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The C-terminus of human glutaminase L mediates association with PDZ domain-containing proteins[1]

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Abstract The enzyme glutaminase in brain is responsible for the synthesis of neurotransmitter glutamate. We used the two-hybrid genetic selection system in yeast to look for interactors of glutaminase in human brain. We have identified two proteins containing PDZ domains, α1-syntrophin and a glutaminase-interacting protein, named GIP, that showed association with human glutaminase L, as deduced from specificity test of the two-hybrid system. The complete GIP cDNA clone has 1315 nucleotides with a 372-base open reading frame encoding a 124-amino acids protein. Glutaminase associates with both PDZ proteins through its C-terminal end; mutagenesis of single amino acids revealed the sequence -ESXV as essential for the interaction. These data suggest the possibility that PDZ domain-containing proteins are involved in the regulation of glutaminase in brain.

Key words: Human glutaminase; Brain; Two-hybrid; PDZ protein; Syntrophin

1. Introduction

Phosphate-activated glutaminase (PAG, EC 3.5.1.2) is a mitochondrial enzyme that catalyzes the hydrolysis of glutamine to stoichiometric amounts of glutamate and ammonia. In most mammal tissues where the enzyme is expressed, it is involved in the generation of energy using glutamine as a major respiratory fuel; thus, many types of tumor cells [1], as well as actively dividing normal cells [2], exhibit high rates of glutamine utilization through this pathway. In the brain, PAG also plays a key role in the synthesis of the excitatory neurotransmitter glutamate, which indeed is used as a precursor of the inhibitory neurotransmitter GABA [3].

Furthermore, the enzyme regulates the cerebral concentrations of glutamine and glutamate, which are very important in processes such as ammonia detoxification [4]. A glutamine–glutamate intercellular cycle between neurons and glial cells has been suggested, in such a way that the glutamate released into the synaptic cleft is transported into the glia, converted to glutamine by glutamine synthetase and shifted back to neurons, where PAG will replenish the released glutamate stores. The location of glutamine synthetase mostly in glial cells [5] and that of PAG in glutamatergic fibers [6,7] support the cycle, although PAG immunoreactivity is present in glial cells too [7]. Within neurons, PAG associates with mitochondria and synaptic vesicles [7] and also appears in a soluble form [8]; results from biochemical studies have also indicated the existence of two PAG populations: a membrane-bound form and a soluble form [9]. These results raise the possibility that a single PAG population works to generate the glutamate needed for metabolic purposes, whereas the second might supply the releasable glutamate transmitter pool [7].

Molecular cloning has given additional evidence to strengthen the view of two PAG isoenzymes being operative in human brain: a kidney-type, K-isoform, has been cloned from human brain [10] and a liver-type, L-isoform, has been cloned from a human breast cancer cell line [11]. The enzymes are the products of separate genes encoded on different chromosomes and both are expressed in human brain [12]. The expression in human brain of two PAG transcripts encoded by different genes rises the intriguing question of which are the physiological functions of both isozymes.

In search for additional cellular factors that may be involved in the regulation of L-PAG in brain, we have used a two-hybrid system approach. In this paper, we describe the isolation of two potential interactors: α1-syntrophin (SNT) and a new glutaminase-interacting protein (GIP), which contain a PDZ protein recognition module [13]. Interestingly, human brain L-PAG may have the potential to interact with PDZ proteins because it possesses a C-terminal end, -ESMV, which matches the consensus sequence X-Ser/Thr-X-Val required for interaction [14], while the K-isoenzyme, which is also expressed in human brain, lacks this motif. In addition to the genetic analyses, direct interaction between PAG, GIP and SNT was shown by in vitro experiments and the essential residues of PAG involved were assessed by site-directed mutagenesis.

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[1] The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AF234997.
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Abbreviations: PAG, phosphate-activated glutaminase; SNT, α1-syntrophin; GIP, glutaminase-interacting protein; GST, glutathione S-transferase; PDZ, postsynaptic density protein PSD-95/SAP90, *Drosophila* tumor suppressor protein DLG and tight junction protein ZO-1

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2. Materials and methods

2.1. Baits construction

The N-terminal (N-PAG) and C-terminal (C-PAG) baits were generated by inserting into the pEG202 (pLexA) vector [15] the coding sequences for amino acid residues 1–392 and 347–602, respectively, of the human glutaminase full-length cDNA cloned from ZR-75 breast cancer cells [11]. The cDNA inserts were subcloned into the pEG202 vector as *Bam*HI–*Xho*I fragments, generated by PCR with the following primers: 5′-CATCAGAAAGTGGATCCTGTCCCGCCTGGG-3′ (sense primer) and 5′-GCCGCAGGAATGCTCGAGGCTGAGG-GTGTTGC-3′ (antisense primer) for N-PAG, and 5′-GCTGTGTT-CTGTGGGGATCCCTTGTGAATCAGGC-3′ (sense primer) and 5′-GTGTGGCCAGCTCGAGCTTTTTATTGAGCAGGG-3′ (antisense primer) for C-PAG. The two baits were sequenced in both directions to confirm the rightness of the constructions.

2.2. Yeast two-hybrid screening

Two-hybrid screening for PAG-interacting proteins was carried out as outlined by R. Brent and colleagues [16], using a Matchmaker two-hybrid system (Clontech). The yeast strain used was EGY48 (partial genotype MATα, his3, trp1, ura3, $LexA_{op\,(\times 6)}$-LEU2) transformed with the reporter plasmid p8op-LacZ. This host strain carries LEU2 and LacZ under the control of LexA operators. Screening was done with a 57 year old human whole-brain cDNA library (Clontech) tagged with B42 transcriptional activation domain in the pJG4-5 (pB42AD) plasmid [15], which allows galactose-dependent expression of cDNA-encoded fusion proteins. The library was first amplified and the final titer was 2.6×10^{10} cfu/ml. We first checked that the baits did not activate the transcription of the reporter genes by themselves, employing colonies of EGY48 transformed with URA3 LacZ reporter plasmid. The selection strains for both baits were then transformed with the library cDNA. Transformation was performed with a variation of the lithium acetate method [17] and the transformation efficiency was always $>10^5$ transformants/μg DNA. Library transformants were plated on Glu-Ura-His-Trp medium, harvested and frozen at −70°C in 1 ml aliquots in glycerol solution. After determining the plating efficiency (10^6 cfu/μl), transformants were plated on −Ura, −His, −Trp, −Leu medium, including galactose and raffinose to select for activation of the LEU2 reporter gene. The Leu+ yeast colonies were further screened by filter assay with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). The Leu+ and LacZ+ clones were restreaked and tested again for their Leu and LacZ phenotypes in a secondary screen. After that, putative positives were also tested against an unrelated protein fused to the DNA-binding domain, to discard false positives that do not show specificity for the target protein [18]. Only those clones that fulfilled the requirements for specificity on these assays were considered and sequenced on both strands with the forward (BCO1) and reverse (BCO2) pJG4-5 sequencing primers, using the dideoxy chain termination method [19] on an automated DNA sequencer ABI model 310 (Perkin Elmer), following the manufacturer's own protocol and reagents. Nucleotide and the deduced amino acid sequences were compiled and homologies determined against the GenBank, SwissProt, Protein Identification Resource and Protein Data Bank using BLAST [20] and the FASTA program of the University of Wisconsin Genetics Computer Group (Madison, WI, USA).

2.3. Generation of glutathione S-transferase (GST) fusion proteins and polyclonal antibodies

Amino acid sequence of residues 347–602 of the predicted C-terminal region of human PAG protein (C-bait), amino acids 49–505 of the human SNT protein [21], and the whole coding sequence of GIP were expressed in *Escherichia coli* as GST fusion proteins (GST-CtPAG, GST-SNT and GST-GIP). For construction of plasmids encoding GST-CtPAG, an *Eco*RI–*Xho*I fragment encompassing nucleotides 1074–1909 was ligated to the *Eco*RI–*Xho*I digested pGEX-6P-1 vector (Pharmacia). An *Eco*RI–*Xho*I restriction fragment of the SNT cDNA clone was also ligated to the *Eco*RI–*Xho*I-digested pGEX-6P-1 vector. For the GIP fusion construct, an *Eco*RI–*Eco*RI fragment containing the open reading frame (ORF) of GIP (nucleotides 64–438) was ligated to the *Eco*RI-digested pGEX-6P-1 vector. Amino acid sequence of the 602 residues of the predicted ORF region of hPAG protein was also expressed in *E. coli* as fusion protein (GST-PAG) as previously described [11]. For protein expression, these constructs and empty pGEX-6P-1 vector were transformed into the *E. coli* BL21(DE3) cells (Stratagene), and the fusion proteins were purified by using glutathione-Sepharose beads, as recommended by the supplier. The purified recombinant proteins were used for hyperimmunization of New Zealand white rabbits and polyclonal antibodies were generated as described elsewhere [22].

2.4. In vitro pull-down assays

The whole coding regions of the human L-PAG and GIP cDNAs were subcloned into the plasmid pcDNA3; the amino acid sequence 52–505 of the human SNT was subcloned into the plasmid pcDNA3, after changing the residue E-52 for a methionine by PCR. Purified plasmids were transcribed and translated using the TnT™-coupled reticulocyte-lysate system (Promega) according to the manufacturer's instructions. In vitro translated [^{35}S]GIP, [^{35}S]SNT or [^{35}S]PAG proteins were incubated, for 2 h at room temperature, with purified GST-PAG, GST-SNT or GST-GIP. Afterwards, GST fusion proteins were pulled-down by using glutathione-Sepharose beads. The beads were washed four times with binding buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 0.1% (w/v) Triton X-100 and the protease inhibitors kit complete® (Roche)) and suspended in sodium dodecyl sulfate (SDS) gel loading buffer [23]. After boiling for 5 min, the eluted proteins were analyzed by SDS–PAGE and fluorography. Gels were exposed to Kodak-OMAT AR films for 3 days at −80°C.

2.5. Mutagenesis of the C-terminal end of PAG

Point mutants were generated using the Quick Change Site-Directed Mutagenesis kit (Stratagene) and the coding region of PAG inserted into the pcDNA3 expression vector. The mutants V602A, M601A, S600A and E599A were generated in the pcDNA3 vector and checked by sequencing.

3. Results

To elucidate the existence of a signaling mechanism controlling glutaminase activity in brain, we have searched for proteins capable of interacting with PAG, using the yeast two-hybrid approach. Initially, the N- and C-termini of the

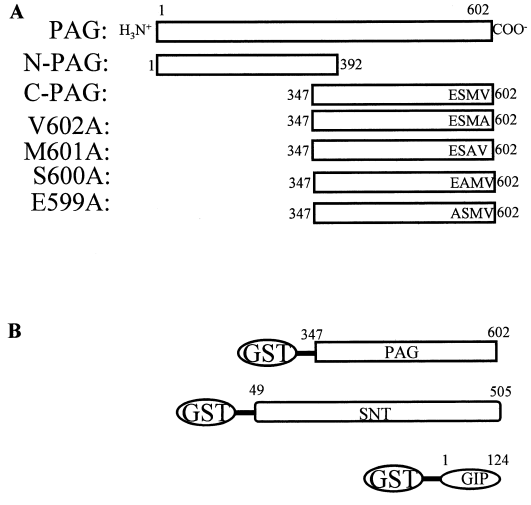

Fig. 1. Baits employed in the two-hybrid screen and constructs used to assess the structural requirements for PAG/GIP and PAG/SNT interactions. (A) Human PAG protein is schematically shown on the top, numbering besides the sequences indicates amino acids of PAG; N-PAG, N-terminal bait; C-PAG, C-terminal bait. Point mutants of the C-terminal bait tested by the two-hybrid assay for interaction with the PDZ clones: single amino acids were changed to alanine at positions 602V, 601M, 600S and 599E giving mutants V602A, M601V, S600A and E599A. (B) Schematic representation of GST fusion constructs tested for in vitro interactions by the GST pull-down assays.

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   1 GCTGCCGGCTTCTCGGAGCGGCGCTGGGCGACCAGAGCAGGGTCGAGATGTCCTACATCCCG
                                                     M  S  Y  I  P    5

  63   GGCCAGCCGGTCACCGCCGTGGTGCAAAGAGTTGAAATTCACAAGCTGCGTCAAGGTGAG
       G  Q  P  V  T  A  V  V  Q  R  V  E  I  H  K  L  R  Q  G  E   25

 123   AACTTAATCCTGGGTTTCAGCATTGGAGGTGGAATCGACCAGGACCCTTCCCAGAATCCC
       N  L  I  L  G  F  S  I  G  G  G  I  D  Q  D  P  S  Q  N  P   45

 183   TTCTCTGAAGACAAGACGGACAAGGGTATTTATGTCACACGGGTGTCTGAAGGAGGCCCT
       F  S  E  D  K  T  D  K  G  I  Y  V  T  R  V  S  E  G  G  P   65

 243   GCTGAAATCGCTGGGCTGCAGATTGGAGACAAGATCATGCAGGTGAACGGCTGGGACATG
       A  E  I  A  G  L  Q  I  G  D  K  I  M  Q  V  N  G  W  D  M   85

 303   ACCATGGTCACACACGACCAGGCCCGCAAGCGGCTCACCAAGCGCTCGGAGGAGGTGGTG
       T  M  V  T  H  D  Q  A  R  K  R  L  T  K  R  S  E  E  V  V  105

 363   CGTCTGCTGGTGACGCGGCAGTCGCTGCAGAAGGCCGTGCAGCAGTCCATGCTGTCCTAG
       R  L  L  V  T  R  Q  S  L  Q  K  A  V  Q  Q  S  M  L  S  *  124

 423   CAGCCACCACCATCTGCGACTCCTGCCTGCCGCCTCTCTGTACAGTAACGCCACTTCCAC

 483   ACTCTGTCCCCATCTGGCTTCTGCTGACCGCTGGGCCCCAGCTCAGAAGGGCTATAGCTG

 543   GTCCCAGAGGCCTGGCCTGGCCTTCCTTCCCTTCTCCCATCCCTGGCCTGGGGCCTCTGG

 603   GACCAGCTTTCTCTCCTGGACACCGAGGATTGGAAATAAGGGCCTGGAGCTGAGTAGTAG

 663   CCAGTCTGCTGTGACCACAGGCTCAGGTCCGACCCTGCTGCTTGGCCACAGCAGTGGCTG

 723   GGCAAGTGGGAACCACTATCTCTTGGGAGCCCCCAAAAGCTGGGAAATGCTGGAGGAACC

 783   AGGCCTTTCCCGCTTTTGCCTGGCTGCAGGGTTCGGCTCCGCCCCTGCCCCCCAGCCCTC

 843   GTGTGTCCACACCGCAGTGCCTCTGCCCCTCGGGGGACTGGACACACATCCTGCCAGAGG

 903   CGCTACGAAGCTTTGCCCAGATGAAGCCAGGTGGGCTCCGCGTTCACTCCCACTCTCCCG

 963   AGGGGTGCTGGCCTCCCCAGGGTTTGCCTTCTTACGGATTTAGACGAGGTTCGAGGCTCA

1023   CCTATCAGGGCAGCTCTCAGGATTGTCATTTTCCTCTTTGCCTGTGGGTTTAACTTTTGT

1083   ATTTTTTTAATCACAAGTTTGATACAAAATGTTTTTATCGTACTCTTTGGAGATGCCCAT

1143   TCTACTTTTGAATTTAGCTTTTACTAATTCGCATCTGGAAGCTCAGCAAGTGCACAAGCC

1203   TTACTTTGGTTACCGTGGAAACCACTGCCACCCCTCCCCGATGTGGTGCGCTCAATAAAA

1263   ATGCTGGAATTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequences of GIP. Deduced amino acids are shown under the coding sequence. Amino acid 1 is the initiation methionine marked in bold and the stop codon is indicated by the asterisk. A polyadenylation consensus sequence is underlined. This sequence has been submitted to GenBank under accession number AF234997.

human PAG were subcloned into the yeast expression vector pEG202, which was designed for yeast two-hybrid interaction cloning [15]. Both were suitable baits because the LexA-PAG constructs had no transcriptional activity. These portions of PAG contained 392 and 256 amino acid residues, respectively (Fig. 1), of the human glutaminase from ZR-75 breast cancer cells [11]; indeed, the C-terminal 256 amino acids were identical to the predicted amino acid sequence of an EST human brain sequence (R15359), which shares 100% identity in the nucleotide sequence with the PAG from breast cancer cells [11]. These constructs (pEG/C-PAG and pEG/N-PAG; Fig. 1) were used to screen a yeast expression library derived from the cDNA of human brain. One million clones were screened, and 37 clones were selected for pEG/C-PAG and 25 for pEG/N-PAG in the primary screening. These clones conferred galactose-dependent phenotypes indicating protein interaction on yeast strain EGY48, and the phenotypes segregated with the plasmids from the cDNA library. Thus, these cDNAs represented candidates encoding PAG-binding proteins. However, after secondary screening the number of positives decreased and, even more, after checking the specificity of the interactions as described in the Section 2, no true positives remained for the N-PAG construct, whereas 19 positives were detected for the C-terminus bait.

Four cDNA clones were randomly selected from the positive pool from the human brain library and were subjected to sequence analysis. Nucleotide sequence of hit 1 showed almost a 100% match to the cDNA of human ubiquitin fusion-degradation protein. The second hit was highly similar (96% identity in 418 nucleotides) to a guanine nucleotide-binding protein, whereas hit 4 was 100% identical to the human SNT, namely to the N-terminal region containing a PDZ domain

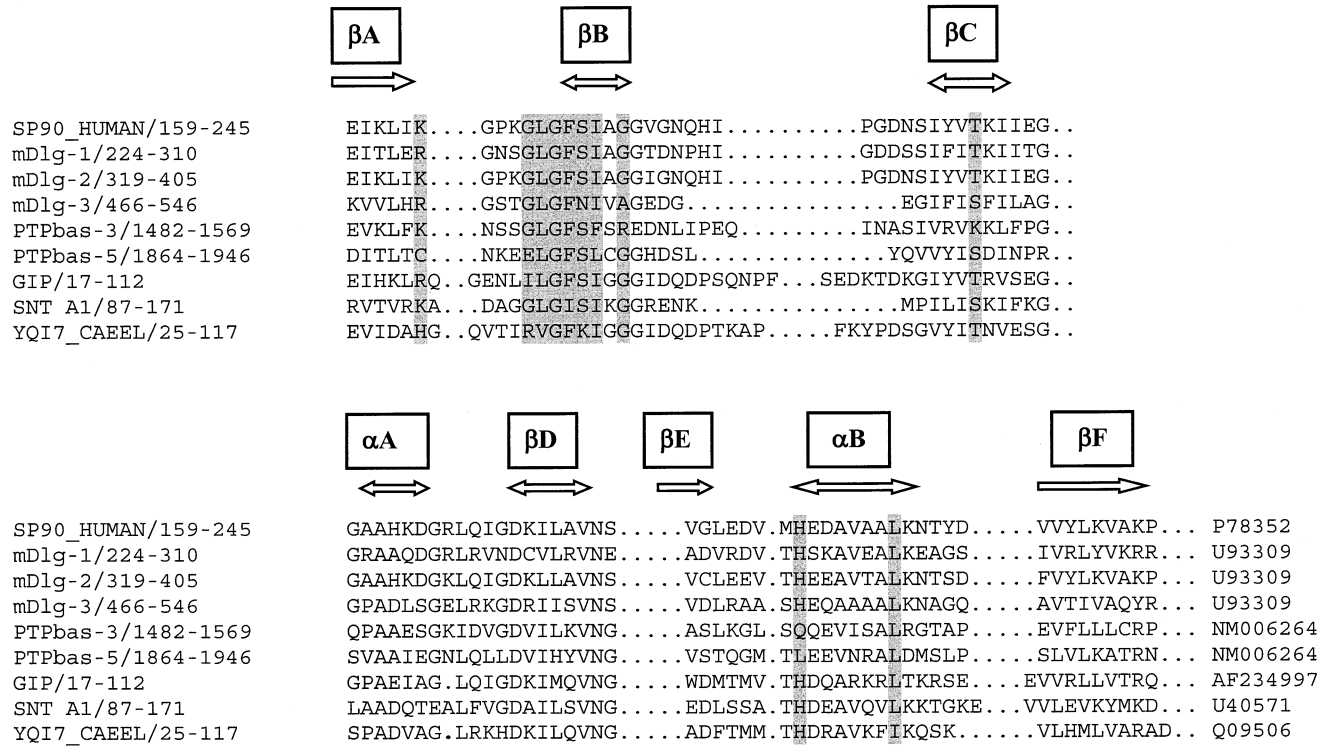

Fig. 3. Multiple sequence alignment of selected PDZ domains. The alignment was done with the program Pfam 3.1 [27]. The predicted secondary structure included at the top of the sequences is taken from those determined in [33,34]. The sequences of GIP, SNT (SNT A1) and a *C. elegans* putative protein (YQI7_CAEEL) similar to GIP are compared with six members of the class I domain of PDZ proteins (target sequence specificity: S/T-X-V/I-COO$^-$ [36]). The name of the proteins, PDZ domain number and the domain limits are indicated at the beginning of the sequences; database accession codes follow the alignment. Essential residues for interaction with the peptide ligand are shown in gray; dots represent insertions/deletions.

[21]. With regard to hit 3, its sequence was shown to be identical to both a human brain clone of unknown function (GenBank accession # U90913) [24], but adding 13 nucleotides in the 5′-end, and to the Tax interaction protein 1, a partial coding sequence isolated from human lymphocytes [25], extending 69 nucleotides at the 5′-end. Hit 3, thereafter referred to as GIP, has 1315 nucleotides (Fig. 2) and contains an ORF of 372 nucleotides that extends from the first translation consensus sequence [26] of an initiation codon at position 48 to the first termination codon at position 420. The entire cDNA contains 47 and 896 nucleotides of 5′- and 3′-untranslated sequences, respectively. Translation of the cDNA results in a predicted protein of 124 amino acids with a relative M_r of 13 735 (Fig. 2).

Although the size of this cDNA agrees with estimates of the size of the GIP mRNA obtained by Northern gel analysis (results not shown), we wanted to assure that the whole 5′-end sequence was present in this clone. For that purpose, the 5′-RACE (rapid amplification of cDNA ends) technique was carried out [11] with human lymphocyte RNA and several antisense primers flanking the 5′-end of GIP; after PCR amplification and Southern blot to detect the GIP-specific products, they were cloned in pGEM-T and sequenced. However, all the GIP clones started with the same 5′-sequence already known and none had additional sequence upstream of the 5′-end (results not shown).

Sequence comparisons between GIP and the NCBI GenBank™ EMBL, DDBJ, PDB sequence databases showed that it was identical to the Tax interaction protein 1 isolated from human lymphocyte in a two-hybrid screen employing the C-terminus of the HTLV-1 Tax oncoprotein as a bait [25]; the only difference is that the GIP sequence adds 8 amino acids at the N-terminus. It is also noteworthy that the deduced amino acid sequence of a putative protein of *Caenorhabditis elegans* (C45g9.7, SwissProt # Q09506) containing 124 amino acids has 63% similarity with GIP. The amino acid sequence of GIP contains a PDZ interaction module encompassing amino acids 17–112; for this reason, apart from the Tax interaction protein and the C45g9.7 hypothetical protein, which showed the best alignments, GIP also displays significant similarity values with other proteins belonging to the PDZ family. Furthermore, hit 4 was the SNT protein, which also contains a PDZ domain inserted in the first pleckstrin homology (PH) domain [21]. In Fig. 3, the multiple alignment of representative PDZ sequences is compared with that of GIP and SNT using the Pfam program [27].

To confirm the interaction between PAG and the PDZ domains of SNT and GIP, in vitro interaction experiments were performed by pull-down assays. GST fusion proteins of GIP, PAG and SNT expressed in *E. coli* (Fig. 4A) were immobilized on glutathione-Sepharose and incubated with their respective interaction partners labeled with [^{35}S]Met. As shown in Fig. 4B, in vitro translated [^{35}S]PAG associates specifically with both GST-GIP and GST-SNT: a strong band was detected which indicates a selective retention of PAG to GIP and SNT, whereas GST alone was unable to retain PAG. Approximately 50–75% of the [^{35}S]PAG input signal was bound by GIP or SNT. Furthermore, the signal correspond-

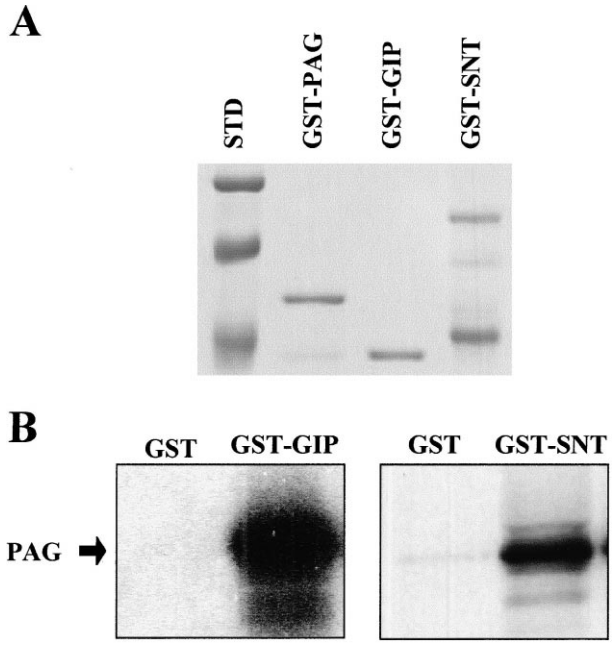

Fig. 4. GST pull-down assays. (A) GST-PAG, GST-GIP and GST-SNT were expressed in *E. coli* and purified by glutathione-Sepharose. The affinity-purified GST proteins were analyzed by SDS–PAGE and stained with Coomassie blue R-250. Standard molecular weight markers are in lane STD, from top to bottom: phosphorylase b (94 kDa), BSA (67 kDa) and ovalbumin (43 kDa). (B) GST-PAG immobilized on glutathione-Sepharose beads was incubated with [^{35}S]Met-labeled, in vitro translated GIP or SNT. Bound proteins were analyzed by SDS–PAGE and visualized by autoradiography. Control lanes with only recombinant GST (without PAG) are also shown.

ing to the [^{35}S]PAG bound decreases to 20–25% of the control values when a competition assay was done in the presence of a four molar excess of cold PAG (results not shown). Similar results were obtained by using GST-PAG to interact with [^{35}S]GIP or [^{35}S]SNT (data not shown). Thus, the binding of PAG/GIP and PAG/SNT seem highly specific interactions.

Since both GIP and SNT have a PDZ interaction domain and PAG has a C-terminus which conforms to the consensus PDZ recognition sequence, it is therefore most likely that the interaction occurs via PDZ modules. Therefore, we then tested whether the PAG C-terminal domain is involved in the interaction; concretely, we analyzed the contribution of individual residues. The analysis was performed with the two-hybrid assay in yeast by comparing the interaction of the C-terminal PAG bait protein with that of mutated forms, where residues at positions 0, −1, −2 and −3 were changed to alanine, giving mutants V602A, M601A, S600A and E599A. In Fig. 5, 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 Fig. 5 (right plate), the mutants V602A and S600A of PAG completely abolish the interaction with GIP, the mutant M601A was without effect, whereas a lower level of interaction was seen with the mutant E599A. Some different results were obtained with the PDZ domain of SNT (left plate): the glutamate at position −3 of PAG was essential for the interaction and the serine at −2 was considerably less critical than for GIP. The last valine (V602) was again essential to maintain the SNT-PAG interaction, as in the case of the pair GIP-PAG. Thus, there is strong evidence that the C-terminal amino acid residues of PAG are key determinants of the interaction.

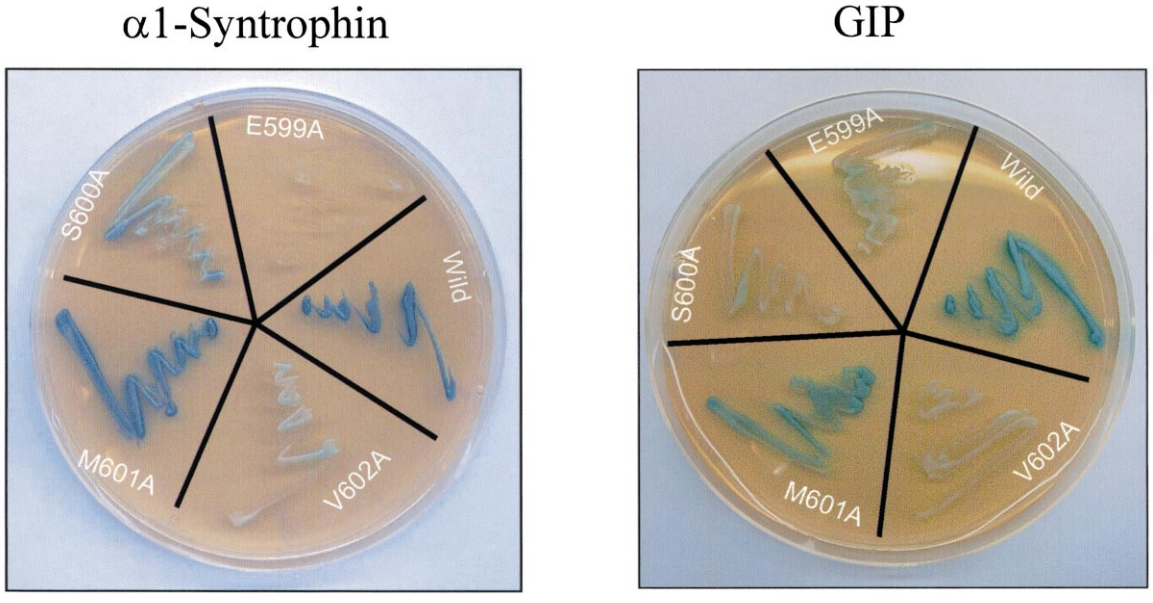

Fig. 5. Analysis of the interaction between PAG mutants and the PDZ clones GIP and SNT using a solid culture β-galactosidase assay. Yeasts were cotransformed with the cDNA of wild-type or point mutants PAG and the cDNA of SNT or GIP. Transformants were plated on Gal/Raf Ura-His-Trp- plates containing X-Gal; the interaction between PAG and SNT or GIP was revealed by the intensity of the blue color developed after reporter gene activation, while white colonies reflect lack of interaction. Control glucose Ura-His-Trp- X-Gal plates and Gal/Raf Ura-His-Trp-Leu- plates were used to confirm the interactions (results not shown). Interaction PAG/SNT, left plate; interaction PAG/GIP, right plate; PAG point mutants are indicated onto the corresponding section of the plate.

4. Discussion

In this study we have shown that the human L isoenzyme of PAG has the potential to interact with two members of the PDZ domain-containing anchoring proteins through its C-terminal motif of ESMV. Furthermore, we describe the isolation of a full-length cDNA clone encoding a novel member of the PDZ family, GIP, while the second interactor isolated, SNT, also contains a PDZ domain. Both cDNAs were isolated from human brain and, interestingly, two isoenzymes of glutaminase are expressed in this tissue: the kidney-type K-PAG and the liver-type L-PAG [10–12].

We designed the C-bait of PAG after complete sequencing of a human brain partial cDNA clone (GenBank accession # AF110331), which has almost 100% identity with the L-PAG cDNA of ZR-75 breast cancer cells, encompassing nucleotides 1070–2408; that is, the C-terminal half and all the 3′-UTR region [11]. Moreover, during the course of this work, a full cDNA sequence corresponding to the L-PAG enzyme has been cloned from human brain and deposited in the database (GenBank accession # AF223944). The protein has a 96% overall identity with our sequence from breast tumor cells, having only two differences – A560V and L581P – in the last C-terminal 256 amino acids used as bait in the two-hybrid screen. Although these changes do not alter the C-termini PDZ consensus sequence of L-PAG, it should be noted that they only appear in the sequence AF223944, because all the putative glutaminase EST from human and mouse, as well as the rat liver enzyme [28], have A and L in those positions in agreement with the breast cancer sequence.

Whereas no real positive was obtained with the first 392 amino acids of PAG (N-bait), the two-hybrid screen with the last 256 amino acids (C-bait) led to the isolation of several positives, and two of them were related by the presence of one PDZ domain: SNT and the new protein GIP. The sequence of GIP is mainly composed of a single PDZ module, which is unusual in this family of proteins; a hypothetical protein C45g9.7 of 124 amino acids in *C. elegans* (SwissProt # Q09506) shares 63% of similarity over 117 amino acids; thus, this genomic sequence would constitute the homolog of GIP in *C. elegans*. Furthermore, this hypothetical protein and GIP represent until now the shortest members of the PDZ family. Syntrophins are a family of intracellular peripheral membrane proteins expressed in skeletal muscle, heart, brain and some other human tissues [21]. SNT has other sequence motifs, apart from the PDZ domain: two PH domains and one syntrophin unique (SU) domain. The clone of SNT isolated in this study was almost complete, containing all the domains except half of the first PH domain, because the sequence lacks the first 48 amino acids. In brain, the PDZ domain of SNT is known to bind to protein kinases [29] and to the neuronal nitric oxide synthase [30]; the interactions have been postulated as a mechanism for selective targeting of these proteins to unique subcellular sites in brain. From our results, it can also be proposed that SNT might contribute to determining the subcellular localization of PAG in certain regions of the brain, thereby creating a mechanism to regulate PAG activity.

The overall amino acid distribution for the PDZ domains does not show many conserved positions, a fact that derives from the large sequence divergence of this fold family [31]. However, techniques of sequence analysis [31,32] and nuclear magnetic resonance and X-ray studies of several PDZ proteins [33,34] have supported evidence about the conserved residues of the PDZ modules and their roles in the binding of the target peptide. As shown in Fig. 3, the PDZ domains of GIP and SNT share the essential residues deduced from studies of structural requirements of the PDZ modules [33,34]. For example, the hydrophobic amino acid residues forming the pocket which accommodate the side-chain of the C-terminal valine (Leu29-Phe31-Ile33-Leu97 for GIP and Leu98-Ile100-Ile102-Leu155 for SNT). A key amino acid is also the αB2 histidine (second residue of the helix αB, nomenclature as outlined in [33]) which is known to play a critical role in PDZ domain target recognition specificity through formation of a strong hydrogen bond between N^{ε} of His and the hydroxyl group of a Ser/Thr residue at position −2 [33,35]. Another structural feature shared by GIP and the second PDZ domain of SP90 (PSD-95 protein) is an unusually large loop connecting βB and βC strands: 13 amino acids in SP90 and 20 amino acids in GIP (Fig. 3). A role in determining the target-binding specificity has been postulated for this loop in the PSD-95 PDZ2 domain [34], which is also longer than normal in the *C. elegans* PDZ protein (Fig. 3).

The interaction between PAG and the PDZ domains of GIP and SNT is supported by clear experimental evidence. It was observed by the two-hybrid assay and also by GST pull-down assays. PDZ domains have been divided into two major groups on the basis of the amino acid selected at the −2 position of the target peptide: group 1 interacts with peptides containing hydroxyl groups (Ser, Thr or Tyr) at position −2, whereas group 2 selects peptides with hydrophobic amino acids at −2, usually Phe [36]. The PDZ domains of both GIP and SNT belong to group 1, which also include the three PDZ domains of the human SP90 [14,33,34], the third and fifth PDZ domains of the phosphotyrosine phosphatase (PTPbas-3 and PTPbas-5) [36], and the three PDZ domains of murine hDlg (mDlg) [36]. It is interesting to note that in a previous two-hybrid screen employing a cDNA library from human lymphocytes and the Tax oncoprotein as bait, a cDNA clone of GIP and a truncated cDNA clone of β1-syntrophin were isolated too [25]. β1-Syntrophin is highly related to SNT and shares a similar domain organization [37]. The C-terminus of Tax oncoprotein, ETEV, is very similar to that of PAG, containing the three essential amino acids: the carboxy-terminal valine, threonine at position −2 and glutamate at −3.

Finally, the experiments of point mutations also reinforced the specificity of the interactions PAG/GIP and PAG/SNT, giving additional information regarding the key residues involved. The C-terminal valine was revealed as absolutely essential for both interactions, while the Met at −1 was irrelevant, in agreement with previous reports which consider selection at this position not being a strict requirement [34]. The other C-termini positions with the highest selection were Ser at −2 for GIP and Glu at −3 for SNT. Although being important to maintain the interaction, a lower degree of selectivity was shown by position −3 for GIP and −2 for SNT. The βB2 Ser residue and βC4 Thr/Ser residues in GIP and SNT seem to be key determinants to select Glu at the −3 position, via hydrogen bond formation [33].

The findings reported in this work suggest that PDZ proteins, which are known to direct the target proteins into functional signaling networks, can interact with L-PAG; such interaction may account for a regulated mechanism of

glutamate synthesis, accumulation, or release in human brain. It is noteworthy that two isoenzymes of PAG are expressed in human brain; the need for two genes and two PAGs to support the single process of glutamate synthesis is unexplained, and identifying the role of each PAG is an important factor in understanding glutamate-mediated neurotransmission. Interestingly, the K-PAG, unlike L-PAG, does not have a PDZ-binding domain and thus PDZ proteins could be important for determining differential cellular or subcellular localization of the two PAG isoforms in the CNS. Further analysis is required to understand the physiological significance of the interaction between L-PAG and PDZ proteins in human brain.

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