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# A BRAIN-SPECIFIC G PROTEIN GAMMA SUBUNIT

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Two different cDNAs for G protein γ subunits have been isolated from mouse brain. One encodes a novel γ subunit, γ4, the expression of which is detected only in brain. A fragment of this cDNA had been isolated previously. The other cDNA encodes γ3, a subunit type previously isolated from bovine brain. The primary structure of the γ3 subunit is conserved completely across species indicating that the diversity in the structure of the γ subunits is of functional consequence. Moreover, γ2, γ3 and γ4, which are predominantly expressed in brain, are more homologous to each other than other γ subunits, indicating that the G protein γ subunits belong to distinct subfamilies similar to the α subunits. 

Heterotrimeric G proteins transduce signals from transmembrane receptors to intracellular effectors [1]. They are made up of three subunits, α, β and γ. About twenty different α subunit types have been identified [2]. Apart from being a relatively large family of proteins, the α subunits also fall into sub-families based on their primary structure. Moreover, there is evidence for the primary structure of α subunit types being conserved across species [2]. There are indications that the β and γ subunits are also families of related proteins. However, the relative size of these families and the diversity in the structure of members of each family are not clear. Five β subunits [3-8] and six γ subunit types have so far been identified [9-18]. Most of the β subunit types are highly homologous in their primary structures and are expressed in a variety of tissues [3-8]. The γ subunits are relatively more diverse. There are indications that the expression of the γ subunit types is differentially regulated in various tissues [9-18].

Recent results have identified a direct role for the G protein γ subunit in specifying receptor coupling of a G protein [19,20]. The characterization of the G protein γ subunit family has achieved special importance in light of these results. Hypothetically, various γ subunits could differentially influence receptor coupling of different G proteins. If this hypothesis is correct, the γ subunit family may be large and many unidentified members may be present especially in cell types that have a variety of signalling pathways. Since neurons are rich in receptors coupled to G proteins [21], we have begun characterizing the diversity in G protein β and γ subunits in the mammalian brain. We report here a γ subunit whose expression can be detected only in brain. The nucleotide sequence of a fragment of this γ cDNA, γ4 has been reported earlier (13).

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## Materials and Methods

Materials. An adult mouse brain cDNA library in λgt10 (from Dr. L.Salkoff, Washington University Medical School) was screened for the γ4 cDNA clone. Restriction endonucleases and Circumvent thermal cycle sequencing kit were from New England Biolabs. T4 DNA ligase was from Boehringer Mannheim. PCR reagents, Taq DNA polymerase, random prime labeling kit were from Life Technologists, Inc. Radiolabelled nucleotides and deoxynucleotides were from Amersham.

cDNA library screening. About a million plaques from the cDNA library were screened using a 100 bp probe that contained a portion of the γ4 sequence [13]. Hybridization was carried out in 6xSSC, 5xDenhardt, 0.5% SDS, 100 μg/ml salmon sperm DNA and a $^{32}$P- random prime labelled probe ($10^6$ cpm/ml) for 16 hrs at 57° C. The filters were washed thrice (15 min each) with 6xSSC, 0.1% SDS at room temperature and then with 2xSSC, 0.1% SDS (room temperature, 15 min each) followed by a final wash at 45° C with 0.1xSSC, 0.1% SDS for 5 min and autoradiographed.

Characterization of cDNA clones. cDNA clones were isolated and the nucleotide sequences of these clones were determined directly by thermal cycle sequencing using end labelled γ4 specific primers DG 23 (5'-GAAGCC TGCATGGACAGGG-3') and DG 24 (5'-CTGGGATGATGAGGGGGTC-3'). Briefly, 0.1 pmoles of the template were annealed to 1.2 pmoles of $^{32}$P- end labelled primer and subjected to 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 40 sec) and extension (72°C, 40 sec). The primer extended products were resolved on a 6% polyacrylamide-urea sequencing gel and autoradiographed. cDNA inserts from four clones whose nucleotide sequences were partially determined in this manner were amplified using λgt10 specific primers containing EcoRI sites: GER1 ( 5'-TATGAATTCTTATGAGTATTTCTTCCAGGGTAA-3' ) and GEF1 ( 5'-TATGAAT TCGAGCAAGTTCAGCCTGGTTAAG-3'). The products were cut with EcoRI and then subcloned into a plasmid. In the case of the novel cDNA, γ4, since the cDNA inserts were isolated by PCR, misincorporation of bases was ruled out by determining the nucleotide sequence of ten independent cDNA clones. Sequences of both strands of cDNAs were determined by the dideoxy method using Sequenase (United States Biochemical). The nucleotide sequences of the ten clones were identical. Analysis of DNA and protein sequences was carried out using MacDNASIS software system (Hitachi).

Northern analysis. A commercially available blot containing poly A$^+$ RNA from different tissues of adult mouse (Clontech ) was probed with a $^{32}$P-labelled 100 bp γ4 probe that was used to screen the library. Hybridization was carried out in Express Hyb hybridization solution (Clontech ) at 68°C for one hour. The filter was washed with 2xSSC, 0.05% SDS for 40 min at room temperature and then with 0.1xSSC, 0.1% SDS for 20 min at 50°C. To eliminate the possibility that the probe was cross hybridizing to a homolog, a 250 bp probe generated from the 3' untranslated portion of the γ4 cDNA was later hybridized to the same blot at high stringency (68°C) and washed at equally high stringency (0.1 x SSC, 0.1% SDS at 65°C for 30 min). The results from hybridization with both these probes were identical. The same blot was stripped and probed with a $^{32}$P-labelled ~700 bp cDNA insert of the mouse γ3 clone under conditions that were employed for the first hybridization with the 100 bp γ4 probe.

## Results and Discussion

Isolation and characterization of a cDNA for γ4. A mouse brain cDNA library was screened with a probe specific to the γ4 cDNA. This probe was generated from single stranded mouse brain specific cDNA using PCR [13]. The PCR primers used were degenerate oligonucleotides specific to conserved residues among the members of the G protein γ subunit type family. The probe encoded 33 amino acids that were unique but homologous to previously identified γ subunit types. Ten clones out of ~$10^6$ cDNA clones hybridized strongly to this probe. These clones were isolated and characterized by thermal cycle sequencing with primers specific to the γ4 cDNA fragment. Since the primers were specific to the protein coding portion of γ4, only cDNA clones that were either γ4 or closely related homologs were expected to yield a nucleotide sequence ladder on performing cycle sequencing. Clones that yielded a nucleotide sequence were purified and the cDNA insert subcloned into a vector for further characterization. One of

these clones contained an insert of size, ~0.6 kb. The nucleotide sequence of this novel cDNA clone is shown in Fig. 1. The seventy five amino acid long protein encoded by this cDNA was unique but homologous to other known G protein γ subunits. At the C terminus, the encoded protein possesses a domain CXXX (C-cysteine and X-any amino acid) characteristic of the G protein γ subunits as well as a variety of other proteins including the small guanine nucleotide binding proteins [22]. The cysteine in this domain is isoprenylated with either farnesyl (C15) or geranylgeranyl (C20) and the last three amino acids proteolytically removed. When the last amino acid is leucine as in the case of γ4, the protein is modified by geranylgeranyl [22].

Expression of γ4. Although the partial cDNA specific toγ4 was isolated using PCR and degenerate oligonucleotide primers from kidney and retina this is a poor indicator of the tissue specific expression of this gene. Degenerate primers tend to amplify cDNAs from a variety of tissues regardless of the level of expression of a transcript due to the sensitivity of PCR [16]. To derive an accurate estimate of variations in the level of expression of the γ4 gene in various tissues, the expression of the γ4 subunit type was examined by hybridizing a blot containing RNAs from a variety of mouse tissues with either the 100 bp PCR fragment specific to the coding portion of the γ4 cDNA or a ~250 bp probe specific to the 3' non coding region of the γ4 cDNA. The results from both these hybridizations were identical. A northern blot hybridized to the 100 bp probe is shown in Fig. 2A. A 3.9 kb transcript specific to γ4 was present only in brain. RNA specific to this subunit type was not detected in any other tissue examined. RNA specific to γ2 is ~4.4 kb long and present in several tissues but especially abundantly in brain [11]. RNA specific to γ3 is ~1 kb long and detectable at high levels in brain and very low levels in testes [13]. The transcript specific to γ5 is 550 bp long and detectable uniformly in various tissues [14,15]. γ7 hybridizes to one or two RNA species of ~4.2 kb length that are present in a

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1    AGTTCAGCCTGGTTAAGTCCAAGCTGCTGTACCACCTCTCAGCAGGGAGTGCAGGAATGA   60
                                                          M  K

61   AGGAAGGCATGTCTAATAACAGCACCACCAGCATCTCCCAGGCCAGGAAAGCCGTGGAGC  120
       E  G  M  S  N  N  S  T  T  S  I  S  Q  A  R  K  A  V  E  Q

121  AGCTGAAGATGGAAGCCTGCATGGACAGGGTGAAGGTCTCCCAGGCTGCCTCAGACCTCC  180
       L  K  M  E  A  C  M  D  R  V  K  V  S  Q  A  A  S  D  L  L
                |-------------------------------------------------

181  TGGCCTACTGTGAAGCCCACGTGCGGGAGGACCCCCTCATCATCCCAGTGCCTGCCTCAG  240
       A  Y  C  E  A  H  V  R  E  D  P  L  I  I  P  V  P  A  S  E
     ----------------------------------------------------|

241  AAAACCCCTTCCGGGAGAAGAAGTTCTTCTGCACCATCCTCTAACACCCATGGCGATGAA  300
       N  P  F  R  E  K  K  F  F  C  T  I  L
                                  #  #  #  #

301  GCGGGCCCTTTCCTGCTGTAACAG  324
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Fig. 1. The nucleotide sequence of the mouse γ4 cDNA. The deduced amino acid sequence is shown below. Some portions of the untranslated regions are not shown. The sequence of the 100-bp partial γ4 cDNA that was used as a probe is underlined. The C-terminal CXXX motif that is posttranslationally modified is denoted with pound signs.

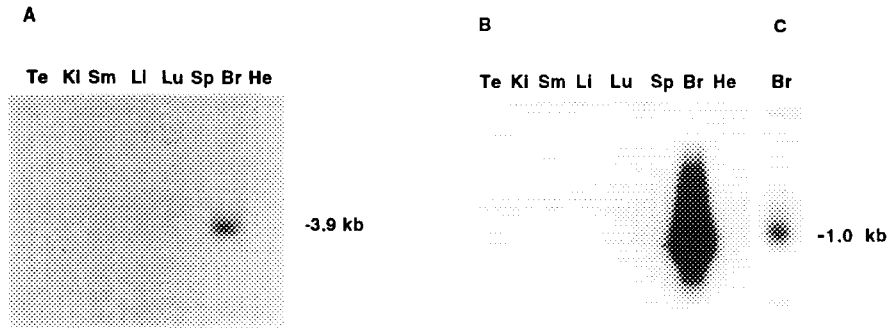


Fig. 2. Northern analysis of mouse γ subunit types. (A) Autoradiogram of a RNA blot with poly adenylated RNAs from various mouse tissues hybridized to the 100-bp γ4 cDNA probe. Exposure of the autoradiogram was for 4 days. (B) Autoradiogram of the same blot probed with the γ3 cDNA. Exposure was for ~16 hrs. (C) 2-hr exposure of the brain RNA lane of the same filter. Te- testes; Ki- kidney; Sm- skeletal muscle; Li- liver; Lu- lung; Sp- spleen; Br- brain; He- heart.

variety of tissues but especially abundantly expressed in brain [15]. The γ6 cDNA has not been fully characterized and its expression in various tissues has not been examined [16]. The size and the tissue distribution of the γ4 RNA is thus different from all the other known γ subunits.

Isolation of mouse cDNA for γ3 and its expression. Three of the other cDNA clones that hybridized to the γ4 fragment were further characterized by nucleotide sequence determination. The three clones were of different lengths but the shared nucleotides sequences were identical. The nucleotide sequence and the amino acid sequence of a γ subunit encoded by one of these cDNAs are shown in Fig. 3. The protein encoded is identical to the bovine γ3 subunit type. RNA blot analysis showed that the pattern of expression of mouse transcripts specific to this subunit type is similar to that of the bovine homolog [13]. That is, the γ3 RNA of ~1 kb length is abundantly expressed in brain and at low levels in testes (Fig. 2B & C).

γ subunit types mainly expressed in brain. The expression of γ4 can be detected only in brain. The expression of γ3 is similar although relatively small amounts of the transcript are detectable in testes. Several of the G protein γ subunits are thus expressed either specifically in the brain or especially abundantly in brain. Examples of the latter type are the subunit types, γ2 and γ7 which are expressed in a variety of tissues but especially abundantly in brain [11,15]. Interestingly, certain α and β subunit types also show a pattern of expression in mammalian tissues which is similar to particular γ subunit types. For instance, the splice variant of the αo subunit type, αob, is expressed mainly in brain and testes [23]. Although quantitatively the levels of expression of γ3 in these two tissues are different from αob, γ3 is nevertheless expressed only in these two tissues. The expression of a newly identified β subunit type, β5 is detected only in brain [8]. This pattern of expression is identical to that of γ4. These similarities in the expression pattern of different G protein subunit types -- α, β and γ -- raise the possibility that the formation of heterotrimers containing certain subunit types may be favoured by differentially regulating the expression of these subunits in various cell types. An extreme example of such regulation occurs in specialized cells--the rod and cone photoreceptors. The α rod, β1 and γ1 subunit types are expressed in rods while the α cone, β3 and a distinct γ subunit


Fig. 3. Nucleotide sequence of mouse γ3 cDNA. The amino acid sequence encoded by the open reading frame is shown below in bold letters. The nucleotides in the bovine γ3 cDNA that are not homologous to mouse γ3 are shown just below the cDNA sequence. As expected, the nucleotide sequence of the γ3 cDNA from the two different species is more conserved in the coding region than in the non-coding regions.

are expressed in cones [24-26]. A distinctly different mechanism that might regulate the type of subunits in a heterotrimer is selective association of certain subunit types. For instance, it has been shown that association between different G protein β and γ subunit types is such that certain combinations are precluded [27,28]. In the yeast two hybrid system γ4 is capable of associating with all known β subunits (β1-β5) but there are indications that its affinity for the different β subunits could be different (K. Yang, V. K. and N. G., unpublished).

G protein γ subunit family. Table 1 shows the relative homologies between different G protein γ subunits that have been identified so far. γ2, γ3 and γ4 are more homologous to each other than the other subunit types especially towards the C terminus (Fig.4). This pattern of homology among G protein γ subunits is reminiscent of the α subunit types which fall into different sub-

Table 1. Percent identity between amino acid sequences of G protein γ subunit types characterized so far [9-15, 28,29]. Homology of γ4 to other γ subunits is highlighted. Note that some subunit types show more homology to each other than to the rest of the types. γ6 is not included since it is not fully characterized.

| | γc | γ2 | γ3 | γ4 | γ5 | γ7 | γ8 |
|---|---|---|---|---|---|---|---|
| γ1 | 64 | 37 | 36 | **32** | 28 | 41 | 36 |
| γc | | 41 | 36 | **34** | 29 | 39 | 33 |
| γ2 | | | 73 | **73** | 49 | 68 | 70 |
| γ3 | | | | **67** | 46 | 56 | 53 |
| γ4 | | | | | **46** | **55** | **60** |
| γ5 | | | | | | 49 | 47 |
| γ7 | | | | | | | 56 |

families based on amino acid sequence homology. In these cases, members of a sub-family may share some common roles in signalling. The last twelve amino acids of γ1 have recently been shown to be involved in directly interacting with a receptor [20]. It is possible that the C terminal tail of G protein γ subunits in general specifically interacts with certain receptors. Comparison of the amino acid sequences of the γ2, γ3 and γ4 proteins at the C terminus indicates that in the processed form (which will lack the amino acids downstream of the cysteine), they are identical (Fig. 4). Other γ subunit types are however distinctly different in the primary structure of this domain. Thus, the pattern of sequence homologies where some γ subunits are identical at the C terminus and others divergent is consistent with a prediction that some γ subunits play the role of directing G proteins with different α subunit types to the same receptor while some other γ subtypes direct G proteins with the same or different α subunits to various receptors. The complete conservation of the amino acid sequence of γ3 from mouse with the amino acid

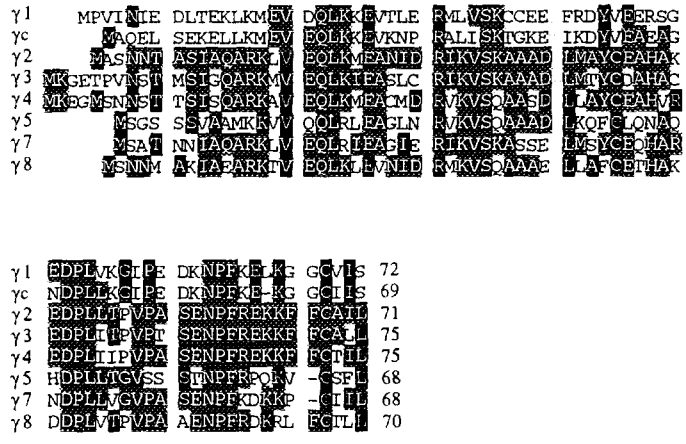

Fig. 4. Alignment of the deduced amino acid sequence of γ4 with the other γ subunit types which are abundant in brain. Note that the twelve residues of γ4, γ3 and γ2 beginning from cys 72 and extending upstream are identical.

sequence of γ3 from bovine tissue, indicates that the differences between the primary structures of γ subunit types are of functional importance since there is evolutionary pressure to retain these differences. Finally, since an antisense oligonucleotide specific to the partial cDNA sequence of γ4 uncouples muscarinic receptor type, m4, from L type calcium channels [13,29], the role of the newly characterized γ subunit, γ4, may be to specify interaction of a G protein with this receptor.

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