequence of

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human LGA was expressed in *E. coli* as a recombinant protein (LGA-(1–602)) and as a fusion protein (GST-LGA) using the vectors pET-3c and pGEX-4T-1, respectively. In all the cases, the purified proteins were used for hyperimmunization of two New Zealand White rabbits for each immunogen, and polyclonal antibodies were generated as described elsewhere (9).

Generation of Antibodies against LGA Peptides—Three non-overlapping peptides 20–24 residues long, corresponding to different regions through the complete human LGA (6), were synthesized. They are noted with two numbers that indicate the position on the primary structure of the first and the last residues of the peptide. They correspond to the following sequence segments: LGA-(311–333), LGA-(462–485), and LGA-(583–602). Before immunization, the peptides were coupled to keyhole limpet hemocyanin using standard methods (9).

Purification of Antibodies—Five to ten milligrams of the recombinant proteins (KGA-(551–669) or LGA-(1–602)) or 3–5 mg of the LGA-(583–602) peptide coupled to a CNBr-activated Sepharose 6MB gel according to the manufacturer’s manual (Amersham Biosciences) and used for affinity purification of their respective antisera as described elsewhere (9). For preadsorption experiments, the working dilutions of the antibodies contained 150 g of the antigenic recombinant protein or peptide.

Preparation of Nuclei—Isolated rat brains (7–8 g) were added to 4 volumes of buffer A (0.25 M sucrose, 3 mM CaCl₂, 1 mM EDTA, 0.5 mM EGTA, and 5 mM Tris-HCl, pH 7.5) and homogenized by 18 strokes in a Teflon/glass homogenizer. All subsequent procedures were carried out as described previously (10). The protein content in each fraction was measured using the Bradford method (11). Cytochrome c oxidase and phosphate-activated glutaminase were assayed by standard enzymic methods (12, 13).

Immunocytochemistry—Adult male rats (Sprague-Dawley) and macaque monkeys (*Macaca mulatta*) were deeply anesthetized and perfused transcardially with phosphate-buffered saline followed by ice-cold fixative solution (4% paraformaldehyde, 75 mM lysine, and 10 mM sodium metaperiodate for the rat and 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% saturated picric acid solution for the monkey). Fixed brains were processed as described previously (15). Free floating sections, after blocking the endogenous peroxidase activity, avidin, biotin, and biotin-binding proteins, were incubated with one of the affinity-purified anti-glutaminase antibodies (anti-KGA at 1:500 or anti-LGA at 1:10) for 48 h at room temperature and processed with the avidin-biotin method using biotinylated anti-rabbit IgG (Vector Labs, 1:500) and ExtrAvidin-peroxidase conjugate (Sigma, 1:2000). Immunoreaction product was visualized with 0.05% diaminobenzidine, 0.03% nickel ammonium sulfate, and 0.01% H₂O₂. For the double immunofluorescence labeling, sections were incubated with anti-KGA and a monoclonal anti-mitochondria antibody (16) and visualized with Alexa 488 goat anti-rabbit IgG and Alexa 568 goat anti-mouse IgG (1:1000, Molecular Probes), respectively. In addition, double fluorescence labeling was carried out with anti-LGA using Alexa 488 anti-rabbit IgG and propidium iodide (4 μg/ml) for 10 min to visualize nuclei. Finally, we performed double immunoperoxidase labeling to co-localize KGA and LGA. For this, sections were incubated first in anti-KGA and processed as described above for single labeling. After incubation with diaminobenzidine/nickel, sections were treated for 45 min with 1% H₂O₂ in phosphate-buffered saline and incubated with anti-LGA. This second labeling reaction was performed with the peroxidase-anti-peroxidase method and visualized only with diaminobenzidine. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and approved by the committee of animal use for research at Málaga University. All efforts were made to minimize the number of animals used and their suffering.

RESULTS

**K- and L-type Glutaminase mRNA Expression in Mammalian Brain**—Fig. 1 shows the regional distribution of both messengers in human brain as assessed by Northern blot analyses using isoform-specific probes. The mRNA encoding LGA was present in all regions of the brain examined (Fig. 1A, middle panel). The strongest signal was seen in poly(A⁺) mRNA extracted from the cerebral cortex; substantial labeling was observed in the cerebellum, and less was observed in the putamen. A weak hybridization signal was also revealed in the lanes containing mRNAs from the medulla and spinal cord when the exposure time was prolonged for more than 36 h (not shown). A similar pattern of expression was found for the KGA mRNA (Fig. 1A, top panel). The transcripts of both isoforms possess very different molecular sizes (~5 and 2.4–2.8 kb for the KGA and LGA mRNA, respectively); this fact rules out the
possibility that the results presented in Fig. 1 may be due to cross-hybridization between the LGA probe and the KGA mRNA and vice versa. This pattern of expression seemed to contrast with that previously reported for the rat (5). Therefore, we next investigated whether other mammalian species showed co-expression of both GA isoforms in brain. Northern blots of mRNA from whole cow, chicken, mouse, rabbit, and rat brain were analyzed. The kidney-type glutaminase cDNA probe detected transcripts in all species, including chicken (Fig. 1B, top panel). When these samples were probed for LGA under stringent conditions, two close bands of 2.4–2.8 kb were detected in all of the mammals analyzed but not in chicken (Fig. 1B, middle panel). Surprisingly, rat brain showed transcripts with the electrophoretic mobility expected for the LGA mRNAs and that did hybridize specifically with the LGA CDNA probe. Because this result was in conflict with the generally accepted idea that, at least in rat, the expression of LGA is restricted to the adult liver, we further investigated the presence of LGA mRNA in rat brain by RT-PCR. A PCR product of the expected size (210 bp) was obtained (Fig. 1C), cloned in pGEMT, and sequenced. Comparison of the sequence of this insert with that of the cDNA for rat LGA (GenBank™ accession no. NM_138904) gave a 100% identity with the exception of a nucleotide duplication at position 24 of the PCR product (Fig. 1D).

**Isoform-specific Antibody Production and Characterization**—To confirm the presence of both glutaminase protein isoforms and to carry out a comparative study of the cellular and subcellular localization of these isoenzymes, we developed a battery of antibodies (see “Materials and Methods”). To assess potential cross-reactivity of our LGA antisera against KGA and vice versa, we immunoblotted proteins from rat liver and kidney samples as well as the recombinant fusion proteins GST-LGA and GST-KGA. The ability to recognize the LGA but not the KGA antigen was used as a criteria to designate a serum as LGA-specific and vice versa (Fig. 2A). Antibodies anti-KGA-(551–669), anti-LGA-(583–602), and anti-LGA-(1–602) were selected for subsequent experiments because they had a higher isoform-specific signal-to-noise ratio than the others. Despite the high degree of similarity between the amino acid sequences of KGA and LGA, anti-LGA-(1–602) specifically recognized the LGA protein and was unable to detect the KGA protein (Fig. 2A, right panel). This specificity was further verified by preadsorption experiments. The immunoreactivity observed against recombinant LGA proteins was completely abolished when the antisera anti-LGA-(1–602) was preincubated with the antigenic protein LGA-(1–602). Moreover, the fusion protein GST-KGA failed to preabsorb the anti-LGA serum (Fig. 2B) but succeeded in preabsorbing the anti-KGA antibody (result not shown).

**Immunolocalization of the KGA and LGA Proteins in Rat and Monkey Brain**—The distribution of the KGA and LGA proteins in rat and monkey brain was studied by light microscopy immunocytochemistry using affinity-purified antibodies. Immunoreactive patterns were the same regardless of the species under consideration. Fig. 3, A–C, shows a high immunoreactivity in the cerebral cortex according to the high mRNA levels found in human brain for both isoforms. Examination under higher magnification revealed a cytoplasmic particulate immunoreactivity pattern for the KGA antigen, strongly suggestive of a mitochondrial localization (Fig. 3, E and F). This location of the KGA protein was confirmed by double immunofluorescence labeling with anti-KGA and anti-mitochondria antibodies using laser scanning confocal microscopy (Fig. 3, J–K). However, LGA immunoreactivity was concentrated in neuronal nuclei (Fig. 3D). The same results were obtained with anti-LGA-(311–333), anti-LGA-(462–485), and anti-LGA-(583–602) (results not shown). Moreover, preadsorption of the antibodies with LGA-(1–602) completely abolished the immunoreactivity in brain sections, confirming the specificity of the antibodies (results not shown). Interestingly, double labeling images (anti-LGA/propidium iodide) indicated that many, but not all, cell nuclei exhibited LGA immunoreactivity (Fig. 3, L–N). Double labeling for KGA and LGA revealed that both isoforms co-localize in numerous cells throughout the brain (Fig. 3, G and H).

**Nuclear LGA Is Catalytically Active**—To assess whether the LGA protein found in the nucleus was a catalytically active enzyme, we performed subcellular fractionation on brain from adult rat. Western blot analysis revealed that LGA was enriched in nuclei relative to crude homogenate (Fig. 4A, right panel), further supporting the specific localization of this protein in the nucleus of brain cells. In contrast, KGA was detected in crude homogenate but was absent from the nuclear fraction, consistent with the notion that KGA is restricted to the mitochondrial compartment (17). Furthermore, cytochrome c oxidase activity, a mitochondrial marker (12), was undetectable when assayed in the nuclear fraction even though high amounts of total protein (up to 255 µg) were used. Nevertheless, a high cytochrome c oxidase activity was detected in crude extracts (not shown). The absence of KGA immunoreactivity and cytochrome c oxidase activity in the nuclear fractions in-
dicated that the purified nuclei were essentially free of mitochondrial contamination. We next investigated the capacity of these purified nuclei to catalyze the hydrolysis of the amide group of L-glutamine. These nuclear preparations exhibited a considerable phosphate-activated specific activity (0.14 μmol/mg of protein/min). As mentioned above, the kidney- and liver-type glutaminases display differential kinetic properties. Therefore, we addressed whether it may be possible to discriminate, by kinetic analyses, the GA activity found in nuclei from that detected in crude homogenates. Fig. 4B shows the effect of glutamate and phosphate concentrations on nuclear GA activity. The sensitivity for activation by phosphate of nuclear glutaminase was consistent with that expected for a LGA enzyme. At low phosphate concentration (10 mM), the glutaminase activity detected in nuclei was 78 ± 3% of that exhibited at high phosphate concentration (200 mM). This percentage was reduced to 28 ± 4% when crude homogenates were assayed. On the other hand, nuclear GA showed a strong and unexpected inhibition by glutamate (Fig. 4B). In contrast, only a slight decrease (18%) in activity was found when a liver crude homogenate was used as control (result not shown).

**DISCUSSION**

For many years the expression of LGA was believed to be restricted to the liver of postnatal animals (4, 18). Recently we have reported the presence of LGA mRNA in human brain (6).
absence and presence of 100 mM glutamate by measuring the ammonia concentrations by measuring the glutamate produced (12). Glutaminase activity was assayed at high (200 mM) and low (10 mM) phosphate concentrations by measuring the glutamate produced (12). Glutaminase activity was also determined at high phosphate concentration in the absence and presence of 100 mM glutamate by measuring the ammonia produced (13). The activity obtained under optimal conditions (200 mM phosphate and without glutamate) was taken as 100%. 100% values in \( \mu \text{mol/mg of protein/min} \) were 0.55 \( \pm \) 0.06 and 0.14 \( \pm \) 0.01 for crude homogenate and nuclei, respectively. Values are expressed as mean \( \pm \) S.E. of three different experiments.

**FIG. 4. Nuclear LGA is catalytically active.** A, representative Western blots showing the distribution of KGA and LGA in brain crude homogenates and nuclei. Proteins (20 \( \mu \)g) from each fraction were subjected to SDS-PAGE and immunoblotting as described under “Materials and Methods.” B, comparison of the kinetic properties of glutaminase from rat brain crude homogenate and from nucleus. Glutaminase activity was assayed at high (200 mM) and low (10 mM) phosphate concentrations by measuring the glutamate produced (12). Glutaminase activity was also determined at high phosphate concentration in the absence and presence of 100 mM glutamate by measuring the ammonia produced (13). The activity obtained under optimal conditions (200 mM phosphate and without glutamate) was taken as 100%. 100% values in \( \mu \text{mol/mg of protein/min} \) were 0.55 \( \pm \) 0.06 and 0.14 \( \pm \) 0.01 for crude homogenate and nuclei, respectively. Values are expressed as mean \( \pm \) S.E. of three different experiments.

In this work, we have confirmed and further extended our initial finding. The occurrence of LGA mRNA in the mammalian brain has been demonstrated by Northern analysis and RT-PCR, while the presence of LGA protein was proved by immunocytochemistry and Western analysis. An earlier report of tissue distribution based on Northern blot analysis showed that LGA was absent in non-hepatic rat tissues (5). The difference with our results was probably due to the fact that total RNA instead of mRNA was used; in addition, blot washes were performed at higher temperatures. In agreement with the presence of mRNA encoding for the LGA enzyme in mammalian brain, we were able to detect the LGA protein in rat and monkey brain.

Since glutamine serves as an oxidative substrate in most cells, the wide distribution of glutaminase throughout the human brain was not unexpected. Nevertheless, glutaminase was highly concentrated in the cerebral cortex, which is consistent with glutamate as a major excitatory neurotransmitter of projection neurons in this area. An excellent correlation between the KGA and LGA expression patterns was observed at the regional and cellular level. The co-expression in the mammalian brain of two GAs raises the intriguing question of why two isoenzymes are needed. In addition to its role in neurotransmission, glutamate participates in multiple metabolic pathways. Therefore, glutamate production must be a process exquisitely regulated to ensure a proper glutamate function.

Although the need for two GAs is so far unexplained, it may represent the biochemical basis to achieve this fine tuning under different physiological circumstances.

Besides the presence of LGA mRNA in mammalian brain, the main finding of this study is the nuclear localization revealed for LGA protein, which was previously thought to be exclusively a mitochondrial enzyme (4). Three arguments support this unexpected location. First, all anti-LGA antibodies used, regardless of whether they were directed to different peptides or elicited against the whole protein, gave the same nuclear pattern of staining in brain of two different species: monkey and rat. On the other hand, preadsorption of the affinity-purified antibodies with the protein/peptide used as immunogen completely abolished the immunoreactivity in brain sections, confirming the specificity of the antibodies. Second, subcellular fractionation on rat brain showed an enrichment of the LGA protein in nuclei with regard to crude homogenate from whole brain. Finally, isolated nuclei exhibited a phosphate-activated glutaminase activity that cannot be assigned to KGA, which was absent from the nuclear preparations.

There are a growing number of examples of proteins that function in more than one subcellular compartment (19). The mechanism by which LGA enters the nucleus at present is unclear because LGA lacks any known targeting signal specific for the nucleus. However, recent findings have established the importance of PDZ domain-containing proteins in localization to the nucleus (20–23), and we have reported the ability of the C terminus of LGA to mediate its association with PDZ domain-containing proteins (24). Therefore, it is tempting to speculate that LGA may reach the nucleus through interactions with other proteins, although this hypothesis remains to be explored. Nevertheless, translocation of a mitochondrial enzyme, lacking a specific nuclear targeting signal, to the nucleus is not without precedent. Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase has been detected in nuclei. Nuclear translocation of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase seems to involve interaction with nuclear hormone receptors through the LXXLL motif present in the mitochondrial enzyme (25). The LXXLL motif is a signature sequence that facilitates the interaction of different proteins with nuclear receptors (26). Strikingly, LGA contains one such consensus motif, LGDGL, at positions 5–9 of the rat protein sequence; whether this short motif is necessary and sufficient to mediate the interaction between LGA and some nuclear receptor is an issue that we are currently addressing.

Interestingly, there is a growing body of evidence pointing to glutamine as a signal molecule involved in gene expression. For example, glutamine, but not glutamate or ammonium, leads to increased levels of Hsp72 mRNA and protein. It is noteworthy that the non-metabolizable glutamine analog 6-diazo-5-oxonorleucine mimics the effects of glutamine on Hsp expression, indicating that glutamine by itself may function as a transcriptional signal (for review, see Ref. 27). However, the identity of the protein target for glutamine remains elusive. In this context, a working hypothesis could be outlined. This is based upon the following circumstantial evidence: (i) the existence of a LXXLL motif in the N-terminal region of LGA, (ii) the ability of the C terminus of LGA to interact with PDZ domain-containing proteins (24), and (iii) the fact that this protein-protein interaction can affect glutaminase activity (14). Therefore, it is tempting to postulate that LGA may form part of a transcriptional complex as a coregulator and that the conversion of glutamine to glutamate, after glutaminase activation, may facilitate the association/dissociation to/from nuclear receptors, particularly in light of the evidence of rapid turnover of receptor interactions (28). In this way, nuclear LGA may bridge the current gap...
between the glutamine signal and the transcriptional machinery. Alternatively, the protein fulfilling this function may be other than glutaminase. In this case, the significance of the nuclear LGA localization could be as simple as being an enzyme controlling in situ the glutamine levels in the nucleoplasm and therefore being indirectly involved in the expression of glutamine-regulated genes. In summary, we have shown that LGA is expressed in the mammalian brain with a nuclear localization and that this nuclear enzyme is catalytically active and displays kinetic properties different from both liver and kidney glutaminases. These findings open a new avenue of research on how glutamine may affect neuronal function.

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