

# Brain Glutamate Decarboxylase Cloned in $\lambda$ gt-11: Fusion Protein Produces $\gamma$ -Aminobutyric Acid

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Glutamate decarboxylase (GAD; E.C. 4.1.1.15) converts glutamate to  $\gamma$ -aminobutyric acid (GABA), the major inhibitory neurotransmitter in the vertebrate central nervous system. This report describes the isolation of a GAD complementary DNA clone by immunological screening of a  $\lambda$ gt-11 brain complementary DNA expression library. The fusion protein produced by this clone catalyzes the conversion of glutamate to GABA and carbon dioxide, confirming its identity as GAD. Antibodies to  $\beta$ -galactosidase remove GAD enzymatic activity from solution, showing that this activity is associated with the fusion protein. In immunoblotting experiments all three available antisera to GAD reacted with the fusion polypeptide and with two major polypeptides (molecular size, 60,000 and 66,000 daltons) in brain extracts.

**B**ECAUSE MOTOR DISORDERS, SEIZURES, and anxiety are often associated with altered concentrations of  $\gamma$ -aminobutyric acid (GABA), the gene for brain glutamate decarboxylase (GAD; E.C. 4.1.1.15) has been suggested as the site of genetic lesions in a wide range of diseases (1,

2). Studies of GAD in disease and development have been hampered by the difficulty of obtaining sufficient enzyme for detailed characterization. We therefore undertook to isolate a recombinant DNA clone containing the GAD coding sequence. Although no amino acid sequence data are available for

Table 1. GAD enzymatic activity in extracts of induced lysogens. *E. coli* strain Y1089, lysogenized with  $\lambda$ GAD or nonrecombinant  $\lambda$ gt-11, was incubated at 32°C until late log phase ( $A_{550} = 0.5$  of growth), and the temperature was then shifted to 44°C for 20 minutes to induce the lytic cycle (6, 7). The IPTG was added and the bacteria were incubated for an additional 60 minutes at 37°C. Bacteria were harvested, resuspended, and frozen (-70°C) in 1/40 of their original culture volume in a buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 2-aminoethylisothiuronium bromide (AET), 0.5 mM pyridoxal phosphate, and 60 mM potassium phosphate, pH 7.1. After being stored and thawed, the cells were lysed by sonication, and debris was removed by centrifugation. Protein concentration ranged from 23 to 35 mg/ml (20). The GAD enzymatic activity was determined as described (21, 22). Production of  $^{14}\text{CO}_2$  was linear with respect to both time of incubation and protein concentration. Enhanced GAD activity in extracts of cells infected with  $\lambda$ GAD depended on IPTG induction. The cell lysate (200  $\mu$ l) was incubated with 4  $\mu$ l of IgG (20 mg/ml, Cappel) to  $\beta$ -galactosidase for 1 hour on ice. Enough Formalin-fixed *Staphylococcus* A (BRL Immunoprecipitin) was added to precipitate all IgG and the mixture was incubated again for 1 hour on ice. The bound antibody-antigen complexes were removed by centrifugation. The supernatant was recovered, reincubated with antibody and Immunoprecipitin, and centrifuged. The second supernatant was then assayed for GAD activity. Immunoprecipitation with antibody to GAD was accomplished similarly with a 1:25 dilution of sheep antiserum to rat GAD (1440-4 from I. J. Kopin) and subsequent treatment with rabbit antibody to sheep IgG (0.74 mg/ml) and Immunoprecipitin. These results were obtained from fusion protein isolated from a single 10-liter culture of  $\lambda$ GAD in *E. coli* Y1089. Partially purified GAD-fusion protein was obtained by fractionation on Sepharose 6B, DEAE-cellulose, and hydroxyapatite. The fusion protein was monitored on the basis of its immunoreactivity with antibody to  $\beta$ -galactosidase and its GAD enzymatic activity. Purified fusion protein was stored in liquid nitrogen. After 4 months of storage, its specific activity fell by a factor of about 10 between the two experiments shown in Table 1. In the control experiments (without antibody), an equal volume of normal goat serum replaced the antibody preparations; the samples were otherwise processed as above.

Extract	GAD activity in extracts*			
	Without antibody		With antibody	
	Activity	Specific activity	Activity	Specific activity
<i>Precipitation of activity with antibody to <math>\beta</math>-galactosidase</i>				
$\lambda$ gt-11 lysogen	414 $\pm$ 12	1.1	378 $\pm$ 22	1.0
$\lambda$ GAD lysogen	7772 $\pm$ 360	20.8	639 $\pm$ 24	1.7
Fusion protein	64463 $\pm$ 3418	246	767 $\pm$ 93	2.9
<i>Precipitation of activity with antibody to GAD</i>				
$\lambda$ gt-11 lysogen	327 $\pm$ 5	1.0	343 $\pm$ 17	1.1
$\lambda$ GAD lysogen	4010 $\pm$ 84	12.7	2013 $\pm$ 33	6.4
Fusion protein	5794 $\pm$ 215	23.5	2555 $\pm$ 50	10.4

\*Enzyme activity is reported as counts per minute (and range) in  $^{14}\text{CO}_2$  per 60  $\mu$ l of reaction mixture, and specific activity as counts per minute per microgram of protein.

GAD, several groups have reported specific antisera to brain GAD (3-5). We have used one of these antisera to screen a bacterial expression library containing brain messenger RNA (mRNA) sequences.

Although immunological screening of bacterial expression libraries allows the efficient isolation of specific complementary DNA's (cDNA's), definitive identification of a cloned cDNA requires additional evidence (6-8). When amino acid sequence data are unavailable, the identity of a clone may be confirmed by characterizing the translation products of hybrid-selected mRNA's (9, 10). These products may differ from cellular polypeptides, however, because of cross hybridization of nonidentical mRNA's, posttranslational alterations or proteolysis of cellular polypeptides, reactivity of antibodies with related but nonidentical epitopes, or impurities in the antibody preparation. Here we report the isolation of a cDNA clone ( $\lambda$ GAD) encoding brain GAD. After initial immunological identification, we used a functional confirmation of identity—the demonstration of GAD activity in the fusion protein encoded by  $\lambda$ GAD.

We constructed a  $\lambda$ gt-11 library of more than  $2 \times 10^6$  recombinant clones with cDNA copied from the polyadenylated RNA of adult cat occipital cortex, a region relatively rich in GABA-containing neurons. The  $\lambda$ gt-11 is a bacteriophage expression vector in which cDNA's are inserted near the 3' end of the gene for *Escherichia coli*  $\beta$ -galactosidase (6, 7). Recombinant phage induced with isopropyl-thio- $\beta$ -D-galactoside (IPTG) produce a fusion protein consisting of an amino terminal  $\beta$ -galactosidase polypeptide linked to a carboxyl terminal polypeptide encoded by the cDNA insert.

Immunological screening was done with a sheep antiserum to GAD, prepared by Oertel *et al.* (4). Bound antibodies were detected with rabbit antiserum to sheep immunoglobulin G (IgG) and  $^{125}\text{I}$ -labeled protein A (11). Initial screening of 400,000 plaques identified 20 immunoreactive candidates, three of which remained immunoreactive in subsequent plaque purifications. DNA isolated from these three clones contained 2.3-

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kilobase (kb) cDNA inserts, which were identical as determined by restriction mapping.

To characterize the fusion protein, we prepared lysogens of  $\lambda$ GAD and  $\lambda$ gt-11 in *E. coli* strain Y1089, induced them with IPTG, and examined the polypeptides of each by electrophoresis and immunoblotting (12, 13). The results (Fig. 1) are consistent with the production of a fusion protein that contains antigenic sites of both  $\beta$ -galactosidase and GAD: (i) antibody to  $\beta$ -galactosidase reacts with the 116-kD  $\beta$ -galactosidase polypeptide in nonrecom-

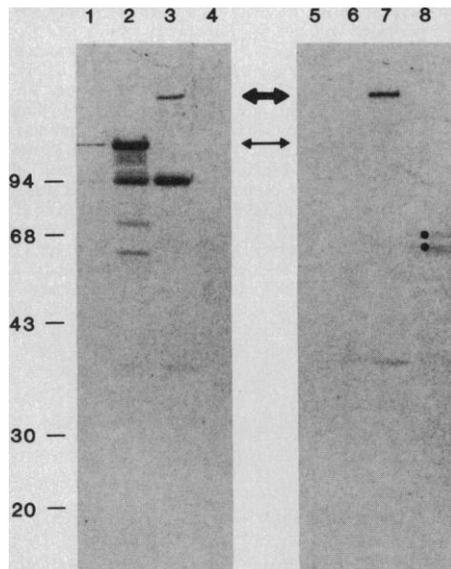


Fig. 1. Immunological verification of GAD and  $\beta$ -galactosidase epitopes in the same polypeptide. Protein was extracted from  $\lambda$ -lysogens as described in the legend to Table 1, except that 50 mM tris-HCl, pH 7.2, replaced potassium phosphate. Brain was homogenized in a solution containing 0.2 mM PMSF, 150 mM NaCl, and 50 mM tris-HCl, pH 8.0, and the protein was extracted. Cell debris was removed by centrifugation. The proteins were separated by electrophoresis in a 10% polyacrylamide gel in SDS (23) and electrophoretically transferred to nitrocellulose (13, 24). The unreacted sites were blocked with bovine serum albumin and gelatin, and the nitrocellulose sheet was cut in half. One half was incubated overnight with a 1:1000 dilution of affinity-purified rabbit antibody to  $\beta$ -galactosidase and the other half with a 1:500 dilution of sheep antibody to GAD; the sheets were then incubated for 1 hour with a 1:1000 dilution of rabbit antiserum to sheep IgG. The sheet was extensively washed, and bound antibodies were detected with  $^{125}$ I-labeled protein A and autoradiography. (Lanes 1 to 4) Detection of  $\beta$ -galactosidase epitopes. (Lanes 5 to 8) Detection of GAD epitopes. Each lane contained the following: lanes 1 and 5, purified  $\beta$ -galactosidase (200 ng); lanes 2 and 6,  $\lambda$ gt-11 lysogen extracts (20  $\mu$ g); lanes 3 and 7,  $\lambda$ GAD lysogen extracts (20  $\mu$ g); and lanes 4 and 8, extracts of cat brain (20  $\mu$ g). A ninth lane (not shown) contained  $^{125}$ I-labeled molecular weight standards. The thin arrow marks the position of wild-type  $\beta$ -galactosidase, and the thick arrow marks the fusion protein produced by  $\lambda$ GAD.

binant  $\lambda$ gt-11 infected cells (lane 2) and with a larger polypeptide (>156 kD) in cells infected with  $\lambda$ GAD (lane 3); and (ii) antibody to GAD does not react with polypeptides in the  $\lambda$ gt-11 control, but does recognize the larger polypeptide in extracts of cells infected with  $\lambda$ GAD (lane 5). Synthesis of the GAD antigen depends on induction with IPTG, an inducer of the lac operon (Fig. 2).

In cat brain extracts, antibody to  $\beta$ -galactosidase does not react with any polypeptide (lane 4), whereas antibody to GAD reacts with both a 60-kD and a 66-kD polypeptide (lane 8). From the size of the fusion polypeptide we estimate that the GAD polypeptide segment is >400 amino acid residues long, corresponding to more than 60% of the 66-kD GAD polypeptide.

The experiments summarized in Table 1 show that the fusion protein produced in *E. coli* by  $\lambda$ GAD catalyzes the decarboxylation of glutamate. Extracts of bacteria infected with  $\lambda$ GAD convert about 20 times as much glutamate to  $\text{CO}_2$  as extracts of cells infected with nonrecombinant  $\lambda$ gt-11. This GAD enzymatic activity can be removed from  $\lambda$ GAD cell extracts by treatment with antibody to  $\beta$ -galactosidase. After immunoprecipitation with antibody to  $\beta$ -galactosidase,  $^{14}\text{CO}_2$  production in the GAD enzymatic assay falls almost to the control level. Immunoprecipitation of control extracts (from bacteria infected with  $\lambda$ gt-11) with antibody to  $\beta$ -galactosidase had no effect on basal production of  $^{14}\text{CO}_2$  in the assay. The limited ability of control cells to convert [ $^{14}\text{C}$ ]glutamate to  $^{14}\text{CO}_2$  could result either from endogenous bacterial GAD or from the oxidative deamination of glutamate and subsequent decarboxylations by Krebs cycle enzymes.

To purify the fusion protein away from bacterial decarboxylases, we performed gel filtration with Sepharose 6B, followed by chromatography on hydroxyapatite and DEAE-cellulose. After approximately 50-fold purification of the fusion protein, approximately 99% of the GAD activity could be removed by antibody to  $\beta$ -galactosidase. This result confirms that the GAD enzymatic activity and  $\beta$ -galactosidase epitopes are physically linked.

GAD enzymatic activity can also be removed with antibody to GAD (Table 1). Treatment with this antibody, however, was less efficient in removing enzyme activity than precipitation with antibody to  $\beta$ -galactosidase. This antibody removes 85% of the GAD enzymatic activity in brain extracts (4). Its lower efficiency with the fusion protein may result from (i) lack of a complete GAD coding sequence in the cDNA, (ii) the masking of epitopes by the  $\beta$ -galac-

tosidase polypeptide, or (iii) altered folding or posttranslational modification in *E. coli*.

To confirm that the partially purified fusion protein catalyzes the production of GABA as well as of  $\text{CO}_2$ , we measured GABA production by high-performance liquid chromatography of phenylthiocarbonyl (PTC)-amino acids on a Waters PICO-TAG reversed-phase column (14). PTC-GABA eluted at 5.8 minutes. In one experiment, for example, uniformly labeled [ $^{14}\text{C}$ ]glutamate was converted to equimolar amounts of  $^{14}\text{CO}_2$  (10,600 dis/min) and [ $^{14}\text{C}$ ]GABA (45,300 dis/min).

Studies of GAD from mice, rats, and humans suggest subunit sizes ranging from 15 to 67 kD, as judged by sedimentation equilibrium under denaturing conditions and electrophoresis of purified GAD in sodium dodecyl sulfate (SDS) (15, 16). Because of this uncertainty, we tested antisera produced to different preparations of purified GAD to ascertain whether they would all react with our fusion protein and if they would recognize the same brain polypeptides. We have used three such antisera in the immunoblotting experiments shown in Fig. 2. These antisera all reacted specifically with the fusion protein produced by GAD.

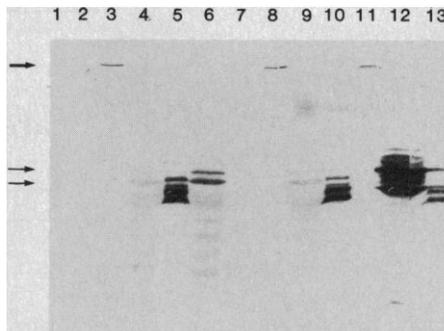


Fig. 2. Immunoblotting of brain extracts with three antisera to GAD. Extracts of lysogens and brain were analyzed by electrophoresis in 10% polyacrylamide gel in SDS followed by immunoblotting as described in the legend to Fig. 1. The nitrocellulose sheet was cut into three parts, and each was incubated with a 1:500 dilution of a different antiserum to GAD: lanes 1 to 7 with sheep antiserum to rat GAD (4), lanes 8 to 10 with sheep antiserum to pig GAD (25), and lanes 11 to 13 with rabbit antiserum to mouse GAD (3). These antisera specifically recognize the fusion polypeptide (thick arrow) and at least two major polypeptides (60 and 66 kD) in cat brain (thin arrows). Although these two polypeptides are weak in lane 7, they can be seen on longer exposures. Similar results were obtained when electrophoresis was performed in 7.5% polyacrylamide. The following extracts were examined: Lane 1,  $\lambda$ gt-11 lysogen (20  $\mu$ g); lane 2,  $\lambda$ GAD lysogen without IPTG induction (20  $\mu$ g); lanes 3, 8, and 11,  $\lambda$ GAD lysogen after IPTG induction (20  $\mu$ g); lanes 4, 9, and 12, cat brain (20  $\mu$ g); lanes 5, 10, and 13, partially purified rat GAD (25) (4  $\mu$ g); lane 6, mouse brain (20  $\mu$ g); and lane 7, human brain (20  $\mu$ g).

All three also recognized two major polypeptides of 60 and 66 kD in human, cat, rat, and mouse brain extracts. These three antisera (two of which have been extensively used to map GABA-containing neurons) seem to recognize overlapping sets of epitopes. The smaller polypeptides recognized by these antisera may represent degradation products of a single 66-kD polypeptide.

The antigenicity and enzymatic activity of the fusion protein establishes the identity of the GAD cDNA. In addition, labeled GAD DNA hybridizes to a single electrophoretic component in polyadenylated RNA of cat and human brain, but not of liver or kidney, a result consistent with the expected distribution of GAD in the brain (1, 2, 17). Furthermore, the pattern of in situ hybridization with single-stranded GAD RNA probes with frozen sections of mouse brain is consistent with the immunocytochemically determined distribution of GABA-containing neurons (18).

Of particular note is the enzymatic activity of the fusion protein, which consists of 1006 amino acid residues of  $\beta$ -galactosidase and at least 400 amino acid residues of GAD, representing two-thirds or more of the total length of one of the brain GAD polypeptides. Apparently the attachment of

the GAD polypeptide segment to the  $\beta$ -galactosidase polypeptide segment is sufficiently flexible to allow the assembly not only of immunologically detectable domains but also of the active site. Direct assays of enzyme activity or ligand binding (19) may be useful for identifying other members of bacterial expression libraries.

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## Dentochronological Separation Estimates for Pacific Rim Populations

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Dental morphology of American Indians, Asians, and Pacific islanders is used with a multivariate statistic to estimate when genetic separation occurred between several populations. These estimates generally match independent estimates of separation. This method, called dentochronology, gives an American Indian fission date from Asians of about 13,000  $\pm$  3,000 years ago, which agrees with archeological data and rules out a European origin because of temporal priority. Polynesians split from Southeast Asians 5,000  $\pm$  2,200 years ago and are not derived from Melanesians. Ainu-Jomon originated in Sundaland 14,000  $\pm$  3,300 years ago. Africans have been separated from Asian-Americans 60,000  $\pm$  6,100 years.

**M**ICROEVOLUTION OF DENTAL morphology was proposed for estimating the separation date of American Indians from north Asians, when the multivariate mean measure of divergence (MMD) value was chronologically calibrated with independent archeological information for both (1). The dental clock for the peopling of the New World by Paleo-Indians, set by Aleut archeological and paleoenvironmental information, indicated that the groups arrived independently in Alaska about 12,000 years ago, giving an MMD of

Aleut to northeast Asian of 0.115733 and a dental microevolution rate of 0.00964 MMD per 1000 years. Dividing the rate into the mean American Indian to northeast Asian MMD of 0.135 suggested Indian-northeast Asian separation about 14,000 years ago. This value agreed with Paleo-Indian archeological findings of Clovis culture in the American Southwest 12,000 years ago (2). A few other comparisons suggested the dentochronological method to be promising. Additional Old World and Pacific dental data were collected

in 1983–84 to further assess the method.

The teeth used here belong to 14,000 crania in 200 human skeletal samples, mostly archeological but some ethnographic or hospital preparations, mainly from the Americas, eastern Asia, and the Pacific Basin. Each sample is characterized by 28 standard crown and root traits, like incisor shoveling, molar and premolar cusp numbers, root numbers of various teeth, and others. Sexes are pooled and counts are based on individuals (3).

The MMD's were calculated for all pairs of comparisons between 85 samples. Those possessing archeological, linguistic, or physical anthropological evidence for common descent are given in Table 1. The rest are excluded for lack of relevant independent information.

Estimating the time of genetic separation between two groups is more difficult than establishing when one of the two initially colonized a new area. Helpful for reconstructing Pacific Rim population history is the 100-m rise in sea level between 16,000 to 12,000 years ago that fragmented and isolated Tasmania–Australia–New Guinea

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