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DIVERSITY AMONG THE BETA SUBUNITS OF HETEROTRIMERIC GTP-BINDING PROTEINS: CHARACTERIZATION OF A NOVEL BETA-SUBUNIT cDNA

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Heterotrimeric guanine nucleotide binding proteins transduce signals from cell surface receptors to intracellular effectors. The alpha subunit is believed to confer receptor and effector specificity on the G protein. This role is reflected in the diversity of genes that encode these subunits. The beta and gamma subunits are thought to have a more passive role in G protein function; biochemical data suggests that beta-gamma dimers are shared among the alpha subunits. However, there is growing evidence for active participation of beta-gamma dimers in some G protein mediated signaling systems. To further investigate this role, we examined the diversity of the beta subunit family in mouse. Using the polymerase chain reaction, we uncovered a new member of this family, Gβ4, which is expressed at widely varying levels in a variety of tissues. The predicted amino acid sequence of Gβ4 is 79% to 89% identical to the three previously known beta subunits. The diversity of beta gene products may be an important corollary to the functional diversity of G proteins.

G proteins are guanine nucleotide binding regulatory proteins composed of three subunits, alpha, beta and gamma. They relay signals from a multitude of cell surface receptors to numerous intracellular effectors including adenylyl cyclases, cGMP-phosphodiesterases, phospholipases and ion channels (for reviews see 1, 2 and 3). The alpha subunit binds GDP which is exchanged for GTP upon interaction with the appropriate activated receptor. This exchange is believed to be accompanied by dissociation of the alpha subunit from the beta-gamma dimer. An intrinsic GTPase activity hydrolyses the GTP to GDP, thereby allowing reassociation of the subunits and termination of the signal.

There is a rapidly growing list of different genes that encode alpha subunits. Nonetheless, the diversity of alpha subunits is much less than the number of different receptors that are coupled to G proteins. There may be more than 100 different receptors that are linked to GTP binding proteins while thus far 16 different alpha subunits have been found (1). The alpha subunit is believed to confer receptor and effector specificity on the heterotrimer. However, there is accumulating evidence for a role of the beta-gamma dimer in determining the specificity of G protein function (4). Indeed, genetic studies with signal transducing systems in yeast provide evidence for an active role of the beta-gamma subunit in coupling to an effector. The binding of GTP to the alpha subunit may serve to

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release and activate beta-gamma function (5,6). In mammalian cells, a role for the beta-gamma subunits in the activation of ion channel function has been suggested (7). This effect may be due to the ability of beta-gamma dimers to activate phospholipase A_2. There is strong evidence from more recent experiments that beta-gamma subunits have regulatory effects on certain types of adenylyl cyclases (8). If beta subunits are integrally involved in influencing receptor or effector function then we might expect a diverse family of these subunits with a relatively wide range of activities.

Initially, two cDNAs specifying highly homologous beta subunits, Gβ1, Gβ2, were found (9,10,11,12). These were correlated to two separable polypeptides with gel migration patterns corresponding to a 35 Kd and a 36 Kd polypeptide (13,14). Recently, a transcript encoding a third highly homologous protein, Gβ3, was described (15). All three of these gene products appear to be distributed in a relatively ubiquitous fashion. This raises the problem that in any reconstitution experiment the beta subunits that are used to interact with alpha subunits represent a mixture of gene products. Furthermore, until we can determine the nature and number of the components that make up the beta-gamma portion of the G protein complexes we will not be able to rigorously assess their functional role. Therefore, in order to examine the diversity among the beta subunits, we used a polymerase chain reaction technique that has, in the past, been successful in revealing the multiplicity in G protein alpha subunits (16). We found a fourth member of the beta subunit family which is expressed at significantly different levels among a variety of tissues.

Materials and Methods

Polymerase Chain Reaction. PCR was performed as described previously (16). cDNA was made from PolyA$^+$ RNA with random hexanucleotide or oligo dT primers using MMLV reverse transcriptase. Conditions were those supplied by the manufacturer (Bethesda Research Labs). The oligonucleotides used for PCR amplification of the cDNA were as follows:

E1, AAGGATCCCARGARGCNGARCARCT; E5, CCGGAATTCCCARTGCATNGCRTADAT;
E8, GACTCGAGTCGACATCGA$(T)_{17}$; E10, GGAATTCGTCTAATATGGACTCCG;
E11, GGAATTCGTTGCAGGCCTTCCG; E12, GGACACACGGGCTACTTG;
E18, GGCTCCAGCTCTTCACTTGAG; E19, GGCTGTAACACGGATTTCTCC

in which R = A or G, D = A, G or T, and N = A, C, G or T. PCR was performed on a Perkin Elmer Cetus thermal cycler. During each cycle, samples were denatured for 1.0 min at 94°C, extended for 1.0 min at 72°C, and the different oligonucleotide pairs were annealed for 0.5 min at the following tempertures: E1+E5, 48°C; E8+E10, 54°C; E11+E12, 50°C; E18+E19, 64°C. Each oligonucleotide was used in the PCR at 10 ng/μl. 35 cycles were performed on approximately 5 ng of cDNA in a 50 μl reaction volume. Inverse PCR was performed on cDNA that had been circularized by intramolecular ligation as follows: 2 μg oligo dT primed cDNA was synthesized with the BRL cDNA synthesis system, diluted into 500 μl ligation mix, ligated with T4 DNA ligase for 2 hours at room temperature, ethanol precipitated and resuspended in 100 μl H_2O. 2 μl of the circularized cDNA was used for PCR. The buffer and Taq polymerase were supplied by Cetus.

Northern Analysis. Total RNA was run on a 1% agarose gel and transferred to Genescreen (Dupont) as described (16). A probe specific to the Gβ4 3'-untranslated regions was made by PCR amplifying the cDNA clone β4-2 with the complement of oligonucleotide E19 (Fig. 1) and the M13-20 primer (New England Biolabs) which is specific to the cloning vector. The amplified product was run on a low melting point agarose gel (Seaplaque, FMC), excised, and labeled by random priming as described (16). The RNA blot was hybridized as previously described (16).

PCR Northern. PCR was performed on cDNA prepared with reverse transcriptase (supplied by BRL) from total RNA as described above. The degenerate oligonucleotides E1 and E5 were used for 35 cycles of amplification. The PCR products were electrophoresed through a 2% agarose gel, blotted to Genescreen and hybridized according to the manufacturer's instructions. These blots were probed with radiolabeled oligonucleotides specific to β4 (E10), β1 (TCACAAACAATATTGATCCA) and β2 (TCACAGCTGGGCTTGACCCA). The oligonucleotides were end-labeled with γ[^{32}P]-ATP as described (17). Blots were hybridized at 42°C and washed at room temperature twice for 5 min in 0.90 M NaCl/0.90 M sodium citrate (6X SSC)/0.1% SDS.

Isolation of cDNAs. The β4-2 cDNA clone (Fig. 1) was isolated from a random primed mouse brain cDNA library in the lambda cloning vector mλJP3 (M.P.S. and M.I.S., unpublished). 10^6 clones were screened with the β4 E18-E19 PCR fragment using standard techniques (17).

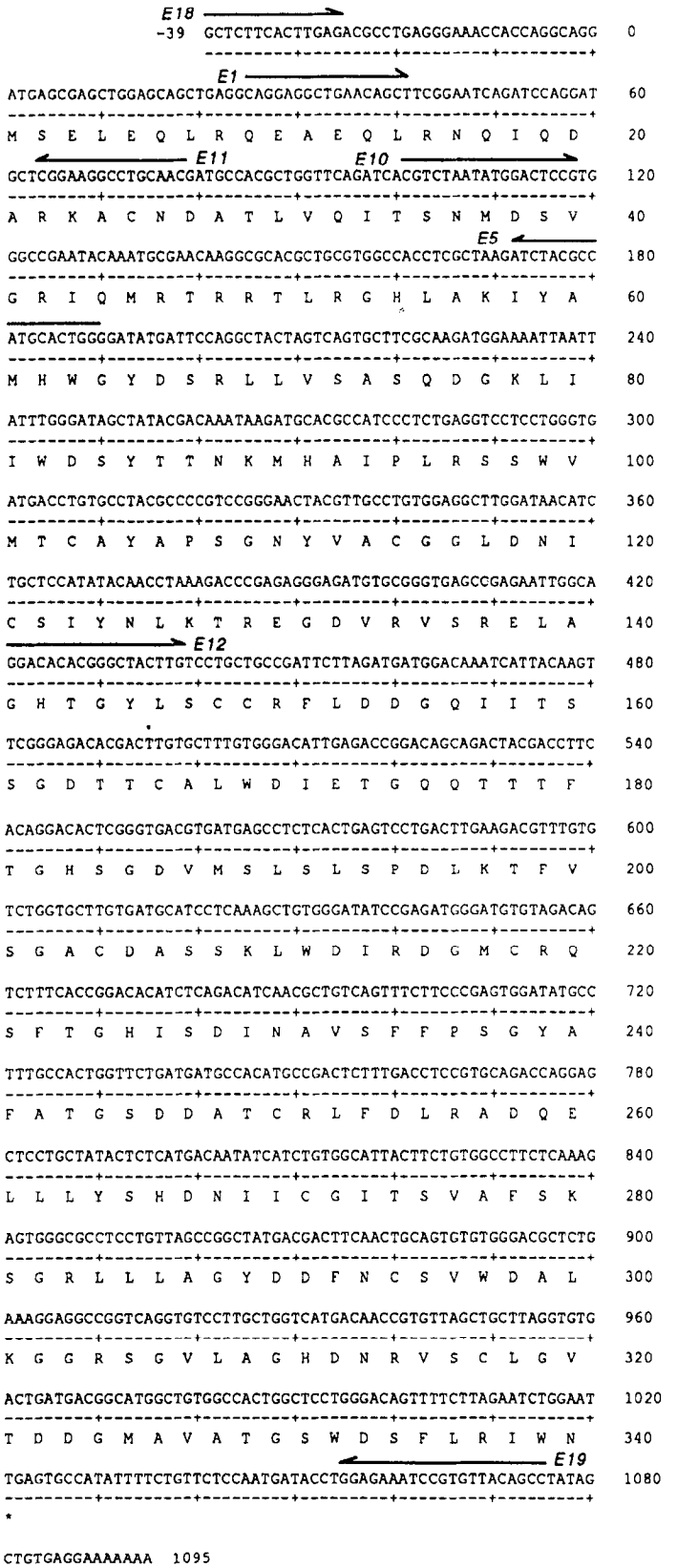
Results and Discussion

To examine beta-subunit diversity the polymerase chain reaction (PCR) was used to amplify cDNA from mouse brain. A set of degenerate oligonucleotide primers (E1 and E5, Fig. 1) was designed for use in the PCR. These primers correspond to two domains in which the amino acid sequences of Gβ1 and Gβ2 are completely conserved. PCR amplified products were separated on an agarose gel, excised and subcloned into a plasmid vector. Analysis of twenty clones revealed three different beta-like sequences. Two of these sequences were classified as the mouse Gβ1 and Gβ2 homologs since the deduced translation products were identical to the bovine beta subunits and the nucleotide sequences differed by only 15% and 7%, respectively. The third sequence, labeled Gβ4, was unique, though clearly related to Gβ1 and Gβ2.

To obtain more of the Gβ4 sequence, a specific primer (E10) was synthesized which, in combination with an oligo dT primer (E8), was used to selectively amplify the 3' end of the Gβ4 cDNA (18). The 5' end of the coding sequence was also obtained by PCR. Circular mouse brain cDNA was generated by intramolecular ligation (see Materials and Methods). This cDNA was used as a template for inverse PCR (19). Two Gβ4 specific primers (E11, E12), directed towards the noncoding regions, were used to amplify the 3' and 5' ends of the coding region as well as the connecting noncoding regions. The PCR product, 1 kb in size, was subcloned and sequenced. Finally, two primers based on the 3' and 5' noncoding regions of Gβ4 were designed (E18, E19). PCR of mouse brain cDNA with these primers resulted in a 1.1 kb product which was subcloned and sequenced. It contained the entire coding region of Gβ4.

In addition, the E18-E19 PCR product was used as a probe for screening a randomly primed mouse brain cDNA library. Approximately 10^6 clones were screened. Four positives were obtained and purified. One clone (β4-2) contains a 2 kb insert which includes the entire coding region. Figure 1 shows the nucleotide sequence and the predicted amino acid sequence obtained from β4-2. The open reading frame encodes a protein of 340 amino acids.

Figure 1. The nucleotide sequence and predicted amino acid sequence of murine Gβ4. The nucleotide sequence was obtained from the clone β4-2 which was isolated from a mouse brain cDNA library. The oligonucleotide primers used for PCR are indicated by arrows above the nucleotide sequence. At position 431, the β4-2 clone contains an adenine instead of a guanine. Another cDNA clone, β4-4, and several PCR products contain a guanine at this position.


E18
E1
E11
E10
E5
E12
E19

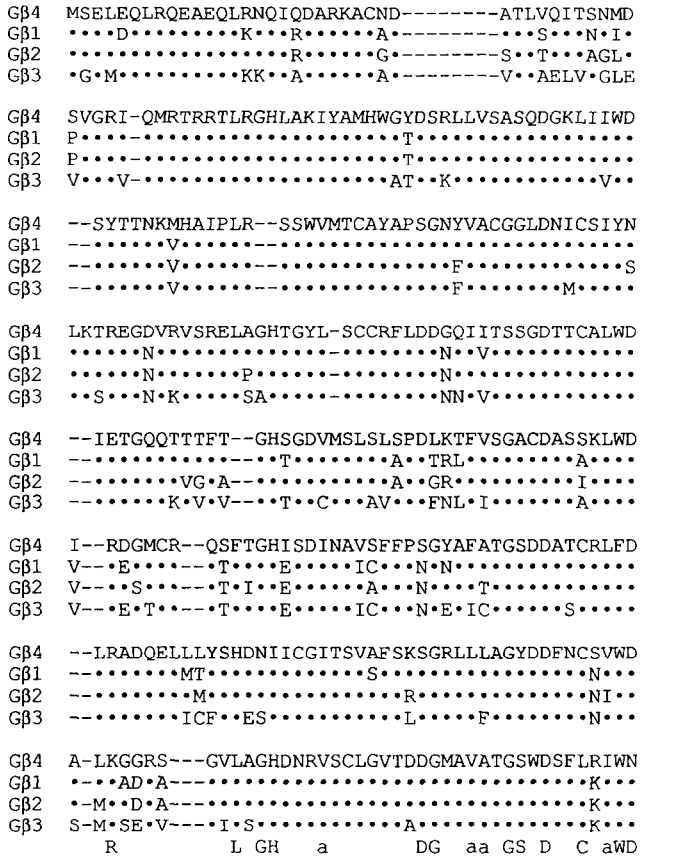


Figure 2. Amino acid sequence comparison of mammalian G protein beta subunits. The sequences are aligned to display the repetitive segmental pattern. Dots represent identities with the Gβ4 sequence. The consensus amino acids in the repeated motif are shown at the bottom of the figure. Aliphatic amino acids are represented by lower case A.

Figure 2 compares the amino acid sequences of Gβ4 to those of Gβ1, Gβ2 and Gβ3. All four beta subunits are closely related; Gβ4 is 89% identical to Gβ1 and Gβ2, and 79% identical to Gβ3. The variation evident among the N-terminal 40 amino acids of the different beta subunits is noteworthy since Cys25 Gβ1 is known to contact Gγ1. Perhaps the surrounding sequence can influence the pairing of beta with gamma, thereby limiting the pairwise combinations of these betas with the growing number of different gamma subunits (20).

Gβ4 belongs to an expanding set of proteins, including the other beta subunits, that conserve a repetitive segmental structure of about 40 amino acids, the WD-40 motif, which is characterized by a tryptophan-aspartate amino acid pair (21). Members of this family include two yeast proteins involved in RNA splicing, PRP4 (22) and PRP17 (1), a protein critical to cell cycle regulation, CDC4 (9), a protein involved in regulating yeast Ras function MSI1 (23), the 12.3 cDNA in chicken which is linked to the major histocompatibility locus (24) and Enhancer of split E(spl), the product of a *Drosophila* neurogenic locus (25). The function of the WD-40 motif is unknown.

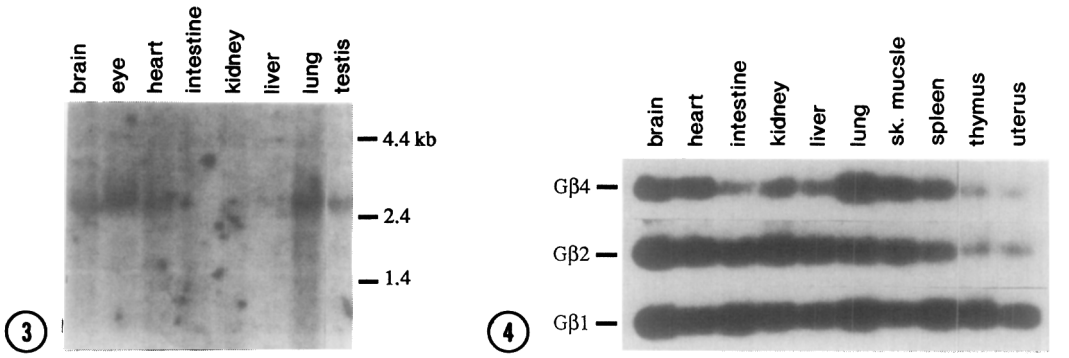


Figure 3. Northern analysis of Gβ4. Probes specific to the 3'-untranslated region of Gβ4 (see Materials and Methods) were hybridized to total RNA from various mouse tissues. 20 μg total RNA was loaded per lane.

Figure 4. PCR Northern. PCR was performed on various mouse tissues using the degenerate oligonucleotides E1 and E5 (Fig. 1). The amplified products were hybridized with radiolabelled oligonucleotides specific to Gβ1, Gβ2 and Gβ4 (see Materials and Methods).

To examine the distribution of the Gβ4 message, Northern analysis was performed on a variety of mouse tissues. A probe was made from the 3'-untranslated sequence of the β4-2 cDNA, and it was hybridized to total RNA (Fig. 3). In contrast to Gβ1, Gβ2 and Gβ3 messages, the levels of the Gβ4 transcript vary significantly among the examined tissues. The Gβ4 message is highest in brain, eye, lung, heart and testis. As a more sensitive assay of tissue distribution, we examined Gβ4, Gβ1 and Gβ2 using PCR Northern analysis (26). The degenerate oligos, E1 and E5, were used to PCR amplify cDNA from numerous tissues. The population of amplified sequences was separated on a gel, transferred to a nylon membrane, and probed with end-labeled oligos specific for each beta. The results of this analysis (Fig. 4) agree with the Northern data. Gβ1 and Gβ2 transcripts show relatively constant levels of expression, whereas Gβ4 varies significantly among the different tissues.

The existence of diverse sets of beta and gamma subunits raises the possibility of combinatorial assembly of G proteins. Perhaps variations in primary sequence of the beta subunits limits the pairwise combinations with gamma subunits. Alternatively, other signals may prevent the "inappropriate" assembly of a particular heterodimer in the cell. However, the availability of a diverse collection of beta-gamma dimers may be essential for a complex multicellular organism. There is evidence to suggest that all beta-gamma dimers are not functionally equivalent (27,28,29). Since beta-gamma is essential for the interactions of the alpha subunit with receptor, a diverse collection of heterodimers may be critical for meeting the specific requirements of the many different receptors that activate a particular alpha subunit. Furthermore, the ability of different beta-gamma dimers to influence the coupling of receptor to alpha subunit could also enable these dimers to modulate the kinetics of a signaling response. If the beta-gamma dimers released upon stimulation of a particular G protein-coupled receptor can influence G proteins that are not coupled to this receptor, then complex signaling networks could exist in which the dynamics of one system are modulated directly by the released beta-gamma dimers from another system. In fact, G_i is postulated to exert its inhibitory action on adenylate cyclase through the effect of free beta-gamma on the stimulatory G

protein, G_s (3). Thus, there may exist G protein networks that are not only capable of transducing signals vertically across the cell membrane but also capable of distributing information horizontally among G proteins within the cell.

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