Gliap – a novel untypical L-asparaginase localized to rat brain astrocytes

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Abstract

L-asparaginases catalyse the formation of the neuroactive amino acid L-aspartate by deamination of asparagine. The major pathophysiological significance of L-asparaginase activity is in its clinical use for the treatment of acute lymphatic leukaemia and neoplasias that require asparagine and obtain it from circulating pools. Here we report the identification and characterization of Gliap, a cytosolic L-asparaginase, which is the founding member of a new group of L-asparaginases in mammalia. Structural modelling suggests that Gliap is an atypical mammalian type-I asparaginase inasmuch as it harbours the active centre of a type-I glycosylasparaginase but, like plant-type asparaginases, lacks their auto-proteolytic site and, in addition, exhibits significant type-II L-asparaginase enzymatic activity. Moreover, in contrast to glycosylasparaginases Gliap is enriched in the cytosolic fraction and not in lysosomes. The protein is particularly abundant in liver, testis and brain. In brain Gliap is exclusively expressed in astrocytes and prominently present in structures reminiscent of glial endfeet. These data suggest that Gliap is involved in astroglial production of the neuroactive amino acid L-aspartate.

Keywords: aspartate, brain, glia, glial endfeet, glycosylasparaginase.


L-aspartate is a neuroactive amino acid that is thought to act as an excitatory neurotransmitter in some brain regions (Gundersen and Storm-Mathisen 2000). Like glutamate, the principal excitatory neurotransmitter in neural tissue, L-aspartate plays an important role in general cell metabolism. L-aspartate is formed in a transamination reaction catalysed by L-aspartate transaminase. This reaction uses the L-aspartate α-keto acid analogue, oxaloacetate, and glutamate as the amino group donor. In brain the major metabolic pathways for L-aspartate production are thought to be the synthesis of a glutamate pool as precursor for L-aspartate, the tricarboxylic acid cycle and the GABA shunt (Gundersen and Storm-Mathisen 2000; Johannessen et al. 2001). L-aspartate, however, can also be formed by deamination of asparagine, which is catalysed by the enzyme L-asparaginase (ASP; L-asparaginase-amidohydrolase, EC 3.5.1.1) and results in the hydrolysis of L-asparagine to ammonia and L-aspartate. Two ASP isozymes have been described in bacteria: a secreted high-affinity enzyme (originally termed ASP II) and a low-affinity cytoplasmic enzyme (originally termed ASP I). The polypeptide chain of the secreted bacterial isozyme consists of 326 amino acids and the active enzyme is a homotetramer (Swain et al. 1993). Type-I ASPs of animals are lysosomal enzymes that exhibit glycosylasparaginase (GA) activity, i.e. they hydrolyse the β-aspartyl-glucosamine bond of asparagine-linked oligosaccharides and convert the asparagine residue into an aspartic acid (Kaartinen et al. 1992). In contrast to cytoplasmic ASPs GAs have a lower substrate affinity and are active as a tetramer only after autoproteolytic cleavage (Xuan et al. 1998). In this paper we describe a novel ASP termed Gliap that seems to be...
Materials and methods

Materials

TNT® Coupled Reticulocyte Lysate System was purchased from Promega (Mannheim, Germany); λZAP cDNA library was obtained from Stratagene (La Jolla, CA, USA); rat multiple tissue northern blot (MTN) blot was from BD Biosciences (Palo Alto, CA, USA); oligodeoxynucleotides were obtained from Invitrogen (Karlsruhe, Germany); pGEX-5X-I vector, glutathione-agarose and the ECL oligodeoxynucleotides were obtained from Invitrogen (Karlsruhe, Germany); Complete (Basel, Switzerland); Protran (Hamburg, Germany); L-aspartic acid protease inhibitors was obtained from Roche (Basel, Switzerland); Protran® nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany); horseradish peroxidase-labelled secondary antibodies were purchased from Dianova (Hamburg, Germany); 1-aspartic acid β-(7-amido-4-methylcoumarin) (AspAMC) was obtained from Bachem (Heidelberg, Germany) and Escherichia coli ASP was obtained from Sigma (Munich, Germany).

Cloning of Gliap cDNA and transcript analysis

The full-length Gliap cDNA was isolated from a rat hippocampus λZAP cDNA library. Northern blot analysis was performed using a rat MTN blot and a 32P-labelled cDNA (corresponding to rat brain homogenate [amino acid residues 76–333 (glutathione-S-transferase/maltose-binding protein fusion proteins and generation of antisera

Three EcoRI-Xhol fragments containing full length or C-terminal parts of rat Gliap [amino acid residues 76–333 (glutathione-S-transferase (GST)-maltose-binding protein (MBP)-G76) and 130–333] were amplified by PCR and ligated into the pGEX-5X-I and pMAL-2c vector. Fusion proteins were expressed in E. coli BL21 cells (Stratagene). Bacteria were lysed in phosphate-buffered saline (10 mM Na2HPO4, 10 mM NaH2PO4, 140 mM NaCl, pH 7.4) containing Complete® protease inhibitors using a French press. Lysates containing GST fusion proteins were adjusted to 1% Triton X-100 and purified on glutathione-agarose according to the manufacturer’s instructions. The MBP fusion proteins were purified as described by the manufacturer. The GST-G76 fusion protein was used to immunize two rabbits and one guinea pig. Specificity of antibodies was tested on immunoblots of crude rat brain homogenate by pre-absorption of the antibody with a Gliap MBP fusion protein or with affinity-purified antiserum. For affinity purification of the antisera, 250 µg MBP-H76 fusion protein was loaded on a preparative SDS-polyacrylamide gel, blotted onto nitrocellulose membranes, stained with PonceauS and the corresponding band was cut out. After blocking the blotted fusion protein with 5% bovine serum albumin (w/v) in Tris-buffered saline (25 mM Tris, 140 mM NaCl, pH 7.6) the filter strips were incubated overnight at 4°C with 1.5 mL antiserum. After extensive washing with 0.1% bovine serum albumin (w/v) in Tris-buffered saline the antibodies were eluted with 0.1 M glycine, pH 2.5, and immediately neutralized with 1 M Tris, pH 8.0.

Western blot analysis, immunoprecipitation, subcellular fractionation and immunolocalization

For western blot analysis tissue was homogenized in 20 mM Tris, pH 7.4, containing Complete® protease inhibitors. Samples were solubilized and 30 µg of total protein were analysed by SDS–PAGE and immunoblot analysis. On western blots, protein bands were visualized by horseradish peroxidase-conjugated secondary antibodies and the ECL system.

For subcellular fractionation rat brain was homogenized in 5 mM Hepes, pH 7.4, 320 mM sucrose containing Complete® protease inhibitors. Cell debris and nuclei were pelleted from the homogenate in a 1000 g centrifugation step (P1). Crude membranes (P2) were obtained from supernatant fluid S1 after a 12 000 g centrifugation. Cytosolic proteins (S3) were obtained after a 100 000 g centrifugation of S2. The pellet (P3) was first extracted with 1% Triton X-100 to get the Triton-soluble fraction (S4-Tx) and the detergent-insoluble pellet was then solubilized in 1% SDS (S5-SDS). Fractions (10 µg) of total protein were analysed by SDS–PAGE and immunoblot analysis. Immunohistochemistry and electron microscopy were performed with affinity-purified antibodies as described previously (Laube et al. 2002).

1-Asparaginase assay and glycosylasparaginase activity

The ASP assays were performed as described by Ylikangas and Mononen (2000). Briefly, after GST purification the protein concentrations were determined photometrically and the amount of undegraded Gliap fusion protein was determined densitometrically from a Commassie-stained gel. The molarity was calculated and set in comparison to the reference ASP from E. coli. The K₅₀ of Gliap was determined by the release of AMC at different total protein amounts (3–30 µg) over a period of 2 h with measurements every 2 min. Using these parameters the fluorescence signal showed a linear increase over time indicating that the Michaelis–Menten equation can be applied. For the calculation of the initial velocity measurements of three assays were averaged. The data were analysed in a direct linear plot as described previously (Henderson 1992) to minimize statistical errors. K₅₀ was calculated as the mean value with SD. The GA activity was determined by the Morgan–Elson reaction according to Reissig et al. (1955) and Tollersrud and Aronson (1989). The substrate X₄-(β-N-acetylglucosaminyl)-l-asparagine was purchased from Bachem (Torrance, CA, USA).

Structural modelling

Structural models were generated by comparative modelling using coordinates from GA structures (http://www.rcsb.org/pdb/9gaf, 1apz). The Gliap sequence was aligned on the template structure using swiss-pdb viewer (Guex and Peitsch 1997). The alignment
was adjusted manually and coordinates were processed for energy minimization and stereochemistry optimization using SWISS-MODEL (Guex and Peitsch 1997). The final coordinates were validated by WHAT-CHECK (Rodriguez et al. 1998) and PROCHECK (Laskowski et al. 1996). Detailed modelling data and coordinates are available at http://www.synprot.de. Also available there is a phylogenetic tree of Gliap and its orthologues generated by using the Fitch–Margoliash method and the program BioEdit.

Results

Cloning and sequencing of a cDNA coding for a 40-kDa mammalian L-asparaginase

Utilizing yeast two-hybrid screening to identify binding partners for the neuronal calcium-binding protein caldendrin (Seidenbecher et al. 1998) we isolated three independent clones sharing a central ASP-like domain. The observed interaction may not be of physiological significance as the two proteins are expressed in different cell types (see below).

Fig. 1 (a) Nucleotide sequence and deduced amino sequence of Gliap. Secondary structure is derived by modelling of Gliap using coordinates from glycosylasparaginase (GA) crystal structures (PDB entries, 9gaf and 1apz). Residues in the vicinity (8 Å) of the substrate-binding site are marked by a box. The precursor loop has the nucleophilic threonine at the position conserved for N-terminal nucleophile (NtN) hydrolases but misses the required aspartate at the cleavage site (vertical arrow). Helices are indicated by rectangles and beta-strands by arrows. Coordinates of the model structure are available at http://www.synprot.de. (b) Topology of Gliap. The characteristic central fold (shaded) of NtN-hydrolases is present in Gliap. This central fold locates the conserved nucleophile (Thr191), all substrate-binding partners and the oxyanion hole (Oxy). Helices are drawn as circles and beta-strands as triangles. (c) The model structure of Gliap depicted as a ribbon presentation. The precursor loop is placed in front of the active centre (Thr191 and Thr202). The active centre constitutes those from bacterial class II asparaginases, while the topology follows the fold of NtN hydrolases. In NtN hydrolases an aspartate at the asparaginase active at the precursor loop fills the binding pocket of the active centre. After cleavage the binding pocket (active centre) is free to bind Asn-terminal peptides. In bacterial type II asparaginases and in Gliap and its relatives a conserved glycine is found at this position, leaving enough space for a single asparagine molecule to access the active centre. (d) Alignment of Gliap (rat)–related sequences. Alignment derived from superimposition of model structures. The sequence of the template GA structure (pdb9gaf) has been added for reference. In NtN hydrolases a precursor loop is autoproteolytically cut (C) at aspartate using the triad HDT leaving the threonine as N-terminal nucleophile at the active centre. In Gliap residues required for autoproteolysis (A) are missing. On the other hand, several active centre residues (X), all substrate-binding residues (B), the oxyanion site (O) and the nucleophile threonine (N) are identical to the reference structure. In the short forms from mouse and human, two active site residues are missing. It remains questionable whether these proteins are active enzymes. NCBI sequence database accessions: ICAD20833, Rn, glial asparaginase (Rattus norvegicus); BAB24431, Mm, gene locus, 241004D18Rik (Mus musculus); AAH16106, Mm, gene locus, 241004D18Rik (Mus musculus); NP_079356, Hs, gene locus, ASRG1L (Homo sapiens); P30362, La, L-asparagine amidohydrolase (Lupinus arboreus); NP_125905, Pa, L-asparagine amidohydrolase (Pyrococcus abyssi); NP_519499, Ra, L-asparaginase precursor protein (Ralstonia solanacearum); Q47898, Cm, W11F mutant GA, pdb9gaf (Chryseobacterium meningosepticum) Type and position of the four residues (thr, thr, asp/glu, ser) which are conserved in glycoasparaginases and bacterial asparaginase II are indicated by an asterisk.
The unique structure of the predicted protein prompted us to clone the full-length cDNA from a rat hippocampus cDNA library. Of a total of six independent and overlapping clones, one clone encompassed 2000 bp and was used to deduce the open reading frame of a novel protein encoding 333 amino acid residues (Fig. 1a). We called this protein Gliap for Glial asparaginase (Accession no. AJ427914). Interestingly, three clones with a much shorter and different 3′-untranslated region were also isolated (Accession no. AJ427915). However, despite the presence of a 3′-terminal poly-A stretch we could not identify a poly adenylation signal or a splice junction in the 3′-prime region of these clones that would clearly indicate that they encode a Gliap splice isoform.

Several expressed sequence tags (GenBank Accession nos. BE101319, AI232205, BI415717, BI407521, AL556649 and BM923692) from rat, mouse and human are either identical or have a high degree of nucleotide identity with corresponding stretches in the Gliap cDNA. Moreover, while this work was in preparation a cDNA from rat testis was published encoding a protein identical to Gliap (Bush et al. 2002; GenBank Accession no. AF329099).

Tissue distribution and subcellular localization of Gliap in brain

In good correspondence to the deduced transcript size from the two types of cDNA clones northern blot analysis revealed two hybridizing bands at 1.2 and 2.2 kb (Fig. 2a). Gliap mRNA was present in brain, kidney, liver and particularly abundant in testis (Fig. 2a). Transcript distribution in brain was examined by in situ hybridization and revealed a broad pattern of Gliap expression in grey and white matter with no major differences in transcript levels between the regions examined (Fig. 2b). Three independent antisera raised in two different species specifically detect a protein doublet of 25/40 kDa in rat brain protein homogenates (Fig. 3a). Neither immunoreactive band was recognized by either of the corresponding pre-immune sera (Fig. 3a) and both could be blocked by pre-absorption of the antibodies with MBP-Gliap fusion protein (not shown). Moreover, an affinity-purified antiserum detects both bands and the immunoreactivity could be blocked by pre-absorption with MBP fusion protein (Fig. 3a). Finally, specificity of the antisera could be further documented by immunoprecipitation with rabbit antisera/subsequent immunodetection with guinea pig antisera and vice versa (not shown).

To verify the proposed open reading frame a coupled cell-free transcription/translation system was used to generate the primary Gliap translation products in vitro. In these experiments a single 40-kDa immunoreactive product was observed that comigrates with the upper Gliap immunoreactive band detected on brain protein blots when the full length Gliap cDNA was used for in vitro translation (Fig. 3b). Control reactions without added Gliap cDNA do not synthesize this polypeptide (Fig. 3b). Subsequent western blotting with protein homogenates from dissected brain regions and different tissues showed a complete match of transcript data and protein expression. Gliap immunoreactivity is abundant in
testis, liver, kidney and brain (Fig. 3c). Subcellular fractionation studies revealed that both protein bands are found in the soluble fraction with only minor amounts in Triton-X100 extracts of the crude membrane fraction (Fig. 3d).

Immunostaining of the cerebellum (Fig. 4a), cortex and hippocampus (not shown) with the affinity-purified antiserum shows that Gliap immunoreactivity was restricted to astrocytes. The peroxidase reaction product was distributed...
in a diffuse pattern throughout the cytoplasm of astrocyte perikarya and processes including the Bergmann glia cells in the cerebellar cortex (Fig. 4a). Gliap immunoreactivity always colocalized to either glutamine synthetase or glial fibrillary acidic protein immunolabel (not shown). In neuropil regions we observed, at the ultrastructural level, immunopositive astroglial processes adjacent to synaptic structures (Fig. 4). Oligodendrocytes, identified by 2¢,3¢-cyclic nucleotide-3¢-phosphodiesterase immunostaining, were free of reaction product (not shown). Gliap immunoreactivity could not be detected in neurons (Fig. 4). Thus, in brain Gliap is exclusively present in all cells that have an astroglial cell lineage (i.e. astrocytes and radial glia). No evidence was found for an association of Gliap with specific cellular organelles, such as lysosomes.
Enzymatic activity of Gliap

To analyse the postulated enzymatic function of Gliap we assayed the L-ASP activity of the GST-Gliap fusion protein fluorometrically using AspAMC as substrate. Utilizing this assay we could demonstrate that recombinant GST-Gliap is able to metabolize AspAMC (Fig. 5). Moreover, under the assay conditions used, the total activity of Gliap was found to be equal to or even higher than that of *E. coli* ASP type-II, which was used as a reference (Fig. 5). Thus, Gliap is fully functional as an L-ASP with a $K_m$ of 2.4 mM ($\pm$ 0.6 mM), which is lower than those of *E. coli* ASP type-II (Fig. 5).

Gliap, however, shows no GA activity. In 2 h 30 µg of protein could not cleave an amount of N-acetylaminoglucone from the substrate lying over the detection level (>1 nmol; data not shown).

Sequence alignment and structural modelling of Gliap

The Gliap cDNA currently shows high sequence homology to 69 NCBI database sequence entries from 35 different species. Alignment of the most homologous sequences (identities from 40–95%; available at http://www.synprot.de; Fig. 1c) shows that all of them share sequence homologies to GA, with the best fit to the structural representative GA from *Chryseobacterium meningosepticum* (PDB entry, 9gaf; Fig. 1c). To gain a better understanding of their three-dimensional structure and putative function we have used the protein databank coordinates of the GA precursor (9gaf) and the aspartate-bound GA (1apz from human) to generate model structures of Gliap and its relatives.

Interestingly, the Gliap family resembles the $\alpha_2\beta_2\alpha$-sandwich fold with 40% identical and 60% similar residues to those five secondary structure elements which are essential for structure and function of N-terminal nucleophile hydrolases (Oinonen and Rouvinen 2000; Fig. 1b). Previous structural analysis of wild-type and mutant GAs has unravelled residues required for auto-proteolysis of the GA precursor, for the substrate transition intermediate binding and the L-asparagine binding site (Tikkanen et al. 1996; Saarela et al. 1998; Xu et al. 1999). Proposed mechanisms of auto-proteolysis have been described in detail (Saarela et al. 1998; Xu et al. 1999), showing that the residues involved in auto-proteolysis and substrate conversion are located in the vicinity of the active centre. It was shown that the Asp151-Thr152 sequence in the precursor loop is an essential requirement for the activation of auto-proteolysis (Saarela et al. 1998). The histidine150 in the His-Asp-Thr triad is proposed to activate Thr152 by deprotonation of the $\beta$-hydroxyl group (Saarela et al. 1998) and the imidogroup of a tryptophan is required for the hydrogen network (Xu et al. 1999). With the exception of the conserved nucleophilic threonine, which is essential for the ASP reaction (Oinonen and Rouvinen 2000), none of these residues are present in the Gliap protein family (modelling data available at http://www.synprot.de). It should be mentioned, however, that plant asparaginases like Gliap also lack crucial residues in their precursor loop, allowing for the possibility that cleavage occurs via a different mechanism than the His-Asp-Thr triad (Borek and Jaskolski 2001).

Discussion

Here we report the cloning and characterization of Gliap, the founding member of a putative new family of ASP in mammalia. Gliap migrates as a protein doublet of 25/40 kDa in protein homogenates from rat brain. At present, the status of the smaller 25-kDa isoform is unclear. The fact that this band can be immunoprecipitated and subsequently immunodetected
with three different antibodies suggests that it might be a processed isoform of Gliap. Moreover, while the current work was in preparation a report was published on an asparaginase-like protein in rat testis that is identical to Gliap (Bush et al. 2002). In this report three immunoreactive bands were found with a Gliap antiserum, two bands corresponding to the 25- and 40-kDa isoforms that we found in brain and a much smaller fragment of about 15 kDa that seems to be only present in testis. Unfortunately, the nature of the 15-kDa isoform was not identified in this study and it should be mentioned that the antibodies used in our experiments will all detect putative N-terminal truncated versions of Gliap and thereby also a C-terminal 15-kDa isoform corresponding to a putative β-subunit. Interestingly, mass spectroscopy revealed that peptides derived from the 25-kDa isoform were all part of the N-terminal region of Gliap in front of the inactive precursor loop (Bush et al. 2002), suggesting that it might represent a proteolytically cleaved N-terminal α-subunit of Gliap. It has to be mentioned, however, that the fusion protein that was used for the determination of the enzymatic activity of Gliap was not substantially degraded during the experiment, making it rather unlikely that proteolytic processing is a prerequisite for the enzymatic activity of Gliap (Landwehr and Kreutz, unpublished observation).

Gliap is capable of utilizing AspAMC as a substrate and its ASP activity is even higher in comparison to a commercially available type-II ASP from E. coli. The $K_m$ of Gliap is clearly lower, which might suggest that larger amounts of substrate have to be used to reach the maximal velocity of the catalytic reaction of Gliap. In our hands, Gliap exhibited no aspartylglucosaminidase activity. Accordingly, no indication was found at the ultrastructural level for an association of Gliap with lysosomes, as has been shown previously for GAs, and no biochemical evidence was found for an association with microsomal fractions which contain lysosomes, as shown using subcellular fractionation methods. In contrast, Gliap is clearly concentrated in the cytosolic fraction. Moreover, although its sequence shows high similarity with GAs in its active centre and it also harbours the Thr at position 191 as the nucleophile involved in the ASP activity, it is not known whether astrocytes have specific requirements for the production of l-aspartate in contrast to other neural cells. Alternatively, Gliap might utilize l-asparagine as one metabolic source for the formation of neuroactive l-aspartate. However, the role of l-aspartate as an excitatory neurotransmitter has been discussed controversially (Gundersen and Storm-Mathisen 2000; Johannessen et al. 2001) and accordingly, the role of l-asparagine as a precursor for the neurotransmitter l-aspartate has been a matter of debate. A large portion of brain (cerebral) l-aspartate is synthesized from metabolites formed through the GABA shunt (Johannessen et al. 2001), indicating that this pathway is localized in interneurones, which is at variance with a role of l-aspartate as an excitatory neurotransmitter.

The major pathophysiological significance of l-ASP activity is in its clinical use for the treatment of acute lymphatic leukaemia and neoplasias that require asparagine and obtain it from circulating pools (Clavell et al. 1986; Asselin 1999). For the pathogenesis of acute lymphatic leukaemia it has been suggested that l-ASP is the enzyme mainly responsible for the synthesis of l-aspartic acid that is necessary for satisfying the living requirements of lymphoid cells. There are currently three preparations of ASP available: (i) E. coli (ASP, Elspar); (ii) the enzyme derived from Erw. chrysanthemi (ERW, Erwinaise) and (iii) pegaspargase (PEG, Oncaspar), the E. coli enzyme modified by covalent attachment of polyethylene glycol. A key limiting factor of l-ASP use has been the development of hypersensitivity to the drug (Asselin 1999). This drawback of l-ASP therapy is based on the development of anti-ASP antibodies that correlates with rapid ASP clearance and a significantly lower response rate. Thus, therapeutic strategies based on a human orthologue of Gliap might provide new therapeutic avenues to maximize the clinical efficacy of ASP as part of acute lymphatic leukaemia therapy.

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