

Molecular cloning and amino acid sequence of brain L-glutamate decarboxylase

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ABSTRACT We used specific polyclonal antibodies against L-glutamate decarboxylase (GAD) to screen a mouse brain cDNA library that was constructed in the expression vector λ gt11. We obtained 1.5×10^6 recombinant DNA clones in the mouse brain cDNA library. One of the clones was positively identified as a GAD clone on the basis of the following results: (i) the clone and its secondary and tertiary clones all reacted strongly with anti-GAD antibodies; (ii) the fusion protein obtained from λ GAD-Y1089 showed good GAD enzyme activity as determined by both CO_2 and γ -aminobutyric acid methods. The GAD clone thus obtained contains GAD cDNA of ≈ 2.6 kilobases that has one internal *EcoRI* site. After GAD cDNA was cut at the *EcoRI* site, two DNA fragments of about 1.6 and 1.0 kilobases were obtained at the 5' and 3' ends, respectively. The cDNA insert was found to be composed of 2632 base pairs, the translation initiation site was assigned to the methionine codon ATG, and the termination site was found to be TGA (positions 2216–2218). Furthermore, the coding region in 2169 base pairs was found to consist of 723 amino acids. The protein has a molecular weight of 83,207 and contains 83 strongly basic, 108 strongly acidic, 226 hydrophobic, and 221 polar amino acids with an isoelectric point of 5.355. The relationship of this GAD cDNA to other forms of GAD is discussed.

γ -Aminobutyric acid (GABA) has been established as a major neurotransmitter in the mammalian central nervous system from physiological, biochemical, pharmacological, and morphological studies (1–3). The rate-limiting step in GABA biosynthesis is the decarboxylation of L-glutamic acid by glutamate decarboxylase (GAD; L-glutamate 1-carboxylase, EC 4.1.1.15) (1, 2) and hence GAD has been used as a specific marker for GABAergic neurons. Although much progress has been made in the identification of GABAergic neurons and their synaptic connectivities (for review, see ref. 3), little is known with certainty regarding the regulation of GAD activity or the expression of the GAD gene. This is partially hampered by the existence of multiple forms of GAD, differing in molecular weight (4), kinetic properties (5–7), and hydrophobic properties (7), and the lack of detailed structural information about those forms of GAD. In this communication, we describe the cloning of the mouse brain GAD gene and the elucidation of a complete nucleotide sequence and the deduced amino acid sequence as a first step toward addressing these questions.[¶] In addition, a comparison of similarities and differences between the mouse brain GAD gene presented in this communication and the feline GAD gene reported previously (8, 9) is included.

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MATERIALS AND METHODS

Construction of cDNA Library. A mouse brain cDNA library was constructed in λ gt11 as described by Young and Davis (10). Briefly, total RNA was extracted from mouse brain, and poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography (11). Poly(A)⁺ RNA was reverse transcribed into double-stranded cDNA, which was inserted into the *EcoRI* site of λ gt11, yielding a library consisting of 1.5×10^6 independent clones.

Screening the cDNA Library with Antibody. About 1.5×10^6 clones were plated on 40 dishes of agar on a lawn of *Escherichia coli* Y1090 and the nitrocellulose filters pre-soaked with isopropyl β -D-thiogalactoside were overlaid onto agar plates. The filter replicas of lysis plaques were first incubated with well-characterized primary antibodies, rabbit anti-purified rat brain GAD (12, 13), followed by incubation with ¹²⁵I-labeled secondary antibodies, goat anti-rabbit IgG, and were then processed for autoradiographic identification (primary screening). The GAD-positive clones identified in the primary screening were recloned and rescreened twice by the procedure described above (secondary and tertiary screening). The GAD-positive clone identified in the primary, secondary, and tertiary screenings was then used for fusion and sequencing studies. The specificity of anti-GAD antiserum was established from extensive immunochemical tests including immunoprecipitation, immunoblotting, immunodot, and immunoelectrophoresis tests (13).

Expression of Fusion Protein in Bacteria. *E. coli* Y1089 was lysogenized with λ GAD or nonrecombinant λ gt11 and induced with isopropyl β -D-thiogalactoside. The fusion proteins thus obtained in the bacterial extract were then assayed for GAD activity by both CO_2 and GABA methods as described (14). Briefly, L-[U-¹⁴C] glutamate was used as a substrate for the GAD assay and the reaction was terminated by the addition of 10 μ M aminooxyacetic acid instead of 1.0 M H_2SO_4 . The amounts of ¹⁴CO₂ and [¹⁴C]GABA formed were determined by the hyamine base (15) and ion-exchange methods (14), respectively.

DNA Sequencing. The cDNA insert was excised by digestion with *EcoRI* and the two fragments obtained, 1.6 and 1.0 kilobases (kb), were subcloned into M13mp18. Overlapping clones were obtained by exonuclease III deletion as described by Henikoff (16). DNA was isolated from M13 plaques and sequenced by the dideoxynucleotide chain-termination method of Sanger *et al.* (17) using the M13 universal primer. Sequencing was done on one strand only, but from multiple clones, so each sequence segment has been confirmed several times. Software developed by

Abbreviations: GABA, γ -aminobutyric acid; GAD, L-glutamic acid decarboxylase.

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^{¶¶}The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55253).

TGC CGC TTC CTT CAC TGT CTG AAG CAA TAC AGT GGT GAT GAG GGT TTC ATG ACA CAG AAC Cys Arg Phe Leu His Cys Leu Lys Gln Tyr Ser Gly Asp Glu Gly Phe Met Thr Gln Asn	1426 460
ATC GCG AAG CAG AAT GAG CAC TGT CTC AAG AAC TTT GAC CTC ACT GAA TAC CGC CAG GTA Ile Ala Lys Gln Asn Glu His Cys Leu Lys Asn Phe Asp Leu Thr Glu Tyr Arg Gln Val	1486 480
CTA AGC GAC CTT TCC ATT CAG ATC TAT CAG CAG CTC ATT AAA ATg CCC gAG GGC TTG CTA Leu Ser Asp Leu Ser Ile Gln Ile Tyr Gln Gln Leu Ile Lys Met Pro Glu Gly Leu Leu	1546 500
CAG CCT ATG ATA GTT TCT GCC ATG TTG GAA AAT gAG AGT ATC CAG GGG CTG TCT GGT GTG Gln Pro Met Ile Val Ser Ala Met Leu Glu Asn Glu Ser Ile Gln Gly Leu Ser Gly Val	1606 520
AGA CCA ACT GGT TAC CGG AAG CGC TCC TCC AGC ATG GTG GAT GGA GAG AAT TCT TTC CAT Arg Pro Thr Gly Tyr Arg Lys Arg Ser Ser Ser Met Val Asp Gly Glu Asn Ser Phe His	1666 540
ACA GTC CTG TGT GAC CAG GGC CTG GAC CCC GAG ATT ATC CTG CAG GTG TTC AAA CAG CTC Thr Val Leu Cys Asp Gln Gly Leu Asp Pro Glu Ile Ile Leu Gln Val Phe Lys Gln Leu	1726 560
TTC TAC ATG ATC AAT GCT GTG ACT CTT AAC AAC CTA CTC CTG CGG AAA GAC GCC TGC TCC Phe Tyr Met Ile Asn Ala Val Thr Leu Asn Asn Leu Leu Leu Arg Lys Asp Ala Cys Ser	1786 580
TGG AGC ACA GGC ATG CAA CTC AGG TAC AAC ATA AGT CAA CTG GAA GAG TGG CTT CGG GGC Trp Ser Thr Gly Met Gln Leu Arg Tyr Asn Ile Ser Gln Leu Glu Glu Trp Leu Arg Gly	1846 600
AAA AAC CTT CAC CAG AGT GGA GCA GTT CAG ACC ATG GAG CCC CTG ATC CAG GCA GCC CAG Lys Asn Leu His Gln Ser Gly Ala Val Gln Thr Met Glu Pro Leu Ile Gln Ala Ala Gln	1906 620
CTC CTC CAG CTG AAG AAG AAA ACC CAC GAG GAT GCT GAG GCC ATC TGC TCT CTG TGC ACC Leu Leu Gln Leu Lys Lys Lys Thr His Glu Asp Ala Glu Ala Ile Cys Ser Leu Cys Thr	1966 640
TCC CTC AGC ACC CAG CAG ATT GTC AAA ATT TTA AAC CTC TAC ACT CCC TTG AAT GAA TTT Ser Leu Ser Thr Gln Gln Ile Val Lys Ile Leu Asn Leu Tyr Thr Pro Leu Asn Glu Phe	2026 660
GAG GAA CCG GTC ACA GTG TCC TTC ATC AGA ACA ATC CAG GCT CAG CTA CAA GAG AGG AAT Glu Glu Arg Val Thr Val Ser Phe Ile Arg Thr Ile Gln Ala Gln Leu Gln Glu Arg Asn	2086 680
GAC CcT CAG CAG CTC CTG CTG GAC TCC AAG CAC GTG TTC CCA GTT CTG TTT CCA TAT AAC Asp Pro Gln Gln Leu Leu Asp Ser Lys His Val Phe Pro Val Leu Phe Pro Tyr Asn	2146 700
CCA TCT GCT CTG ACC ATG GAC TCG ATC CAC ATC CCG GCC TGT CTC AAC CTG GAG TTT CTC Pro Ser Ala Leu Thr Met Asp Ser Ile His Ile Pro Ala Cys Leu Asn Leu Glu Phe Leu	2206 720
AAT GAA GTC TGA G GATGCGTGT TCCGAGGCGA GCGAGAAGGA AGCATGTGCT GTCAGCCGAG Asn Glu Val Ter	2269 723
AGAATGCTAG GTGTGTTAAA TATTCCAGCG TAGATCAAAC CATGTTAGAG ACTGGCGGGA CGACAGAACT	2339
AAACAGCGGG GTGCACAGTT GTCGCCAATG CTGCTCAGAA AACACCCGGA AGTGGATTG TTAAGCTGT	2409
GCTTTCAGGT TAAACCAAGA CACGTCAGAA CGAACAGCCA CTCTGCAGCT CCAGTCGCCA TATAAAAATG	2479
CCAGTTCTAC AGAGTGGAAAG TGCCTAGCTT TGATCTTTGT ATATATCTTG AGAATGTTC AACTGAGATA	2549
ATATTA AAAA CACATGACGT AAATTGCCCT TGTGGTCTT TCAAGAAATG ATGGGACTAA TAACCATAAG	2619
ATTGACAGGA ATT	2632

FIG. 1. Nucleotide sequence of mouse brain GAD cDNA and its deduced amino acid sequence. The secondary structure deduced from the amino acid sequence according to Garnier *et al.* (19) is indicated. ~, α -Helix; -, β -sheet; ·, turn. Vertical bar indicates the position of the first nucleotide of every 10-nucleotide segment.

Devereux *et al.* (18) was used for sequence analysis and determination of protein secondary structure.

RESULTS

Screening of cDNA Library. In the primary screening, among 1.5×10^6 clones there were 25 positive clones identified by

antibodies against rat brain GAD. However, only 1 clone was positively identified as the GAD clone in secondary and tertiary screenings. The GAD clone thus obtained contains GAD cDNA of ≈ 2.6 kb, which has one internal *EcoRI* site. After the GAD cDNA was cut at the *EcoRI* site, two DNA fragments of about 1.6 and 1.0 kb were obtained at the 5' and 3' ends, respectively.

Table 1. Assay of GAD activity in the fusion protein

Sample	GAD activity	
	$^{14}\text{CO}_2$, cpm $\times 10^{-3}$	$[^{14}\text{C}]\text{GABA}$, cpm $\times 10^{-3}$
$\lambda\text{GAD-Y1089}$ extract	4.9 \pm 0.3	20.1 \pm 1.2
$\lambda\text{gt11-Y1089}$ extract	2.3 \pm 0.2	2.5 \pm 0.2
Mouse brain extract	8.0 \pm 0.6	30.4 \pm 1.8

Values represent means \pm SD from eight assays.

Assay of Fusion Proteins for GAD Activity. GAD activity was measured in $\lambda\text{GAD-Y1089}$ and in $\lambda\text{gt11-Y1089}$. Mouse brain extract was included as a positive control. GAD activity obtained by the CO_2 method for $\lambda\text{GAD-Y1089}$, $\lambda\text{gt11-Y1089}$, and mouse brain extract was $(4.9 \pm 0.3) \times 10^3$, $(2.3 \pm 0.2) \times 10^3$, and $(8.0 \pm 0.6) \times 10^3$ cpm of $^{14}\text{CO}_2$, respectively, whereas the GABA method gave $(20.1 \pm 1.2) \times 10^3$, $(2.5 \pm 0.2) \times 10^3$, and $(30.4 \pm 1.8) \times 10^3$ cpm of $[^{14}\text{C}]\text{GABA}$, respectively, as shown in Table 1.

cDNA Nucleotide Sequence and Its Corresponding Amino Acid Sequence. The nucleotide sequence and the deduced amino acid sequence for mouse brain GAD are shown in Fig. 1. The cDNA insert was found to be composed of 2632 bp, the translational initiation site was assigned to the methionine codon ATG (positions 47–49), and the termination site was found to be TGA (positions 2216–2218). Furthermore, the coding region in 2169 bp was found to consist of 723 amino acids. The protein has a molecular weight of 83,207 and contains 83 strongly basic, 108 strongly acidic, 226 hydrophobic, and 221 polar amino acids with an isoelectric point of 5.355. The conformation of the protein deduced from the primary structure by the method of Garnier *et al.* (19) shows 51% helix, 15% β -sheet, 14% turn, and 20% coil. The segments of amino acid sequence that contain α -helix, β -sheet, and turn are indicated in Fig. 1. The amino acid composition of mouse brain GAD deduced from the nucleotide sequence is shown in Table 2, together with those reported in the literature for GAD from human (20), rat (21), and feline brain (9).

DISCUSSION

The identification of the GAD clone is based on two criteria: First, the clone reacts strongly with anti-GAD antiserum in

Table 2. Comparison of amino acid composition of mouse brain GAD with the reported values for human, rat, and feline GAD

Residue(s)	Mouse	Human	Rat	Feline
Asp + Asn	82	59	52	52
Thr	33	39	26	38
Ser	49	41	37	43
Glu + Gln	136	69	46	57
Pro	21	17	28	24
Gly	24	45	43	45
Ala	38	47	28	37
Cys	12	10	12	13
Val	36	34	37	30
Met	21	4	7	17
Ile	34	34	33	31
Leu	96	55	55	56
Tyr	17	15	13	18
Phe	18	26	20	27
Lys	51	34	43	40
His	19	6	27	16
Arg	32	28	39	32
Trp	4	—	—	10

the primary screening as well as in the secondary and tertiary screenings. The specificity of anti-GAD antiserum has been established through extensive tests, including immunodiffusion, immunoprecipitation, immunoelectrophoresis, Western immunoblotting, and immunohistochemical tests. Indeed, the anti-GAD antiserum produced in the authors' laboratory has been widely used for immunohistochemical identification of GABAergic neurons and their synaptic connectivities, and the results obtained from those studies agree well with the known distribution of the GABAergic system in many regions of the mammalian central nervous system (for review, see ref. 3). Second, the fusion protein does contain good GAD activity measured by both the CO_2 and the GABA methods. Furthermore, both the fusion protein and the mouse brain preparation produce CO_2 and GABA in a stoichiometric relationship (1:4), whereas no such stoichiometric relationship between CO_2 and GABA is observed for $\lambda\text{gt11-Y1089}$. It is not surprising that GAD activity in the fusion protein is somewhat lower than that in the mouse brain. This may be due to the fact that there are several forms of GAD in the mouse brain and only one of them is expressed in the fusion protein. Alternatively, the cloned cDNA does not contain the entire coding sequence. This issue cannot be resolved at the present time because of lack of amino acid sequence data from the purified GAD protein.

The GAD clone reported here has little homology with that reported by Kobayashi *et al.* (9). This may be due to the difference in the cDNA library and the antibodies used in identification of GAD-positive clones. In the present study, we used well-characterized anti-GAD antiserum directed against purified rat brain GAD (12, 13) to screen the mouse brain cDNA library, while Kobayashi *et al.* (9) used anti-GAD antiserum directed against partially purified GAD (5) to screen the cDNA library constructed in feline occipital cortex (8). It may also represent different genes encoding different forms of GAD. There are numerous reports in the literature suggesting the presence of multiple forms of GAD in mammalian brain. Spink *et al.* (7) reported the presence of α , β , and γ forms of GAD in porcine brain and four molecular forms of GAD in rat brain (6) that differ in their hydrophobic and kinetic properties. Wong *et al.* (4) reported that GAD in the developing brain differs from that of the adult brain in molecular weight as well as in immunological properties. Recently, Chang and Gottlieb (22) reported the presence of cytosolic and membrane-bound GAD in rat brain that showed some differences in NaDodSO₄/PAGE. We have also reported the presence of two forms of GAD with different affinity toward the coenzyme pyridoxal phosphate (15). Previously, we reported that mouse brain GAD has a molecular weight of $85,000 \pm 2000$, which is in good agreement with the molecular weight of 83,207 calculated from the amino acid sequence reported here. It is of interest that rat brain GAD is similar to mouse brain GAD in its isoelectric point (pI 5.4 vs. 5.355) (12). Also, one of its subunits is of molecular weight similar to that of mouse brain GAD— $80,000 \pm 4000$ (12). Furthermore, from kinetic studies we have also shown (24, 25) that GAD preparations from both mouse and rat brain are highly sensitive to sulfhydryl agents [e.g., 5,5'-dithiobis(2-nitrobenzoic) acid and *p*-chloromercuribenzoic acid], mercapto compounds (e.g., 3-mercaptopropionic acid), dicarboxylic acids (e.g., glutaric acid), and carbonyl trapping agents (e.g., aminoxyacetic acid), suggesting that mouse brain GAD and rat brain GAD are probably closely related proteins. It is interesting to note that the composition of cysteine, lysine, arginine, and histidine in human (20), rat (21), and feline (9) GAD as reported in the literature is similar to that of the mouse brain reported here (see Table 2). Another similarity among GAD from various species is the low content of aromatic amino acids—e.g., tryptophan, phenylalanine, and tyrosine—and the high content of acidic

amino acids—e.g., glutamic and aspartic acids. The hydrophobic character of mouse brain GAD as determined by the method of Kyte and Doolittle (23) indicates no strong hydrophobic region similar to feline GAD (9) and is compatible with the fact that this particular form of GAD is a soluble enzyme. Hence, it is reasonable to suggest that despite little homology in the sequence between mouse brain GAD and feline brain GAD, they probably have similar active sites and conformation. Future studies involving the use of site-specific mutagenesis and microsequencing (26) of purified GAD proteins should provide important information regarding the functional regions of the GAD molecule and the molecular nature of GAD isozymes.

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