

Multiplicity of Glutamic Acid Decarboxylases (GAD) in Vertebrates: Molecular Phylogeny and Evidence for a New GAD Paralog

Peter T. Bosma,*† Mercedes Blázquez,*† Martin A. Collins,* John D. D. Bishop,‡ Guy Drouin,§ Imants G. Priede,* Kevin Docherty,† and Vance L. Trudeau*¹

*Department of Zoology, University of Aberdeen; †Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, U.K.; ‡Marine Biological Association of the U.K., The Laboratory, Plymouth, U.K.; and §Department of Biology, University of Ottawa, Ontario, Canada.

The evolution of chordate glutamic acid decarboxylase (GAD; EC 4.1.1.15), a key enzyme in the central nervous system synthesizing the neurotransmitter gamma-aminobutyric acid (GABA) from glutamate, was studied. Prior to this study, molecular data of GAD had been restricted to mammals, which express two distinct forms, GAD₆₅ and GAD₆₇. These are the products of separate genes and probably are derived from a common ancestral GAD following gene duplication at some point during vertebrate evolution. To enable a comprehensive phylogenetic analysis, molecular information of GAD forms in other vertebrate classes was essential. By reverse transcriptase-polymerase chain reaction (RT-PCR), partial nucleotide sequences of GAD were cloned from brains of zebra finch (*Taeniopygia guttata*), turtle (*Trachemys scripta*), goldfish (*Carassius auratus*), zebrafish (*Danio rerio*), and armoured grenadier (*Coryphaenoides (Nematonurus) armatus*, a deep-sea fish), and from the cerebral ganglion plus neural gland of *Ciona intestinalis*, a protochordate. Whereas GAD₆₅ and GAD₆₇ homologs were expressed in birds, reptiles, and fish, only a single GAD cDNA with equal similarities to both vertebrate GAD forms was found in the protochordate. This indicates that the duplication of the vertebrate GAD gene occurred between 400 and 560 million years ago. For both GAD₆₅ and GAD₆₇, the generated phylogenetic tree followed the general tree topology for the major vertebrate classes. In turtle, an alternative spliced form of GAD₆₅, putatively encoding a truncated, nonactive GAD, was found. Furthermore, a third GAD form, which is equally divergent from both GAD₆₅ and GAD₆₇, is expressed in *C. (N.) armatus*. This third form might have originated from an ancient genome duplication specific to modern ray-finned fishes.

Introduction

The amino acids gamma-aminobutyric acid (GABA) and glutamate are the most abundant neurotransmitters in the brain, both playing essential roles in synaptic plasticity (Nakanishi 1992; Luddens, Korpi, and Seeburg 1995; Mori and Mishina 1995; Staley, Soldo, and Proctor 1995) and neuroendocrine function (Brann and Mahesh 1994; Trudeau 1997). Defects in amino acid neurotransmission are the cause of several pathologies, including neurodegradation and epilepsy (Nakanishi 1992; During 1994; During, Ryder, and Spencer 1995; Luddens, Korpi, and Seeburg 1995; Mori and Mishina 1995). Gamma-aminobutyric acid is considered to be a classical inhibitory neurotransmitter, whereas most of the excitatory synapses in the central nervous system use glutamate as their neurotransmitter. Of major importance for amino acid-mediated neurotransmission is the control of GABA synthesis in the brain by the enzyme glutamic acid decarboxylase (GAD; EC 4.1.1.15), which catalyzes the formation of GABA from glutamate (Erlander and Tobin 1991; Martin and Rimvall 1993). In mammals, GAD exists in

at least two forms with molecular weights of approximately 65 (GAD₆₅) and 67 kDa (GAD₆₇). The amino acid sequences of GAD₆₅ and GAD₆₇ are less similar (~65% identity) than are sequences of each form in different species (~95% identity; Erlander et al. 1991; Bu et al. 1992; Suzuki et al. 1995). GAD₆₅ and GAD₆₇ are the products of two separate genes in mammals. Both have differing functions depending on their distribution in the neuron and also on their interaction with the co-enzyme pyridoxal 5'-phosphate (PLP; e.g., Erlander et al. 1991; Kaufman, Houser, and Tobin 1991; Bu et al. 1992; reviewed by Martin and Rimvall 1993). GAD₆₅ and GAD₆₇ are also expressed in nonneural tissues, but little is known about the physiological role of GABA in these tissues (reviewed by Tillakaratne, Medina-Kauwe, and Gibson 1995).

To date, two molecular forms of vertebrate GAD have been sequenced only from the brains of a few mammalian species (Julien, Samana, and Mallet 1990; Katarova et al. 1990; Erlander et al. 1991; Bu et al. 1992; Lee et al. 1993; Suzuki et al. 1995; Mitshushima et al. 1996), whereas sequence information of the GADs in the cat is restricted to the larger form (Kobayashi, Kaufman, and Tobin 1987). Immunocytochemical and mRNA expression data also suggested the presence of GAD₆₅ and GAD₆₇ in the brains of birds (Legay, Pelhate, and Tappaz 1986; Åhman, Wågberg, and Mattsson 1996), yet only a partial sequence of chicken GAD₆₅ is presently available (Åhman, Wågberg, and Mattsson 1996). In contrast to the duality of GAD in higher vertebrates, only one GAD has originally been reported in poikilothermic vertebrates such as the trout and frog, using mammalian anti-GAD antibodies in Western blot analysis (Legay, Pelhate, and Tappaz

¹Present address: Department of Biology, University of Ottawa, Ontario, Canada.

Abbreviations: GAD, glutamic acid decarboxylase; GABA, gamma-aminobutyric acid; RT-PCR, reverse transcriptase-polymerase chain reaction; PLP, pyridoxal 5'-phosphate.

Key words: glutamic acid decarboxylase, GAD, gamma-aminobutyric acid, neurotransmitter, molecular phylogeny, chordate evolution.

Address for correspondence and reprints: Vance L. Trudeau, Department of Biology, University of Ottawa, P.O. Box 450 Stn. A, Ottawa, Ontario, Canada. K1N 6N5. E-mail: vtrudeau@science.uottawa.ca.

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1986). The limited data on nonmammalian GADs precluded any phylogenetic analysis of the evolutionary origins of the mammalian GADs.

We undertook the sequencing of GAD cDNAs from a reptile, a bird, several fish, and a protochordate. The results indicate that GAD₆₅ and GAD₆₇ genes are expressed in the central nervous system of the major vertebrate classes. We also present the first phylogenetic analysis of this enzyme and demonstrate the existence of alternative splicing in the transcript of a turtle GAD₆₅ gene and the presence of a third GAD gene in a deep-sea fish.

Materials and Methods

Animals and Tissue Collection

Zebra finches (*Taeniopygia guttata*), red-eared slider turtles (*Trachemys scripta*), and zebrafish (*Danio rerio*) were purchased from a local pet shop. Goldfish (*Carassius auratus*) were purchased from a commercial supplier (Mount Parnell, Penn.). These animals were killed by decapitation, and nervous tissue (zebra finch and turtle: two brains; zebrafish and goldfish: four brains) was excised, pooled on dry ice, and stored at -80°C . One specimen of the armoured grenadier (*Coryphaenoides (Nematonurus) armatus*), an abyssal, scavenging, macrourid fish living at depths of >2500 m (Priede et al. 1991), was collected by trawl in the Porcupine Seabight (northeast Atlantic) during an expedition in August 1996. The brain was dissected, frozen immediately to -70°C , transported to a shore-based laboratory, and used for the initial cloning of grenadier GAD forms. The protochordate *Ciona intestinalis* was collected near Plymouth, U.K., and 10 cerebral ganglia with the associated neural glands were dissected, pooled on dry ice, and stored at -80°C .

RNA Extraction, Reverse Transcriptase-Polymerase Chain Reaction, and DNA Sequencing

Total RNA was isolated from frozen nervous tissue using RNazol (Biogenesis, Poole, U.K.), and further extraction of poly(A)⁺ RNA was performed with the Oligotex mRNA mini kit (Qiagen, Crawley, U.K.). First-strand cDNA was synthesized from 100 ng poly(A)⁺ RNA with Moloney murine leukemia virus reverse transcriptase and oligo (dT) primer (RAP-PCR kit, Stratagene, La Jolla, Calif.) and stored at -30°C until used. Partial GAD-related cDNAs were amplified by a PCR strategy adapted from Cram et al. (1991) by using two degenerate oligonucleotide primers (Gibco/BRL, Paisley, U.K.) that correspond to DNA sequences encoding conserved amino acid sequences of the known GAD₆₅ and GAD₆₇ genes. The sense primer was 5'-(A/G)AC(A/T/G)GC(A/C)AA(C/T)AC(T/C/G)AA-(C/T)ATGTT(C/T)AC(A/T/C)TATGA-3'; the antisense primer was 5'-CATCAT(C/T)TT(A/G)TG(A/C/T/G)GG(A/G)TTCCA(A/C/T/G)GT-3'. The antisense primer contains the complementary nucleotide sequence encoding the highly conserved putative PLP-binding site NPHK (Martin and Rimvall 1993). For each species, a 4- μl aliquot of cDNA was amplified in

a 50- μl PCR volume in a Hybaid Touchdown thermal cycler (Hybaid/Life Sciences International, Basingstoke, U.K.) by the following program: 5 min denaturation at 94°C , then 10 cycles of denaturation at 94°C for 30 s, annealing at 43°C for 30 s, and extension at 72°C for 1 min, followed by 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min. After the last cycle, further extension was performed at 72°C for 5 min. Amplified products of the expected size (~ 600 bp) were extracted from a 1% agarose gel with the QIAquick gel extraction kit (Qiagen), ligated into the PCR 2.1 cloning vector (Original TA cloning kit; Invitrogen, Leek, The Netherlands), and subsequently transformed into competent cells (One Shot competent cell kit; Invitrogen). Single colonies were cultured, and plasmids were recovered with the Plasmid mini-kit (Qiagen). All procedures were done according to the instructions of the manufacturers. For each new GAD form, both strands of four to seven different cloned inserts were sequenced using an ABI 377 automated sequencer (P. E. Applied Biosystems, Warrington, U.K.). Nucleotide sequences (excluding the oligonucleotide primers) were submitted to FASTA for comparison to known sequences accessible in the EMBL and GenBank databases.

GAD Phylogeny and Relative-Rate Tests

Comparison of the nucleotide and deduced amino acid sequences, as well as of the phylogenetic analysis of the vertebrate GADs, was performed with the interactive sequence analysis software available from the University of Wisconsin Genetics Computer Group (GCG). For the phylogenetic study, GAD amino acid sequences were aligned with CLUSTAL W (Thompson, Higgins, and Gibson 1994). Our partial GAD amino acid sequences were aligned with the equivalent regions of previously sequenced vertebrate and *Drosophila melanogaster* GAD genes. These sequences were obtained from the EMBL and GenBank databases (accession numbers are as follows: human GAD₆₅ and GAD₆₇—M81882 and M81883, pig GAD₆₅ and GAD₆₇—D31848 and D31849, mouse GAD₆₅ and GAD₆₇—L16980 and Z49976, rat GAD₆₅ and GAD₆₇—M72422 and X57572, cat GAD₆₇—M18629, zebrafish GAD₆₅—AF01726, and *D. melanogaster* GAD—X76198). The phylogenetic analyses were performed on the amino acid sequence alignment shown in figure 1 but without the truncated turtle GAD₆₅ sequence. Maximum-likelihood trees were calculated using PUZZLE version 4.0, with the *D. melanogaster* GAD sequence as the outgroup, the Dayhoff model of amino acid substitutions, and eight gamma-distributed rates estimated from the data set (Strimmer and von Haeseler 1996). Statistical confidence of the phylogenetic position of the grenadier GAD3 sequence was evaluated by calculating the standard error of the difference in log likelihood between different user-specified trees with PROTML version 2.2 and PUZZLE version 4.0 programs (Adachi and Hasegawa 1996, Strimmer and von Haeseler 1996). Two phylogenies were considered significantly different when the difference in log likelihoods between the best tree and those being

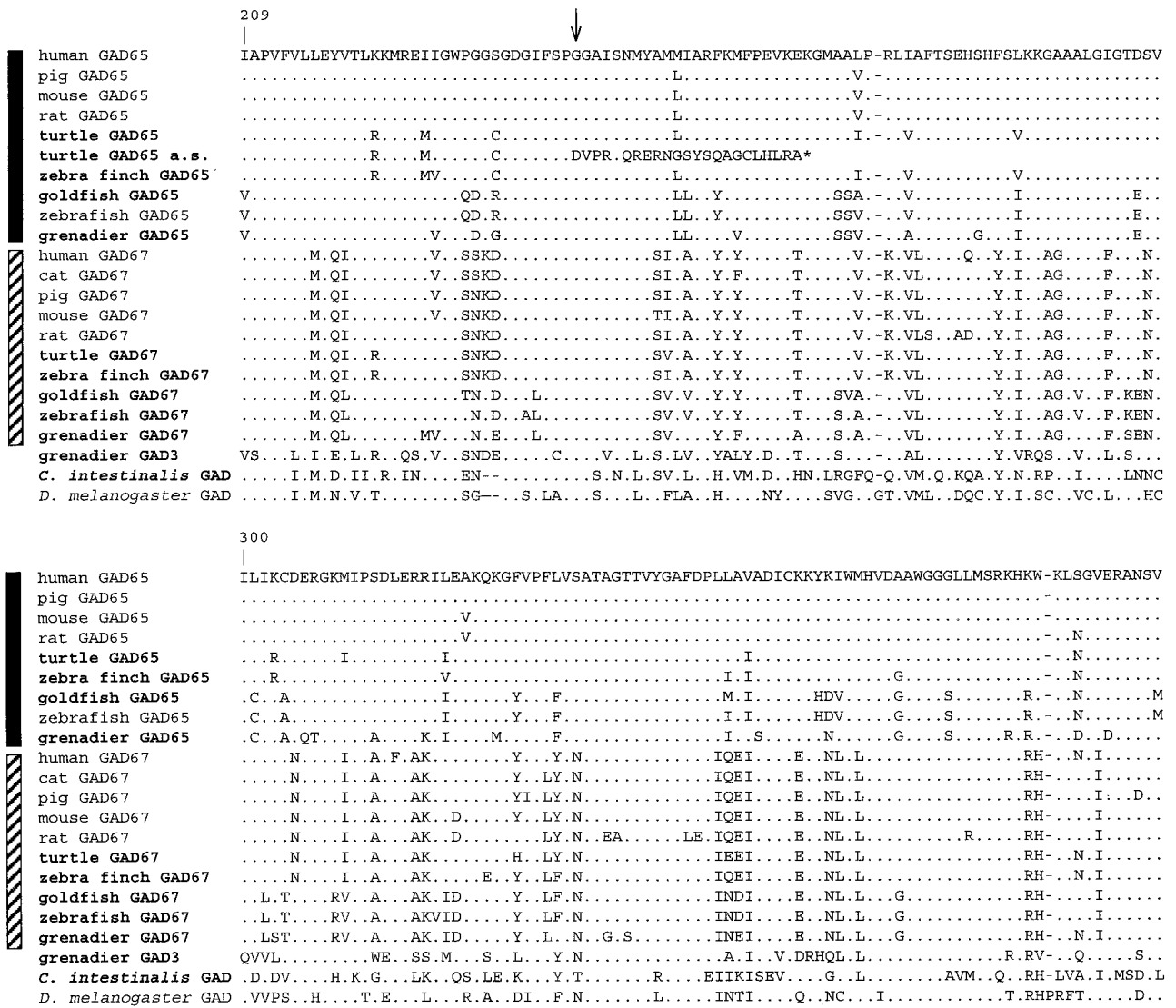


FIG. 1.—Alignment of deduced amino acid sequences used in this study. Dots indicate amino acid identities with human GAD₆₅; sequence gaps are indicated by dashes. Numbers indicate the amino acid positions in the human GAD₆₅ sequence. Names of GADs cloned in this study are in bold. The arrow indicates the position of an intron between exons 6 and 7 in human GAD₆₅ and GAD₆₇. Turtle GAD₆₅ a.s., alternatively spliced variant of the GAD₆₅ form in this species; the asterisk indicates a translational stop codon. GAD₆₅ sequences are preceded by a black bar, while GAD₆₇ sequences are preceded by a hatched bar.

tested was more than 1.96 times the standard error (Kishino and Hasegawa 1989).

The DNA sequences were too divergent to use the relative-rate test method of Li and Tanimura (1987). Relative-rate tests were therefore performed on the aligned amino acid sequences shown in figure 1 using the one-degree-of-freedom method of Tajima (1993) and the relative-rate test method of Robinson et al. (1998) as implemented in the program RRTree version 0.6 (available at ftp://pbil.univ-lyon1.fr/pub/datasets/MBE98/).

Results

Multiple GAD Forms in Vertebrate Brain

The expression of GAD was studied using RT-PCR in zebra finch, turtle, goldfish, zebrafish, armoured gren-

adier, and *C. intestinalis*. Twelve new partial sequences were obtained from the six species examined. All clones showed highest similarities with vertebrate GAD₆₅ or GAD₆₇ sequences in the EMBL and GenBank databases. The sequences (excluding the oligonucleotide primers) were 547 bp in length, except for *C. intestinalis*, which was 541 bp. These sequences represent approximately one third of the total length of the coding region, and in-frame deduced amino acid sequences were compared with the equivalent regions of GAD₆₅ and GAD₆₇ from other vertebrates, and with *D. melanogaster* GAD (fig. 1). GAD₆₅ and GAD₆₇ homologs were found in goldfish, grenadier, zebra finch, and turtle, whereas only a GAD₆₇ homolog was cloned and sequenced from zebrafish brain. A third sequence of 500 bp was obtained from turtle, which was identical to the turtle GAD₆₅ homolog,

apart from a missing 47-bp stretch. This 47-bp DNA sequence is flanked with the consensus splicing sites 5'-CCTG/GTGG..CAAG/ATGT-3', thus strongly suggesting that the 500-bp clone is the result of alternative splicing during RNA precursor processing. This leads to a shift in the reading frame, causing multiple stop codons in the new sequence, which putatively encodes a truncated GAD₆₅ peptide that lacks the PLP-binding site (fig. 1). In addition to the GAD₆₅ and GAD₆₇ sequences, a third GAD sequence (GAD3) was identified in the grenadier brain, which is equally divergent from the GAD₆₅ and GAD₆₇ forms (fig. 1). The expression of three GAD forms in the brain of armoured grenadier was confirmed in three more specimens by RT-PCR, using specific primers for each form (not shown). One GAD sequence was cloned from cerebral ganglion plus neural gland of *C. intestinalis* (fig. 1). Its overall identity with the other GADs is the lowest of all forms, yet the deduced amino acid sequence of *C. intestinalis* GAD still shows approximately 80% similarity (including the conservative amino acid substitutions) with both human GADs (fig. 1).

GAD Phylogeny and Relative-Rate Tests

The amino acid alignment of the 22 GAD sequences (excluding the alternatively spliced turtle GAD₆₅ sequence) shown in figure 1 was used to determine phylogenetic relationships. The tree shown in figure 2, rooted with the *D. melanogaster* GAD, shows the phylogenetic relationships between these GAD sequences. For both GAD₆₅ and GAD₆₇, the branching order for the different species is similar and follows the general tree topology for the major vertebrate classes (Colbert and Morales 1991, pp. 1–15). In contrast, neither grenadier GAD3 nor *C. intestinalis* GAD group as GAD₆₅ or GAD₆₇. Instead, *C. intestinalis* GAD is clearly an outgroup to GAD₆₅ and GAD₆₇, whereas grenadier GAD3 appears to have originated at the same time as GAD₆₅ and GAD₆₇. We used the one-degree-of-freedom method of Tajima (1993) to test whether the three grenadier amino acid sequences evolved at the same rate. With the *C. intestinalis* sequence as the outgroup, the χ^2 values for GAD₆₅ versus GAD₆₇, GAD3 versus GAD₆₅, and GAD3 versus GAD₆₇ were 0.03, 1.58, and 1.20, respectively. Since none of these values are significant at the 0.05 level ($\chi^2 < 3.84$), these three genes therefore evolved at substantially the same rate. We also used the relative-rate test method of Robinson et al. (1998) to determine whether the 9 GAD₆₅, the 10 GAD₆₇, the grenadier GAD3, and the *C. intestinalis* sequences evolved at different rates. These tests were performed both with and without topological weights based on the tree topology shown in figure 2. In all cases, and using the *D. melanogaster* sequence as an outgroup, these tests showed that these four groups of sequences did not evolve at significantly different rates from one another (results not shown). Although, in figure 2, the grenadier GAD3 sequence is shown to have originated at the same time as the GAD₆₅ and GAD₆₇ sequences, this phylogenetic position is not well supported. The phylogenetic position of the grenadier GAD3 sequence was evaluated with the

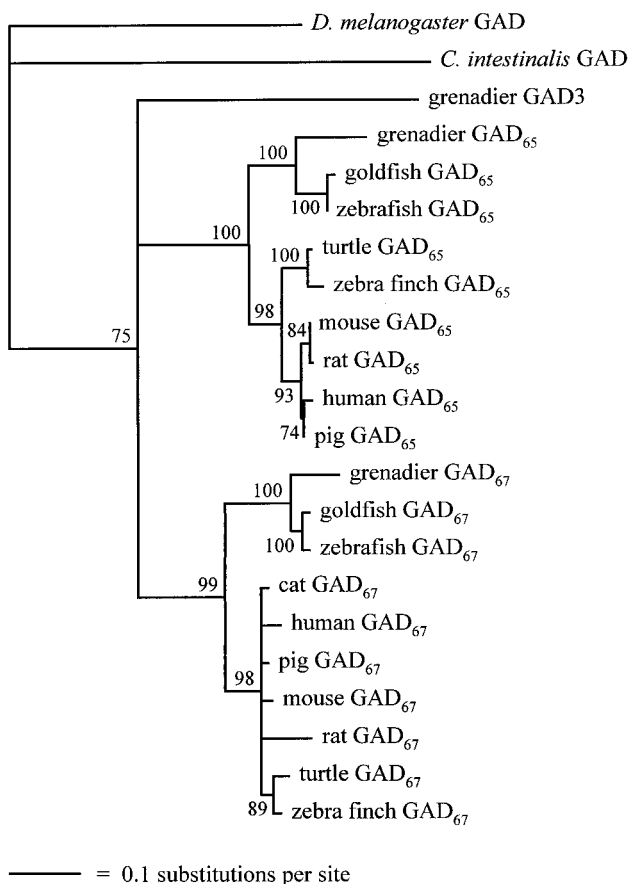


FIG. 2.—The phylogenetic tree for vertebrate GAD. Maximum-likelihood analyses were performed with the PUZZLE software in GCG. Support values are shown next to their respective nodes.

Kishino-Hasegawa test as implemented in the PROTML and PUZZLE programs (Adachi and Hasegawa 1996; Strimmer and von Haeseler 1996). The PROTML analyses, which used the Jones, Taylor, and Thornton (1992), the Poisson, or the Dayhoff models of amino acid substitutions, showed that trees in which the GAD3 sequence originated at the same time as the GAD₆₅ and GAD₆₇ sequences (trichotomy), in which the GAD3 sequence was a sister group to GAD₆₅ sequences, or in which the grenadier GAD3 sequence was a sister group to GAD₆₇ sequences were all as likely (results not shown). The PUZZLE analyses, using the Dayhoff model of amino acid substitutions and eight gamma-rate categories, also found no significant difference between the three topologies tested with the PROTML program (results not shown). In contrast, both PROTML and PUZZLE analyses showed that topologies in which the grenadier GAD3 sequence was a sister group to either the fish GAD₆₅ or the fish GAD₆₇ sequences were significantly worse than the topology shown in figure 2 (results not shown).

Data Deposition

The sequences reported in this paper have been submitted to GenBank. Accession numbers are as follows: zebrafish GAD₆₇—AF042374; goldfish GAD₆₅ and GAD₆₇—AF043265 and AF043266; grenadier GAD₆₅,

GAD₆₇, and GAD3—AF043267, AF043268, and AF043269; zebra finch GAD₆₅ and GAD₆₇—AF043270 and AF043271; turtle GAD₆₅, GAD₆₅ (alternative splicing form), and GAD₆₇—AF043272, AF043273 and AF043274; and *C. intestinalis* GAD—AF043275.

Discussion

We showed that multiple forms of GAD are expressed in the brain of birds, reptiles, and fishes. In vertebrates, the expression of two molecular forms of GAD in the brain had only been established previously in mammals (Julien, Samana, and Mallet 1990; Katarova et al. 1990; Erlander et al. 1991; Bu et al. 1992; Lee et al. 1993; Suzuki et al. 1995; Mitshushima et al. 1996), while GAD₆₅ and GAD₆₇ expression was recently shown in neurons of the developing zebrafish embryo (Martin, Heinrich, and Sandell 1998). The presence of GAD₆₅ and GAD₆₇ mRNA in zebra finch unambiguously confirmed that two different GAD genes are expressed in avian brain (Legay, Pelhate, and Tappaz 1986; Åhman, Wågberg, and Mattsson 1996). In addition, the present study showed, for the first time, the expression of GAD₆₅ and GAD₆₇ genes in a reptile. Furthermore, the expression of GAD₆₅ and GAD₆₇ in the brain of armoured grenadier and goldfish extends the findings in zebrafish embryos (Martin, Heinrich, and Sandell 1998) to include two other teleost genera.

The determination of a substantial part of GAD nucleotide sequences in these vertebrate classes and in a protochordate enabled us to perform a comprehensive analysis of the evolutionary relationships between the chordate GADs. The phylogenetic tree indicates that GAD₆₅ and GAD₆₇ genes are present in the genome of the major vertebrate classes. Although only a single GAD form has previously been isolated from cats (Kobayashi, Kaufman, and Tobin 1987), our phylogenetic analyses show that this is a GAD₆₇ homolog and suggest that a GAD₆₅ gene product should also be present in this species.

Previous hypotheses on the origin of the two forms of GAD were based only on mammalian sequences. The high sequence similarities between GAD₆₅ and GAD₆₇ and the exon/intron structure of the two mammalian GAD genes suggested that both GADs were derived from a common ancestral GAD gene by gene duplication (Erlander et al. 1991; Bu et al. 1992; Lee et al. 1993; Bu and Tobin 1994). The expression of GAD₆₅ and GAD₆₇ genes in all the vertebrate classes studied here indicates that such a gene duplication must have occurred before the divergence of teleosts from the main vertebrate lineage about 400 million years ago. Our phylogenetic tree indicates that the single GAD form found in *C. intestinalis*, a member of a protochordate lineage (ascidians) that diverged from vertebrate chordates after the Cambrian explosion about 560 million years ago, is probably the only GAD in this species and is a derivative of the archetypal chordate GAD form. This implies that the gene duplication of the single ancestral GAD gene occurred along the lineage leading to chordates, after the branching off of ascidians.

Interestingly, there are many examples of genes that duplicated early in vertebrate evolution. The list includes genes from several homeobox gene families, such as *Otx* (Williams and Holland 1998), *Pax* (Wada et al. 1998), and *Hox* gene clusters (Sharman and Holland 1998; reviewed by Holland and Garcia-Fernández 1996; Sharman and Holland 1996), a gene encoding the DNA-binding protein HMG (Sharman, Hay-Schmidt, and Holland 1997), and genes of the neuropeptide Y family (Larhammar 1996). On the basis of phylogenetic analyses, Sharman and Holland (1996) suggested that the widespread gene duplications in vertebrates have occurred in two phases. The first phase, during the origin of vertebrates (550–450 million years ago), was probably a duplication of only a subset of the genome, whereas the second phase (450–400 million years ago) was presumably a complete genome duplication through tetraploidization. It is possible that the multiplicity of vertebrate GAD, a key enzyme in the central nervous system, has its origin in these gene duplication events. This is supported by the notion that these gene duplications may have allowed the evolution of specific, or more complex, vertebrate characteristics of developmental mechanisms and body plans, such as the possession of a backbone and a highly complex brain (Holland and Garcia-Fernández 1996). In contrast to the vertebrate brain, the neural tube in ascidians is quite simple, although it can be subdivided into three regions that are homologous to the vertebrate forebrain, midbrain, and hindbrain regions (Wada and Holland 1996; Wada et al. 1997; Wada et al. 1998). With regards to GAD, the possession of multiple genes for this enzyme has likely provided increased control over GABA synthesis in the nervous system, thus contributing to the evolution of a more complex brain in vertebrates.

The site of the alternative splicing in the turtle GAD₆₅ exactly matches the site of the intron between exons 6 and 7 in the coding region of the human GADs (fig. 1), supporting the notion that the exon/intron organization of GAD₆₅ and GAD₆₇ forms has been conserved during vertebrate evolution (Bu and Tobin 1994). It is not known if the alternative splicing of turtle GAD₆₅ occurs as part of the removal of the intron between exons 6 and 7, or if it is excised in a separate event using the consensus splicing sites that flank the 47-bp sequence. Without the PLP-binding site, the resulting truncated turtle GAD₆₅ protein will likely be enzymatically inactive, and a possible function remains to be elucidated. Several other examples of alternative splicing of brain GAD mRNA have been described previously. In embryonic rat and mouse brains, truncated GAD₆₇ forms are encoded by alternative spliced mRNAs that have an extra exon inserted that contains an early stop codon in frame (Bond, Wyborski, and Gottlieb 1990; Behar et al. 1994; Szabo, Katarova, and Greenspan 1994). Splicing events have also been reported for nonneural GAD (Karlsen et al. 1991; Michelsen et al. 1991; Tillakaratne et al. 1992; Li et al. 1995).

To our knowledge, the expression of three distinct molecular forms of GAD in one species has not previously been established. A potential GAD3 gene was

mapped in the genomes of human and mouse on chromosomes different from those containing the genes for GAD₆₅ and GAD₆₇ (Edelhoff et al. 1993). However, the cDNA fragment used as the hypothetical GAD3 gene, obtained by rapid amplification of cDNA ends of human pancreatic islet cDNA, showed 98% identity with MSE55 (Bahou, Campbell, and Wicha 1992), a marrow stromal/endothelial cell protein (Lernmark and Karlson, personal communication). Furthermore, a molecule with GAD enzyme activity has been cloned from a mouse brain cDNA library (Huang et al. 1990). However, this protein shows only 17% identity with other GADs and does not contain the NPHK consensus sequence for the PLP-binding site and thus seems unrelated to any of the GAD forms cloned in the present study.

How can the expression of a third, distinct GAD gene in the grenadier brain be explained? Our analyses show that the grenadier GAD3 sequence evolved at substantially the same rate as the other GAD sequences. In addition, although we were unable to distinguish between tree topologies in which the GAD3 sequence originated at the same time as GAD₆₅ and GAD₆₇, was a sister group to GAD₆₅, or was a sister group to GAD₆₇, the possibilities that GAD3 arose as an early duplication on the branches leading to either fish GAD₆₅ or fish GAD₆₇ sequences were not supported by our maximum-likelihood analyses. Hence, our analyses indicate that GAD3 originated before the separation of teleosts from the main vertebrate lineage and is the result of an ancient gene duplication. This would imply that the ancestral GAD gene took part in both gene duplication events during the origin and early evolution of vertebrates (Sharman and Holland 1996), which resulted in four GAD genes being present in the early vertebrate genome. Our results suggest that two of these genes are silent and perhaps have been lost altogether in most extant vertebrate lineages, although it is possible that a third GAD form remains to be found in other vertebrate classes. Recently, Wittbrott, Meyer, and Scharl (1998) suggested that fish, by maintaining seemingly redundant multiple copies of genes, gained the evolutionary advantage of a "flexible genome," which allowed them to rapidly adapt to changing environmental challenges. Within this context, the GAD3 paralog may have been one of the necessary pre-adapted genes that allowed the ancestor of the armoured grenadier to migrate into the abyss ~80 million years ago. Thus, two consecutive GAD gene duplications followed by the loss of a single gene could explain the presence of three distinct GAD mRNAs in the brain of armoured grenadier. However, our maximum-likelihood analysis of the grenadier GAD3 protein sequence did not resolve its evolutionary relationship with the GAD₆₅ and GAD₆₇ sequences. GAD3 sequences from other species will therefore have to be sequenced in order to answer this question.

In conclusion, we have presented evidence for the expression of multiple GAD genes in the brain of birds, reptiles, and fish, while only a single GAD could be cloned from a protochordate. In addition to GAD₆₅ and GAD₆₇, a third GAD paralog was found in the brain of a fish. The resulting phylogenetic tree shows that GAD,

which synthesizes one of the most abundant and important neurotransmitters in the central nervous system, is a powerful marker to study the evolution of vertebrate species.

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