Identification of a GABA<sub>B</sub> Receptor Subunit, gb2, Required for Functional GABA<sub>B</sub> Receptor Activity* 

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G protein-coupled receptors are commonly thought to bind their cognate ligands and elicit functional responses primarily as monomeric receptors. In studying the recombinant γ-aminobutyric acid, type B (GABA<sub>B</sub>) receptor (gb1a) and a GABA<sub>B</sub>-like orphan receptor (gb2), we observed that both receptors are functionally inactive when expressed individually in multiple heterologous systems. Characterization of the tissue distribution of each of the receptors by in situ hybridization histochemistry in rat brain revealed co-localization of gb1 and gb2 transcripts in many brain regions, suggesting the hypothesis that gb1 and gb2 may interact in vivo. In three established functional systems (inwardly rectifying K<sup>-</sup> channel currents in Xenopus oocytes, melanophore pigment aggregation, and direct cAMP measurements in HEK-293 cells), GABA<sub>B</sub> mediated a functional response in cells coexpressing gb1a and gb2 but not in cells expressing either receptor individually. This GABA<sub>B</sub> activity could be blocked with the G protein-coupled receptor antagonist CGP71872. In COS-7 cells coexpressing gb1a and gb2 receptors, co-immunoprecipitation of gb1a and gb2 receptors was demonstrated, indicating that gb1a and gb2 act as subunits in the formation of a functional GABA<sub>B</sub> receptor.

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Metabotropic GABA<sub>B</sub> receptors were first distinguished pharmacologically by Hill and Bowery (1). Kaupmann et al. (2) recently cloned two alternatively spliced forms of the GABA<sub>B</sub> receptor, termed gb1a and gb1b, which belong to the G protein-coupled receptor superfamily and are most closely related to the metabotropic glutamate receptors. Although native GABA<sub>B</sub> receptors are reported to activate inwardly rectifying K<sup>-</sup> channels (Kir) (3), recombinant gb1a receptors coexpressed with Kir channels in Xenopus oocytes failed to be functionally active (4, 12). A recent report has shown that recombinant GABA<sub>B</sub> receptors fail to couple to effector pathways in a variety of non-neuronal and neuronal cell types, suggesting that additional cellular component(s) are required (4). Failure to show GABA<sub>B</sub> receptor function coupled with previous reports that GPCRs can undergo dimerization (5–8), suggested that heterodimerization may be important for GABA<sub>B</sub> receptor function. Furthermore, receptor heterodimerization appears to rescue function of mutated or chimeric muscarinic and adrenergic receptors (9). We report here that coexpression of gb1 and gb2 receptors are necessary for the formation of functional GABA<sub>B</sub> receptors and result in heterodimerization.

EXPERIMENTAL PROCEDURES

Expression Constructs for gb1a and gb2 Receptors—The murine gb1a (mgb1a) cDNA was constructed from two expressed sequence tags (IMAGE Consortium clone identification numbers 472408 and 319196), combined by standard PCR methods, and subcloned into the pcNeo (Stratagene) and pcDNA3.1 (Invitrogen) vectors. The rat gb1a receptor was obtained by PCR of rat brain cDNA using degenerate nucleotide primers based on the published sequence (Ref. 2; GenBank<sup>TM</sup> accession number Y10369) and subcloned into pcDNA3.1.

Two independently derived cDNAs of human gb2 (GenBank<sup>TM</sup> accession numbers AF069755 and AF056085, the former having previously been called GBP51 (10)) were used to make expression constructs. A N-terminal FLAG-tagged hgb2/pcDNA3.1 construct encoding a modified influenza haemagglutinin signal sequence (MKTIALP(LYP)FA) followed by an antigenic FLAG (DYKDDDDK) epitope was generated by PCR (10). The hgb2 construct used for expression in 293 cells was the coding sequence from GenBank<sup>TM</sup> accession number AF056085 with the C-terminal splice variant of GenBank<sup>TM</sup> accession number AF095723 inserted into pcDNA3.1 in a manner similar to that for the rat gb1a.

In Situ Hybridization Histochemistry—Adjacent coronal rat brain sections were hybridized with labeled antisense and sense riboprobes directed against rgb2 (GenBank<sup>TM</sup> accession number AF058795) or rgb1 as described previously (11). Probes were generated by amplification of rgb2 with JC216 (T3 promoter and bases 1172–1191) and JC217 (T7 promoter and the complement of bases 1609–1626) or with JC215 (T3 promoter and bases 2386–2405) and JC219 (T7 promoter and the complement bases 2776–2793) or by amplification of rgb1a with JC160 (T3 promoter and bases 631–648) and JC161 (T7 promoter and the complement of bases 1024–1041). For colocalization experiments, probes were either labeled with digoxigenin (rgb1a) or fluor (rgb2). Detection of the radiolabeled rgb2 probe was performed using emulsion after detection of the digoxigenin-labeled rgb1 probe on the same brain slices.

Melanophore Functional Assay—Growth of Xenopus laevis melanophores and fibroblasts and DNA transfections by electroporation were...
Performing as described previously (13). To monitor the efficiency of transfection an internal control GPCR was used (pcDNA1-cannabinoid 2). For Gβγ-coupled responses (pigment aggregation), cells were preincubated in the presence of 100 μM forskolin and 100 μM GABA in the absence of forskolin and 100 μM GABA in the presence of 10 μM forskolin. Wild-type HEK-293 cells were tested with 250 μM GABA and 250 μM GABA in the presence of 10 μM forskolin. Data are presented as the percentage of total cAMP synthesized in the presence of forskolin only. The data presented are from single representative experiments that have been replicated twice.

**RESULTS AND DISCUSSION**

**Immunoprecipitation and Immunoblotting of GABAB Receptors—**

COS-7 cells (ATCC) were cultured and transiently transfected with FLAG-hgb2 receptor DNAs alone and in combination using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Membranes prepared from these cells were digoxigenin-solubilized, and mgb1a/FLAG-gb2 heterodimers were immunoprecipitated with either a mouse anti-FLAG M2 antibody (Kodak IB) targeting the FLAG-hgb2 receptor or anti-gb1 receptor rabbit polyclonal antibodies 1713.1–1713.2 using previously described conditions (6). The immunoprecipitates were then washed and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with either the anti-FLAG or anti-gb1 antibodies using previously reported conditions (16) and as described below.

**RESULTS AND DISCUSSION**

**Tissue Distribution of gb1 and gb2 Receptor mRNAs—**

Northern blot analysis revealed that many brain regions, including cortex, show overlapping expression patterns for gb2...
and gb1 receptor mRNA (10). *In situ* hybridization in adjacent coronal sections of rat parietal cortex using ³⁵S-labeled antisense riboprobes indicates that mRNAs for both gb1 and gb2 receptors are coexpressed in this brain region (Fig. 1, A and B). No hybridization signal was detected with the control ³⁵S-labeled sense gb1 and gb2 riboprobes (data not shown). To examine expression at the cellular level, digoxigenin-labeled gb1 and radiolabeled gb2 riboprobes were hybridized to the same sections (Fig. 1, C and D). Overlay of the gb1 and gb2 hybridization signals revealed that for both receptors are generally expressed in the same neurons (Fig. 1E). In other major brain regions, including the hippocampus, thalamus, and cerebellum,³ gb2 and gb1 receptor mRNAs are colocalized with ≥95% of gb2 expressing cells also expressing gb1. This suggests that their coexpression may be required for activity in these neurons.

**Recombinant gb1 Receptors Require Coexpression of gb2 for Functional Activity**—In melanophores transiently cotransfected with the mbg1a and hgb2 receptors, GABA mediated a dose-dependent pigment aggregation response with an IC₅₀ value of 3–7 μM (n = 3), which is absent in mock-transfected cells and cells transfected with the mbg1a or hgb2 alone (Fig. 2). The GABA-mediated inhibitory activity represented 42–56% (n = 3) of a control Gᵢ-coupled CB2 cannabinoid receptor response (Fig. 2, inset). GABA activity could be inhibited by the CGP71872 antagonist (Fig. 2). Functional activity required coexpression of gb1a and gb2 for GIRK activation in *Xenopus oocytes*. A, representative current families of Kir 3.1/3.2. Currents were evoked by 500-ms voltage commands from a holding potential of −10 mV, delivered in 20 mV increments from −140 to 60 mV. B, in a protocol designed to measure the effects of various receptors on Kir currents, oocytes were held at −80 mV (a potential where significant inward current is measured). Expression of mgb1a or FLAG-gb2 receptors alone with or without Gₛ₁α resulted in no modulation of current after GABA treatment. Coexpression of mgb1a and FLAG-hgb2 receptors followed by treatment with 100 μM GABA resulted in stimulation of Kir 3.1/3.2. Shown are representative traces from at least three independent experiments under each condition.
sometimes observed with rgb1a-50/rgb1a and hgb2-42/hgb2, these effects were relatively small (0–20% inhibition; Fig. 3) when compared with those observed with the coexpressing cells. Neither baclofen nor GABA in the absence of forskolin had any effect on cAMP synthesis (Fig. 3). In addition, wild-type HEK-293 cells did not exhibit baclofen- or GABA-mediated inhibition of forskolin-stimulated cAMP synthesis (Fig. 3). These data demonstrate that the functional GABAB receptor is heterothetic for both the gb1 and gb2 receptors for signaling via adenylyl cyclase.

Native functional GABAB receptors have been reported to couple to Kir3 (3). Co-expression of the mbg1a and hgb2 with Kir 3.1/3.2 resulted in a significant stimulation of Kir current in response to GABA (301 ± 20.6% (n = 3) increase over control current) measured at −80 mV, which could subsequently be washed out with control solution (Fig. 4) or blocked with the CGPT1872 antagonist (data not shown). Modulation of Kir 3.1/3.2 was not seen in oocytes expressing mbg1a or hgb2 individually even in the presence of GABA (Fig. 4). The dependence of functional GABAB receptor activity on the coexpression of gb1a and gb2 suggests that these receptors may undergo heterodimerization.

gb1a and gb2 Form a Heterodimer—Immunoblot analysis revealed selective expression of mbg1a monomers and homodimers in mbg1a and mgb1a/FLAG-gb2 expressing cells and expression of FLAG-gb2 receptors in FLAG-gb2 and mgb1a/FLAG-gb2 expressing cells (Fig. 5, lanes 1–8). To demonstrate the existence of gb1a-gb2 heterodimers, we utilized a different co-immunoprecipitation and immunoblotting strategy. Anti-gb1 receptor antibodies were used to blot receptors immunoprecipitated with anti-FLAG antibodies (Fig. 5, lanes 9–12). No mbg1a immunoreactivity was detected in samples prepared from mock vector transfected cells, FLAG-gb2 expressing cells, and mbg1a receptor expressing cells as expected because these species could not be immunoprecipitated with the anti-FLAG antibody and detected with the anti-gb1 antibody (Fig. 5, lanes 9–11). Immunoreactive ~250 kDa species (representing the mbg1a-gb2 heterodimer) and ~130-kDa species (representing the mbg1a monomer) were detected only in cells coexpressing the mbg1a and FLAG-gb2 receptors, demonstrating that gb1a and gb2 can only be co-immunoprecipitated as part of a complex (Fig. 5, lane 12). Similar demonstration of gb1a-gb2 heterodimerization was obtained when coexpressed receptors were immunoprecipitated first with anti-gb1 antibodies followed by immunoblotting with the anti-FLAG antibody (Fig. 5, lane 16). The ~250-kDa species represents the heterodimer, whereas the ~130-kDa species represents the FLAG-gb2 monomer. No FLAG-gb2 immunoreactivity was detected in samples prepared from mock vector transfected cells, FLAG-gb2 expressing cells and mbg1a receptor expressing cells as expected because these species could not be immunoprecipitated with the anti-gb1 antibody and detected with the anti-FLAG antibody (Fig. 5, lanes 13–15). The gb1a-gb2 heterodimer, which is stable in SDS, might result from SDS-resistant intermolecular transmembrane interactions as reported for the formation of p2-adrenergic and dopamine D2 receptor homodimers (5, 6). The monomer presumably results from partial disruption of protein-protein binding domains (Sushi Repeats) or C-terminal a-helical domains in mbg1a. Disulfide bonds may also contribute to dimer formation as has been reported for the structurally related metabotropic glutamate receptor (7).

We have shown in three disparate expression systems that coexpression of the gb1 and gb2 receptors is required for functional GABAB receptor activity. That the two receptors are co-immunoprecipitated is consistent with the formation of heterodimers. We cannot rule out the possibility that either receptor may function as a monomeric or homodimeric receptor in the appropriate native cells. Indeed there are regions of the brain, such as caudate/putamen, where gb1 mRNA is abundant and gb2 mRNA is undetectable (Fig. 1, A and B). In such regions gb1 would either require a different subunit or function as a monomer or homodimer. However, our in situ hybridization analysis indicates that in brain regions expressing gb2, gb1 is expressed in a majority of the same neurons, suggesting that native receptors are heterodimers in these cells. GPCRs are commonly thought of as monomeric receptors. However, in light of our functional data and in situ hybridization findings, it seems appropriate to consider the gb1 and gb2 receptor proteins to be subunits of a functional GABAB receptor.

Addendum—During the editorial review of this manuscript, similar work from four independent groups was published reporting the formation of functional GABAB receptor heterodimers (19–22).

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