Glutaminase: A multifaceted protein not only involved in generating glutamate

Javier Márquez *, Amada R. López de la Oliva, José M. Matés, Juan A. Segura, Francisco J. Alonso

Departamento de Biología Molecular y Bioquímica, Laboratorio de Química de Proteínas,
Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain

Received 13 October 2005; received in revised form 27 October 2005; accepted 27 October 2005
Available online 3 March 2006

Abstract

The protein glutaminase has been traditionally considered as a mitochondrial enzyme, playing a key role in the energy and nitrogen metabolism of mammalian cells. However, new experimental evidence in the last few years has challenged this simplified view. The recent discovery of novel extramitochondrial localizations, the identification of potential protein interacting partners, the existence of multiple transcripts for mammalian glutaminase genes, and the presence of signature sequences and protein motifs on its sequence support the notion of glutaminase being a multifaceted protein, which may be involved in other functions besides glutamate generation from glutamine. In this short review, we will briefly summarize recent works on glutaminase proteins in mammals, with particular emphasis in brain studies. This experimental evidence will then be used to highlight new potential roles for this classical metabolic enzyme.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Glutaminase; Glutamate; PDZ-proteins; Neurones; Protein–protein interaction; Nucleus; Mitochondria

1. Introduction

Phosphate-activated glutaminase (EC 3.5.1.2) hereinafter referred to as GA, catalyses the hydrolytic deamidation of glutamine to glutamate and ammonia (Krebs, 1935). In mammals, the enzyme needs inorganic phosphate for in vitro activity and plays a key role in several physiological processes, such as renal ammoniagenesis, hepatic ureagenesis and synthesis of neurotransmitter glutamate in brain (Curthoys and Watford, 1995). Glutaminase also initiates glutamine catabolism which is very important for the cell’s bioenergetics (Kovacevic and McGivan, 1983).

In humans, there are two genes encoding glutaminase isoforms in different chromosomes: one gene is located in chromosome 2 and encodes the kidney-type (K) isozyme; the second locus is located on chromosome 12 and codes for the liver-type (L) isozyme (Aledo et al., 2000). The human K gene spans 82 kb. Its genomic sequence was split into 19 exons after comparison with available human cDNAs (Porter et al., 2002).

At least two different transcripts arise from this gene: the KGA mRNA formed by joining exons 1–14 and 16–19, and the alternative spliced transcript GAC mRNA which uses only the first 15 exons, omitting exons 16–19 (Elgadi et al., 1999; Porter et al., 2002).

The human L gene, located on chromosome 12, has a length bigger than 18 kb and split into 18 exons (Pérez-Gómez et al., 2003). Although located in different chromosomes, the human K and L genes share a considerable degree of sequence similarity. Apart from the additional exon present in the human K gene, the main differences in the coding sequences of both genes are located at exons 1 and 18. Exon 1 shares 62.5% similarity, but it codes for 129 amino acids in KGA and only for 61 amino acids in LGA, accounting for the 67 extra amino acids of KGA protein at the N-terminal. The sequences encoded by exon 1 contain the signals involved in mitochondrial targeting and translocation processes (Shapiro et al., 1991; Gómez-Fabre et al., 2000). Likewise, exon 18, which codes for the C-terminal region of both proteins, shows the lowest sequence similarity (29.4%). This region of the human LGA protein has been demonstrated recently to be involved in the recognition of PDZ (PSD95/Dlg/ZO1 domains)-interaction modules (Olalla et al., 2001). Therefore, the most significant differences between

* Corresponding author. Tel.: +34 952 132024; fax: +34 952 132041.
E-mail address: marquez@uma.es (J. Márquez).
human LGA and KGA exons are located in regions involved with organelle targeting and protein–protein interactions, which may help to explain their differential function and regulation. Conversely, exons 3–17 of both mRNA transcripts have the same length and show a high sequence similarity. Altogether, these data suggest that GA genes may have evolved from a common ancestral gene, arising by gene duplication and divergent evolution, as has been previously noted for the rat genes (Porter et al., 2002).

2. Glutaminase expression in mammals

In the classical view of glutaminase expression in mammals, there were two main isoenzymes traditionally denoted liver and kidney-types, according to the nomenclature already seen for the glutaminase genes. In rat, LGA was believed to be present only in liver mitochondria from adult animals (Smith and Watson, 1990), whereas K-type enzymes were found in rat kidney, brain, small intestine, lymphocytes and foetal liver (Cutroff and Watson, 1995). Both enzymatic forms of glutaminase display different kinetic, immunological, and molecular characteristics (Kovacevic and McGivan, 1983). The enzymatic inhibition by the end-product glutamate (Glu) has been a hallmark to differentiate between KGA (strongly inhibited) and LGA (not inhibited) isoforms.

This classical pattern of glutaminase expression in mammals has been recently challenged by new experimental evidence. Thus, the LGA isozyme has been demonstrated to be expressed in other tissues apart from adult liver, like brain, pancreas, and breast cancer cells (Gómez-Fabre et al., 2000). On the other hand, the K-type isoform seems to be ubiquitous and is found in all other tissues with glutaminase activity. Moreover, simultaneous expression of both isoenzymes is a more frequent event than previously thought, as has been demonstrated in human tissues (Aledo et al., 2000) and cancer cells (Turner and McGivan, 2003; Pérez-Gómez et al., 2005). The third isozyme, named GAC, arises by alternative splicing of the K gene, having a distinct C-terminal region. This GAC isoform was first isolated from a human colon carcinoma cDNA library (Elgadi et al., 1999), but recently it has been reported to be present also in rat kidney and pig renal cells (Porter et al., 2002). The GAC isoform has been only characterized at the transcript level; thus, information concerning the protein product is not available. The existence of alternative spliced forms of the L gene seems very likely (Campos et al., 2003), as happens with the K gene, even though mRNA transcripts of different lengths have not yet been characterized for a single species.

In addition to fulfilling key tissue-specific physiological roles, GA is also overexpressed in a variety of experimental tumours and human malignancies (Pérez-Gómez et al., 2005; Szeliga et al., 2005). Furthermore, new therapeutic strategies, aimed at inhibition of GA expression by antisense technology, have shown promising results in animal tumour models (Lobo et al., 2000). However, the mechanisms by which GA expression is regulated in tumours remain unknown, making it difficult to ascertain if GA overexpression is a cause or an effect of the malignant transformation.

3. Glutaminase in brain

The amino acid glutamate (Glu) is the major excitatory neurotransmitter in the vertebrate CNS (Collingridge and Lester, 1989), and hence its synthesis and breakdown in brain has received considerable attention. Glutamate is utilized as a releasable transmitter or for general metabolism and the amino acid glutamine (Gln) plays an important role in both processes (Fonnum, 1984; Erecinska and Silver, 1990). Glutaminase is both an important contributor to transmitter pools of Glu (Nicklas et al., 1987) and the main Gln-utilizing enzyme of the brain (Kvamme, 1984). A glutamine–glutamate shuttle between neurons and glial cells has been postulated, based on the preferential localization of glutamine synthetase and glutaminase enzymes in astrocytes and neurones, respectively (Norenberg and Martínez-Hernández, 1979; Aoki et al., 1991; Laake et al., 1995). However, evidence for the involvement of several other enzymes in both the synthesis and degradation of glutamate has been also reported (Palaiologos et al., 1988; Erecinska and Silver, 1990; Waagepetersen et al., 2005).

We first described the simultaneous expression of LGA and KGA mRNA transcripts in human brain (Aledo et al., 2000; Gómez-Fabre et al., 2000). The regional distribution of both glutaminase transcripts in human brain was studied by Northern blot analysis using isoform-specific probes (Olalla et al., 2002). The mRNA encoding LGA was present in all regions of the brain examined. The strongest signal was seen in cerebral cortex. A weak hybridization signal was also revealed in medulla and spinal cord. A similar pattern of expression was found for the KGA mRNA. Furthermore, co-expression of KGA and LGA transcripts in brain was also demonstrated in other mammalian species like cow, mouse, rabbit and rat, while in contrast no signal for LGA was found in chicken.

Since glutamine serves as an important precursor for biosynthesis of neurotransmitter glutamate in neurons, as well as an oxidative substrate in most cells, the wide distribution of glutaminase throughout the human brain was not unexpected. Nevertheless, GA was highly concentrated in the cerebral cortex, which is consistent with glutamate as a major excitatory neurotransmitter of projection neurons in this area. The presence of both glutaminase protein isoforms at the cellular and subcellular levels was studied in monkey and rat brains by immunocytochemistry (Olalla et al., 2002). The study was focused on cerebral regions associated with the glutamatergic neurotransmission, where the immunolabel was more intense, concretely in cerebral cortex, hippocampus, cerebellum and striatum. A strong immunoreactivity was seen in the cerebral cortex according to the high mRNA levels found in human brain for both isoforms. The staining was seen in all cortical layers in both species, rat and monkey. The immunolocalization revealed a cytoplasmic particulate immunoreactivity pattern for the KGA antigen, strongly suggestive of a mitochondrial localization; however, LGA immunostaining was concentrated in neuronal nuclei (Olalla et al., 2002).

The location of KGA protein was confirmed by double immunofluorescence labelling with anti-KGA and anti-mitochondria antibodies using laser scanning confocal microscopy.
The mitochondrial localization of this isoform had been previously reported by other authors in mammalian brain using immunocytochemistry (Aoki et al., 1991; Laake et al., 1999). A predominant mitochondrial location for KGA is also consistent with data from subcellular fractionation studies using mammalian cells from brain (Roberg et al., 1995) and non-brain origin (Aledo et al., 1997). However, the nuclear localization revealed for LGA protein was completely unexpected. Double labelling images with anti-LGA antibodies and propidium iodide to stain nuclei indicate that many, but not all, neuronal cell nuclei exhibited LGA immunoreactivity. The same nuclear pattern of staining was seen in brain of two different species: monkey and rat (Olalla et al., 2002). LGA was previously thought to be exclusively a mitochondrial enzyme (Curthoys and Watford, 1995).

4. LGA protein: motifs and interaction partners

The co-expression of two GAs in the mammalian brain raises several questions with sound physiological meanings; for example, what is the rationale for having two glutaminase isoforms, which are the functions that LGA fulfil in nuclei, and how is LGA targeted to the nucleus. The LGA protein structure may give us some useful clues to get insights into these subjects.

Most mitochondrial proteins are encoded in the nucleus, synthesized in the cytoplasm, and transported into the mitochondria either post- or co-translationally. Although no consensus sequence has been found for mitochondrial localization, sequences with shared characteristics have been described. For the human LGA protein (Fig. 1), we have predicted a mitochondrial targeting signal located at amino acids 1–14, because this sequence has the requirements for a mitochondrial targeting signal (amino acids 1–14, Go´mez-Fabre et al., 2000), a propensity to form amphiphilic α-helix, an abundance of basic and hydrophobic residues and a lack of acidic amino acids (Gómez-Fabre et al., 2000).

Whereas LGA seems to have structural determinants needed for mitochondrial targeting, the mechanism by which LGA enters the nucleus is unclear because LGA lacks a discernible classical nuclear localization signal. However, LGA has other motifs that may be essential for its nuclear import. For example, it has a consensus motif for interaction with nuclear receptors (Fig. 1). The consensus sequence of the nuclear receptor box is LXXLL: human LGA possesses the sequence LGDLL on exon 1 which conforms to such consensus motif. This sequence motif allows for specific interaction with nuclear receptors and is primarily found in co-activators of nuclear receptors (Heery et al., 1997). We are currently addressing whether this short motif is necessary and sufficient to mediate the interaction between LGA and some nuclear receptors. Nuclear translocation of a mitochondrial enzyme containing a mitochondrial targeting sequence, but lacking a specific nuclear targeting signal, is not without precedent: mitochondrial 3-hydroxy-3-methylglutarylCoA synthase has been detected in nuclei and its nuclear translocation seems to involve interactions with nuclear hormone receptors through its LXXLL motif (Meertens et al., 1998).

Glutaminase L has been endowed with other consensus motifs and domains that might support its role as a multifunctional protein. In the C-terminal region, for example, the last four amino acids, ESMV, matches the consensus sequence X-Ser/Thr-X-Val required for interactions with PDZ proteins (Fig. 1). So, we decided to search for interactors of LGA that may be involved in the regulation of this protein in brain. A yeast two-hybrid genetic screen was performed to identify protein interaction partners of human LGA. We devised a strategy based on the design of two different baits, corresponding to the N-termini and C-termini of the glutaminase protein sequence. A human brain cDNA library was screened. After checking the specificity of the interactions, no real positives were isolated for the N-terminus bait while 19 hits were obtained for the C-terminal bait, which contained the last 256 amino acids of the LGA sequence. Several of these cDNAs were cloned and sequenced; two of them, alpha-1-sytropin (SNT) and GIP (glutaminase-interacting protein) coded for PDZ domain-containing proteins (Olalla et al., 2001).

The interaction between glutaminase and the PDZ domains of GIP and SNT was confirmed by in vitro interaction experiments using pull-down assays (Olalla et al., 2001). Thus, the binding of LGA/GIP and LGA/SNT seem highly specific interactions. We also tested the contribution of individual residues in the C-terminal domain of glutaminase. The analysis was performed with the two-hybrid assay in yeast by comparing the interaction of the C-terminal LGA bait protein with that of mutated forms, where residues at positions 0, −1, −2 and −3 were mutated to alanine giving mutants V602A, M601A, S600A and E599A. In Fig. 2, the results are monitored following the β-galactosidase assay in the plate, which indicates whether the interaction occurs or not and the strength of the interaction. As can be seen in both plates, the C-terminal valine was essential to maintain the interaction of LGA with both GIP and SNT, while the serine at position −2 and the glutamate at position −3 were also critical for GIP and SNT interactions, respectively. In contrast, the mutant M600A was without effect. Thus, there is strong evidence that the C-terminal amino acid residues of LGA are key determinants of the interaction.

Fig. 1. Schematic illustration of the human LGA protein showing the main signature sequences and motifs characterized and/or identified by sequence analysis. Starting from the N-terminal end, the following domains are shown: mitochondrial targeting signal (amino acids 1–14, Gómez-Fabre et al., 2000), nuclear receptor interaction box (72–76, Olalla et al., 2002), glutaminase domain (177–463, Pfam database, Bateman et al., 2004), ankyrin repeats (518–551 and 552–572, Pfam database, Bateman et al., 2004), and PDZ protein recognition module (599–602, Olalla et al., 2001).
The PDZ module is a peptide motif of approximately 90 amino acids initially found in proteins associated with synaptic, septate and tight junctions (Ponting et al., 1997). They mediate cellular protein–protein interactions, serve important roles in protein targeting and are scaffolds for assembling multiprotein complexes that facilitate the integration of signalling components and pathways. Some PDZ domains interact with the carboxyl terminus of ion channels and receptors and thus provide a mechanism for channel clustering (Kornau et al., 1995). The amino acid sequence of GIP is mainly composed of a single PDZ interaction module (Olalla et al., 2001). This protein was originally isolated as an interactor of the Tax-1 oncoprotein involved in the Rho signalling pathway: it allows transactivation of the c-Fos serum response element (RSE) through complex formation with RhoA and rhotekin (Reynaud et al., 2000). Interestingly, GIP has been also shown to participate in the regulation of transcription: the protein physically interacts with β-catenin inhibiting its transcriptional activity, being a critical component of the β-catenin regulatory network (Kanamori et al., 2003). Syntrophins are a family of intracellular peripheral membrane proteins expressed in skeletal muscle, heart, brain and some other human tissues (Ahn et al., 1996). The PDZ domain of α-1-syntrophin is known to bind the neuronal nitric-oxide synthase (nNOS) and to the water channel, aquaporin-4; the interactions have been postulated as a mechanism for selective targeting of these proteins to unique subcellular sites (Adams et al., 2001). These observations indicate that SNT works as a modular adaptor protein that can link signalling enzymes and regulate their subcellular localization.

The interaction of LGA with PDZ proteins may suggest a molecular mechanism to explain its nuclear translocation. In this regard, several PDZ domain-containing proteins have been reported to localize to the nucleus (Poulat et al., 1997; Hsueh et al., 2000). Moreover, a recent work demonstrated that selective interactions of target proteins with distinct PDZ-domain proteins determine their subcellular localization, including recruitment to the nucleus (Kausalya et al., 2004). From our results, it can be proposed that SNT or GIP might contribute to determining the subcellular localization of LGA in certain regions of the brain, thereby creating a mechanism for nuclear targeting and/or regulation of LGA function. Nevertheless, the physiological relevance of the interaction between LGA and PDZ proteins still remains to be elucidated.

Bioinformatics analysis of the LGA sequence identifies another protein–protein interaction motif presents in its primary structure: two ankyrin repeats in the C-terminal region (Fig. 1). Ankyrin repeats are about 33 amino acids long consisting of a β-turn and two antiparallel α-helices and have been found in proteins of diverse function such as transcriptional initiators, cell-cycle regulators, cytoskeletal, ion transporters and signal transducers (Sedgwick and Smerdon, 1999). The ankyrin-repeat proteins mediate many important protein–protein interactions in virtually all species. Of particular relevance in this context is the existence of transcription factors (GA-binding protein) and transcriptional regulators (IκB protein family, ANCO proteins) whose ankyrin repeats are essential functional domains for proper transcriptional activity (Batchelor et al., 1998; Sedgwick and Smerdon, 1999; Zhang et al., 2004). Furthermore, ankyrin-repeat motifs are critically required for nuclear localization of signalling proteins.
enzymes (Hozumi et al., 2003) and transcriptional cofactors (Sedgwick and Smerdon, 1999), being able to functionally substitute for a classical nuclear localization signal (Sachdev et al., 1998). Therefore, the involvement of this modular motif in the nuclear import of LGA will also require further attention.

5. LGA as a multifunctional protein

Very recently, a third location for LGA has been described in human neutrophils (Castell et al., 2004). In these cells of the immune system, LGA was shown to be present in the cell surface. Subcellular fractionation revealed that the enzyme was enriched in the secondary granules and could be released into cell culture medium upon stimulation with the phorbol ester PMA. The presence of LGA in these leukocytes was related with their bactericidal action through a glutamine-dependent mechanism of superoxide production, while its secretion to the extracellular space suggested a role in the anti-inflammatory response by regulating interleukin-8 production. These results point towards a cell-specific subcellular location of LGA and increase the number of potential roles this protein may fulfill. Furthermore, the fact that secretion of LGA is achieved after PMA stimulation might suggest a mechanism mediated by protein kinase C.

Taking into account the results here presented, several working hypothesis concerning new functions of glutaminase can be put forward. First, LGA may form part of a transcription-regulating complex as a co-regulator, based on the existence of a nuclear receptor interaction signal in its protein sequence and on its ability to interact with PDZ proteins. Second, the significance of the nuclear LGA localization could be as simple as being an enzyme controlling in situ the glutamine/glutamate levels in the nucleoplasm and hence being indirectly involved in the expression of glutamine/glutamate regulated genes. The amino acid regulation of gene expression in mammals has become an important area of research. Glutamine may function as a cellular signal and its availability has been shown to regulate expression of a certain number of genes (Bungard and McGivan, 2004 and references therein). And third, LGA may be targeted to the plasma membrane to interact with unknown macromolecular components. Even more, LGA can be secreted through activation of specific signalling cascades; in the extracellular space, LGA may regulate glutamine level which, in turn, has a modulating effect on cytokine production in cells of the immune system.

The idea of one protein-one function has become too simple because many proteins are found to have two or more different functions. Multifunctional proteins is a mechanism to rise the number of functions by “reusing” proteins for an additional purpose. In this way, a single polypeptide may perform several roles at different times and/or places, increasing organism complexity without increasing the number of genes. There are an increasing number of proteins having more than one role in an organism: well known examples include phosphoglucone isomerase and glyceraldehyde-3-phosphate dehydrogenase (Jeffery, 1999). Thus, glutaminase may be added to the growing list of moonlighting proteins, that is, proteins having a second (or third) function. Moonlighting refers to a single protein that has multiple functions that are not because of gene fusions, splice variants or multiple proteolytic fragments (Jeffery, 2003). Glutaminase behaves very much like a moonlighting protein: a metabolic mitochondrial enzyme that may fulfil other functions in nucleus, in the cell surface or in the extracellular space. Further work will be needed to elucidate whether these new functions are distinct from its role as an enzyme.

6. Concluding remarks

LGA is characterized by several conserved modules, including two ankyrin repeats, a nuclear receptor interaction box and one PDZ domain, elements indicating that LGA may serve multiple purposes. Interestingly, LGA contain some unique motifs not shared by the KGA, the other GA family member expressed in brain. It is noteworthy that two isoenzymes of GA are expressed in human brain; the need for two genes and two GAs to support the single process of glutamate synthesis is unexplained, and identifying the role of each GA is an important factor in understanding glutamate-mediated neurotransmission.

New research points towards elucidation of the new functions of LGA. To address this issue, it would be important to ascertain whether the subcellular location of LGA depends not only on the cell type but also on the developmental state or growth conditions of the cell. Clearly, further studies are necessary to reveal the physiological significance of the interaction between LGA and PDZ proteins, like GIP and SNT. In this sense, the regional, cellular and subcellular localizations of GIP in mammalian brain will help to clarify whether GIP is an in vivo interaction partner of LGA that regulates its targeting to other organelles and/or its catalytic activity.

Acknowledgements

This work was supported by Grant SAF2004-02339 from the Ministry of Education and Science of Spain. We are indebted to the private company HUTESA, S.A. (Fuente de Piedra, Málaga, Spain), the Confederación de Empresarios de Málaga (CEM) and the Foundation of the University of Málaga for additional sponsoring of our research. A.R.L.O. was a predoctoral fellow from the Ministry of Education and Science of Spain. Thanks are due to Dr. J.A. Campos-Sandoval and Dr. L. Olalla for their comments and help with the graphic work.

References


