A Single Subunit (GB2) Is Required for G-protein Activation by the Heterodimeric GABA\textsubscript{B} Receptor*

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Although G-protein-coupled receptors (GPCRs) have been shown to assemble into functional homo or heteromers, the role of each protomer in G-protein activation is not known. Among the GPCRs, the \(\gamma\)-aminobutyric acid (GABA) type B receptor (GABA\textsubscript{B}R) is the only one known so far that needs two subunits, GB1 and GB2, to function. The GB1 subunit contains the GABA binding site but is unable to activate G-proteins alone. In contrast the GB2 subunit, which does not bind GABA, has an heptahelial domain able to activate G-proteins when assembled into homodimers (Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prézeau, L., and Pin, J.-P. (2001) EMBO J. 20, 2152–2159). In the present study, we have examined the role of each subunit within the GB1-GB2 heteromer, in G-protein coupling. To that end, point mutations in the highly conserved third intracellular loop known to prevent G-protein coupling were introduced into GB1 and GB2. One mutation, L686P introduced in GB2 prevents the formation of a functional receptor, even though the heteromer reaches the cell surface, and even though the mutated subunit still associates with GB1 and increases GABA affinity on GB1. This was observed either in HEK293 cells where the activation of the G-protein was assessed by measurement of inositol phosphate accumulation, or in cultured neurons where the inhibition of the Ca\textsuperscript{2+} channel current was measured. In contrast, the same mutation when introduced into GB1 does not modify the G-protein coupling properties of the heteromeric GABA\textsubscript{B} receptor either in HEK293 cells or in neurons. Accordingly, whereas in all GPCRs the same protein is responsible for both agonist binding and G-protein activation, these two functions are assumed by two distinct subunits in the GABA\textsubscript{B} receptor: one subunit, GB1, binds the agonists whereas the other, GB2, activates the G-protein. This illustrates the importance of a single subunit for G-protein activation within a dimeric receptor.

G-protein-coupled receptors (GPCRs)\textsuperscript{1} are coded by the large gene family of the animal genomes. These receptors detect and transduce across the plasma membrane, the information carried by a large variety of message molecules, from photons to glycoprofins and from amino acids to ions, with a very high specificity (1). Accordingly, GPCRs are involved in a large variety of physiological and pathological processes, and as such constitute the targets of a large number of therapeutic drugs. All these receptors have an heptahelial domain (HD) formed by seven transmembrane helices interconnected by three extracellular and three intracellular loops. The intracellular face of the receptor contacts the heterotrimeric G-proteins and stimulates them upon agonist activation. Indeed, the second and third intracellular loops have been shown to play a critical role in G-protein activation by forming a pocket in which the C-terminal tail of the \(\alpha\)-subunit of the G-protein binds (2–4).

Within the last decade, a number of studies reported that several GPCRs can form oligomers, either homo or heterooligomers (5). Such protein complexes, when constituted of different subunits, can have specific properties, not shared by the corresponding receptors alone. Indeed, the heteromers can have a specific pharmacology (6, 7), desensitization properties (7, 8), or even can activate specific intracellular pathways (9). However, the respective role of each subunit of the oligomer in G-protein activation is not known.

Among the various GPCRs, the GABA\textsubscript{B} receptor is the only one identified so far that requires two subunits, GB1 and GB2, to be functional. This is true both in heterologous expression systems (10–14) and in neurons (15). Such a characteristic of the GABA\textsubscript{B} receptor makes it an excellent model to examine the respective role of each subunit in G-protein coupling.

Each GABA\textsubscript{B} receptor subunit shares sequence similarity with the metabotropic glutamate (mGlu), the calcium sensing (CaS), and some putative pheromone and taste receptors. All these receptors have been shown to form dimers (16–18), and all are composed of two main domains, a HD involved in G-protein coupling (19–21) and a large extracellular domain (ECD) where the agonists bind (22–27). But why are two distinct subunits required to get a functional GABA\textsubscript{B} receptor? Indeed, GABA\textsubscript{B} agonists likely bind on GB1 only (26, 27) although they are unable to activate it. The GB2 subunit is required for the correct insertion of the GB1 subunit in the plasma membrane but also for the correct functioning of the receptor (28–30). We recently reported that the GB2-HD can couple to G-protein when associated in homodimers, but not the GB1-HD (31). Such data did not exclude a direct coupling of the GB1-HD when associated with GB2-HD within the hetero-

\(\gamma\)-aminobutyric acid; HEK, human embryonic kidney; GABA, \(\gamma\)-aminobutyric acid; GFP, green fluorescent protein; HA, hemagglutinin; HRP, horseradish peroxidase; Tricine, \(N\)-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HD, heptahelial domain.

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The abbreviations used are: GPCR, G-protein-coupled receptor; IP, inositol phosphate; HEK, human embryonic kidney; GABA, \(\gamma\)-aminobutyric acid; GFP, green fluorescent protein; HA, hemagglutinin; HRP, horseradish peroxidase; Tricine, \(N\)-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HD, heptahelial domain.

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mer. Here, the specific role of the GB1-HD and GB2-HD in G-protein activation by the heteromeric GABA<sub>B</sub> receptor was examined. Our data show that the GB2-HD plays a critical role within the heteromer for G-protein activation.

**MATERIALS AND METHODS**

**Construction of Point-Mutated Receptors**—cDNAs encoding wild-type GB1 and GB2 were described previously (31). Site-directed mutagenesis of sequences encoding amino acids of the i3 loop of GB1 and GB2 was performed on a BamHI-SalI fragment from pRK-BR1a cloned into pBlueScript SK(−) Vector (Stratagene, La Jolla, CA) and NdelBstEndI-XbaI fragment from pCI-Neo-BR2 cloned into pBlueScript SK−, using the QuikChange site-directed mutagenesis kit (Stratagene). For each mutagenesis, two complementary 30-mer oligonucleotides (sense and antisense; Genaxis Biotechnologie, Nimes, France) were designed to contain the desired mutation in their center. The authenticity of each point mutation and the absence of undesired ones were confirmed by DNA sequencing. Subsequently, a short fragment surrounding the mutation was subcloned in place of the corresponding wild-type fragment of pRK-BR1a or pRK-BR2.

**Cell Culture and Transfection**—Human embryonic kidney (HEK293) cells were cultured and transfected by electroporation as described previously (32). After electroporation, the cells were plated on polyornithine-coated dishes. Serum, culture media, and other solutions used for cell culture were from Invitrogen (Cergy Pontoise, France).

**Western Blot**—The Western blotting protocol was already described (31). 10 µg of membrane were loaded on a Tricine-SDS gel for electrophoresis and transferred on nitrocellulose membranes (Amersham Biosciences). After incubation in phosphate-buffered saline/milk 5%, the membranes were incubated with the anti-GB1 antibody (Sigma) or the monoclonal anti-HA antibody (1/3000, Roche Diagnostics) at room temperature for 2 h. After washing, the membranes were incubated overnight at 4 °C with the anti-mouse HRP antibody (Amersham Biosciences). Signals were revealed using an ECL chemiluminescent assay (Amersham Biosciences).

**Ligand Binding on Intact HEK293 Cells**—Ligand binding experiments were performed on intact HEK293 cells as previously described. Briefly, cells were plated after electroporation the day before the experiment. Thus, the cells on ice were washed with ice-cold binding buffer (20 mM Tris-Cl, pH 7.4, 118 mM NaCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 4.7 mM CaCl<sub>2</sub> and insulin) and incubated in the presence of [125I]CGP64213 with or without unlabeled ligands at the indicated concentration. The incubation was terminated by washing with ice-cold buffer. The cells were disrupted with 0.1 M NaOH (400 µl), and the bound radioactivity was counted and normalized to the amount of protein in each well. Nonspecific binding was determined in the presence of 1 mM GABA.

**RESULTS**

**Generation and Characterization of GB1 and GB2 Mutants**—Like any other G-protein-coupled receptors, the GABA<sub>B</sub>-like receptors, such as the mGlu and Ca<sub>S</sub> receptors contact the G-protein α-subunit via their second and third intracellular loops (i2 and i3 loops, respectively) (19–21, 36). As shown in Fig. 1, the i3 loop is very well conserved among the receptors of this family, and several residues in this loop have already been shown to play a critical role in G-protein coupling in both mGlu1 and Ca<sub>S</sub> receptors. Among these, Arg-796 and Phe-802 of Ca<sub>S</sub>(Arg-775 and Phe-781 of mGlu1) which were mutated into Trp and Ala, respectively, generate receptors unable to activate G-protein upon agonist activation (21, 36, 37). As shown in Fig. 1 the general characteristic of these residues (basic and hydrophobic respectively) is conserved in both GB1 and GB2 subunits suggesting that they play a similar role in the Ca<sub>S</sub> and mGlu1 receptors. To identify the respective role of GB1 and GB2 in G-protein coupling of the GABA<sub>B</sub> heteromer, these residues (Lys-791 and Ile-798 of GB1; Arg-679 and Leu-686 of GB2) were mutated into different residues. The following mutants were generated: GB1-L791W; GB1-L791D; GB1-I798F; GB2-R679W; GB2-R679D; GB2-L686S, and GB2-L686F. All these constructs were tagged at their N-terminal end with either a HA or c-Myc epitope, as previously described (29, 31).

Western blot analysis showed that all mutated receptors were expressed in HEK293 cells (Fig. 2A). Binding experiments performed on intact cells with membrane impermeable high affinity GB1 radioligand [125I]CGP64213 indicated that any GB1 mutants were correctly targeted to the plasma membrane when co-expressed with the wild-type GB2 (Fig. 2B) but not when expressed alone (data not shown). Because GB1 only reaches the cell surface when associated with GB2 (28–30), these data indicate that the mutations in GB1 do not affect the correct folding of this subunit nor its association with GB2. Similarly, [125I]CGP64213 binding can be detected on intact cells expressing any of the mutated GB2 subunits co-expressed with the wild-type GB1 (Fig. 2B). This indicates that these mutated GB2 subunits are still able to bring GB1 to the cell surface, like the wild-type GB2.

**The Mutation L686P in GB2 Suppresses the Functional Coupling of the Heterodimer G<sub>qa</sub>**—We then analyzed the effect of the mutations in the i3 of GB1 and GB2 on the G-protein coupling of the heteromeric receptor. To that aim, various combinations of wild-type and mutated GB1 and GB2 subunits were co-expressed with the chimeric Go protein G<sub>qa</sub> in HEK293 cells. This chimeric G-protein α-subunit corresponds to Go<sub>a</sub> in which the last 9 C-terminal residues have been replaced by those of Go<sub>q</sub>. This allows this G-protein construct to couple many G<sub>qi</sub>-coupled receptors to PLC (2), including the heteromeric GABA<sub>B</sub> receptor (32). As shown in Fig. 3, GABA-
induced IP formation was not affected when Lyn-791 of GB1 or Arg-679 of GB2 were mutated into Trp or Asp. Only a 10-fold decrease in GABA potency (10-fold increase in the EC_{50} value, see Table I) was noticed with GB2-R679W and GB2-R679D mutants, GB2-L686P and GB2-L686S (by 94 and 83%, respectively), together with the wild-type GB2. Indeed, the maximal induced IP formation was not affected when Lys-791 of GB1 or Arg-679 of GB2 were mutated into Trp or Asp. Only a 10-fold decrease in GABA potency (10-fold increase in the EC_{50} value, see Table I) was noticed with GB2-R679W and GB2-R679D mutants.

As mentioned above the GB2-L686S and GB2-L686P mutants were able to bring the wild-type GB1 subunits to the cell surface, but it remains possible that the loss of GABA response in cells co-expressing these GB2 mutants and GB1 is because of an instability of the heteromers at the cell surface, or to the absence of allosteric interaction between the two subunits required for function. It has been reported that the association of GB2 with GB1 increases agonist affinity on GB1 by a factor 10 (10, 12, 31). As shown in Fig. 4 and in Table I, both GB2 mutants were able to increase GABA affinity on GB1 like the wild-type GB2, indicating that they not only bring GB1 at the cell surface, but also still allosterically interact with GB1 at the cell surface. Indeed, the K_{i} value of GABA in displacing ^{125}I-CGP64213 binding on intact cells expressing the different subunit combinations. Because GB1 reaches the cell surface only when it is co-expressed with GB2, the binding on GB1 indicates that the heteromer GB1+GB2 is formed and gets to the cell surface. The total binding of the wild-type is 3243 ± 750 cpm, and the nonspecific binding 238 ± 39 cpm (n = 12). Values correspond to the amount of bound radioactivity per well expressed as a percentage of wild-type maximal binding. Data are the mean ± S.E. of 6-12 independent experiments done in triplicates.

**Fig. 1.** Alignment of the i3 loop of GABA\_B receptor subunits and related receptors. Highlighted in black are residues conserved within this family of receptors. The consensus sequence of the conserved residues in this loop is indicated at the bottom, with b, basic; a, acidic; h, hydrophobic; and X, any residue. These include the sequences of GB1 from human (swall accession number: NP_001461), rat (CAA71398), mouse (AAD22194), Drosophila melanogaster (Q9V3Q9) and Caenorhabditis elegans (from the cosmids Y41G9), of GB2 from human (O75899), rat (P31421), 6 (P35349), and 8 (NP_071538) from rat, from Catfish (CaF, Q9VPS7), the metabotropic glutamate receptors subtypes 1 (P23385), 2 (P31421), 6 (P35349), and 8 (NP_071538) from rat.

**Fig. 2.** Expression of the wild-type and mutant GABA\_B subunits. A, the expression of the different wild-type and mutated GB1 and GB2 subunits was analyzed by Western blot analysis. The proteins were detected with the monoclonal anti GB1 antibody for GB1 subunits and with monoclonal anti-HA antibody for the GB2 subunits. B, the expression at the cell surface of the mutated or wild-type GB1 subunits was determined by measuring the total [^{125}I]-CGP64213 binding on intact cells expressing the different subunit combinations. Because GB1 reaches the cell surface only when it is co-expressed with GB2, the binding on GB1 indicates that the heteromer GB1+GB2 is formed and gets to the cell surface. The total binding of the wild-type is 3243 ± 750 cpm, and the nonspecific binding 238 ± 39 cpm (n = 12). Values correspond to the amount of bound radioactivity per well expressed as a percentage of wild-type maximal binding. Data are the mean ± S.E. of 6-12 independent experiments done in triplicates.
GABA B receptors do not naturally couple to the artificial Gqi9 protein. Indeed, in neurons, GABAB receptors couple to Gi/o proteins and as a consequence inhibit adenylyl cyclase or Ca2+ channel activity, or activate G-protein inwardly rectifying K+ channels (38). It is possible that GB2 plays a critical role in the coupling to Gqi9 in HEK293 cells, but not to Gi/o in neurons. We therefore examined the ability of our wild-type and mutated GB1 and GB2 subunits to form a functional GABA B receptor able to inhibit Ca2+ channels in cerebellar granule cells. In these neurons, depolarization to 0 mV induced activation of a Ba current (IBa), which is mediated by various types of Ca2+ channels (35, 39).

Under our culture conditions, the GABAB selective agonist baclofen was unable to inhibit the voltage-activated IBa in control or GFP-expressing neurons (Fig. 5a). Moreover, expression of either GB1 or GB2 subunits alone was also not sufficient to generate a GABAB receptor able to inhibit IBa. In contrast in neurons co-expressing both subunits, baclofen induced a 20 ± 2% inhibition (n = 8) of IBa (Fig. 5b). A similar effect of baclofen was also observed in neurons expressing GB1-I798P and GB2 (Fig. 5c), 19 ± 3% inhibition (n = 9). However, baclofen did not affect IBa in neurons expressing GB1 and GB2-L686P subunits (Fig. 5d), even though both subunits where correctly expressed at the cell surface as revealed by immunostaining of intact cells with the HA antibody (data not shown).

### Table I

<table>
<thead>
<tr>
<th>Subunit Combination</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
<th>K&lt;sub&gt;e&lt;/sub&gt; (nM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tr>
<td>GB1-ASA</td>
<td>22.6 ± 3.9</td>
<td>32.0 ± 6.4</td>
<td>NR</td>
</tr>
<tr>
<td>GB1 + GB2</td>
<td>22.4 ± 3.9</td>
<td>2.9 ± 0.9</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>GB1-K791W + GB2</td>
<td>22.9 ± 2.8</td>
<td>3.6 ± 0.7</td>
<td>1.4 ± 1.0</td>
</tr>
<tr>
<td>GB1-K791D + GB2</td>
<td>17.2 ± 4.6</td>
<td>*</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>GB1-I798S + GB2</td>
<td>16.4 ± 2.2</td>
<td>*</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>GB1-I798P + GB2</td>
<td>21.3 ± 2.3</td>
<td>*</td>
<td>1.9 ± 1.1</td>
</tr>
<tr>
<td>GB1 + GB2-R679W</td>
<td>14.2 ± 1.2</td>
<td>*</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>GB1 + GB2-R679D</td>
<td>25.0 ± 0.1</td>
<td>*</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>GB1 + GB2-L686S</td>
<td>18.7 ± 1.2</td>
<td>*</td>
<td>105.3 ± 5.8</td>
</tr>
<tr>
<td>GB1 + GB2-L686P</td>
<td>19.2 ± 2.3</td>
<td>*</td>
<td>3.4 ± 0.9</td>
</tr>
</tbody>
</table>

*Numbers not determined. NR, no response.

## Affinity potency values of CGP 64213 and GABA on wild-type and mutated GB1 and GB2 subunits

The K<sub>i</sub> of CGP64213 and GABA was determined according to displacement of [125I]-CGP 64213 binding experiments performed on intact cells expressing the indicated subunits combinations (as described in "Material and Methods"). EC<sub>50</sub> values of GABA as determined from displacement curve of IP3 production. Values are means ± S.E. of at least three independent determinations.

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**GB2 Coupling to G-protein in the GABA<sub>B</sub> Receptor**

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**Fig. 3.** G-protein coupling of the wild-type and mutated GB1 and GB2 subunits. A, dose-response curves of GABA on cells expressing the GB1 mutants with the GB2 wild-type subunit. B, dose-response curves of GABA on cells expressing the GB2 mutants with the GB1 wild-type subunit. Values correspond to the percentage of the maximal agonist-induced IP formation measured in cells expressing the wild-type receptor (GB1<sub>WT</sub>GB2). In those cells, GABA induced a 6.4-fold increase in the basal IP formation. Values are means ± S.E. of at least three experiments performed in triplicates.

**Fig. 4.** Displacement curves of [125I]-CGP64213 with GABA on cells expressing the different mutant receptors. The values correspond to the amount of bound radioactive [125I]-CGP64213 displaced by increasing concentrations of GABA on the different receptor mutants. Specific [125I]-CGP64213 binding is plotted as the percentage of the binding obtained in cells expressing the wild-type GABA<sub>B</sub> receptor. Values are means ± S.E. of at least three experiments performed in triplicates.
GABAB receptor: GB1 being responsible for agonist binding data better define the specific role of each subunit within the equivalent mutation in GB1 does not prevent coupling. These I798P (GB1), or GB1 and GB2-L686P (GB2) (b). The current was measured over a 500-ms depolarizing step to 0 mV in the absence or in the presence of baclofen (100 μM). Scale bars: 200 pA and 200 ms.

**DISCUSSION**

In the present study we identified a single residue within the i3 loop of GB2 that plays a critical role for the coupling of the heteromeric GABA<sub>B</sub> receptor to G-proteins, either to the chimeric G<sub>qi9</sub> protein in HEK293 cells, or to native G-proteins inhibiting Ca<sup>2+</sup> channels in primary neurons. In contrast, the equivalent mutation in GB1 does not prevent coupling. These data better define the specific role of each subunit within the GABA<sub>B</sub> receptor: GB1 being responsible for agonist binding and GB2 playing a pivotal role in G-protein activation.

GABA<sub>B</sub> receptor subunits are part of the so-called Family 3 GPCRs. These receptors do not share the sequence similarity with the other rhodopsin-like or secretin receptor-like GPCRs. One of the characteristic of these receptors is their short and highly conserved i3 loop, in which a signature has been identified (F/L/Y/L)-N(E/D/-)X-X(R/K) at the bottom of TM6 (Fig. 1). Among these residues, the first hydrophobic residue has been shown to play a critical role in G-protein coupling in both mGlu1 and CaS receptors because its mutation into either Ser, Pro in mGlu1 (21), or Ala, His, Glu, Leu, or Val (but not Tyr or Trp) in CaS (36) receptor abolished coupling of these receptors to PLC. In the GB1 and GB2 subunits, this Phe residue is replaced by an Ile and a Leu respectively. Our data revealed that this residue in GB2 also plays a critical role in G-protein coupling because its mutation into either Ser or Pro largely decreases and abolishes, respectively, coupling of the heteromeric receptor to either G<sub>qi9</sub> in HEK293 cells or inhibition of Ca<sup>2+</sup> channels in neurons. It may appear surprising that the replacement of Phe in the CaS receptor by Ser, as found in the GB2 subunit prevent CaS receptor functioning. This may be due to a different environment of the Leu residue in the CaS and GB2 proteins. However, the mutation of the Ile residue found at that position in the i3 loop of GB1 into either Ser or Pro (but also into Ala, data not shown) did not affect coupling of the GABA<sub>B</sub> receptor either in HEK293 cells or in neurons.

Another residue within the i3 loop is highly conserved among the family 3 GPCR, this is the Arg of the conserved sequence K/T/S/I/R at the bottom of TM5 (Fig. 1). This Arg of the CaS receptor was found to be mutated into a Trp in families suffering from hypocalciuric hypercalcemia (37). This mutation of either the CaS or mGlu1 receptor was found to prevent coupling to G-proteins (21, 37). Here we show that the mutation of this residue in either GB1 (Lys-791) or GB2 (Arg-679) into Trp or Asp did not modify the ability of the heteromeric receptor to activate G<sub>qi9</sub>. Indeed, only a decrease in the potency of GABA was noticed with the GB2-R679W and R679D mutants, consistent with a decrease in G-protein coupling efficiency (no change in the GABA affinity nor receptor expression level being observed with these mutants). When a careful analysis of the same mutation introduced in mGlu1 was performed, we found that it resulted in an increase in the basal activity of the receptor, indicating that this mutation does not prevent mGlu1 receptor coupling to G-protein but rather stabilize an active state of the receptor. It is possible that the loss of function observed for the Arg→Trp mutants of the CaS and mGlu1 receptors by others results from the desensitization of the receptor due to a high basal activity, rather than from a total uncoupling to G-proteins. Taken together, these data show that the conserved basic residue at the bottom of TM5 does not play a critical role in G-protein coupling, but rather modify the coupling efficacy of the receptor to the G-protein.

These data show that GB2-HD plays a crucial role in G-protein coupling in the heteromer. So what is the role of the GB1-HD in GABA<sub>B</sub> receptor function? Our data demonstrate that GB1-HD cannot activate G<sub>qi9</sub> or the native G-protein responsible for Ca<sup>2+</sup> channel inhibition in neurons. We cannot exclude however, that GB1 or GB1-I798P can activate another type of G-protein. Alternatively, it is also possible that GB1 coupling to G-proteins also requires GB2 coupling. This would be the case if the simultaneous interaction of G-proteins with GB1 and GB2 would be required for G-protein activation. However, the simplest hypothesis would be that GB1 does not couple to the G-protein α-subunit. Another possibility is that GB1 only interacts with the βγ dimer. This could explain our recent data showing that GB1-HD improves the G-protein coupling efficiency of the GB2-HD (31). Of interest, it has been reported recently that GPCRs could act as a lever to tilt the βγ-subunit on the α-subunit to stimulate the GDP-GTP exchange (40). This model, as well as the respective size of the heterotrimeric G-protein and a dimeric GPCR (41), will very well fit with the possibility that one subunit in a dimeric GPCR interacts with the G-protein α-subunit whereas the other interacts with the βγ-subunit. However, although a direct specific cross-linking approach revealed a close contact of rhodopsin with the α-subunit of transducin, no cross-linking was obtained between rhodopsin and the βγ dimer (42, 43). Finally, it remains possible that GB1 does not contact the heterotrimeric G-protein at all and that it plays a different role in the transduction mechanism of the GABA<sub>B</sub> receptor, possibly by interacting with other intracellular proteins such as the ATP4 protein (44–46).

Several sets of data reported recently indicate that within the heteromeric GABA<sub>B</sub> receptor, only the GB1 subunit binds the agonists (15, 26, 27). More recently, the GB2-HD has been shown to contain enough molecular determinants for activation of G<sub>qi9</sub> when associated into homodimers. Indeed, the co-expression of a chimeric construct containing the GB1-EC and the GB2-HD together with the wild-type GB2 forms a functional GABA<sub>B</sub> receptor that can activate a same set of G-proteins as the wild-type heteromer (31). In contrast, no coupling to any tested chimeric G-proteins could be detected with a receptor containing only GB1-HDs. These data did not however exclude the possibility that GB1-HD can couple to G-proteins when associated with the GB2-HD, or in its natural environment. Our present data show that the mutation of a single residue in the i3 loop of GB2 is sufficient to prevent coupling of the heteromeric GABA<sub>B</sub> receptor both to G<sub>qi9</sub> in

<sup>2</sup>S. Restituito and J.-P. Pin, unpublished observation.
HEK293 cells and to native G-proteins involved in GABA\(_B\) receptor mediated Ca\(^{2+}\) channel inhibition in neurons. As discussed above, the simplest hypothesis to explain our data is that the GB1-HD does not couple to G-proteins. This makes the GABA\(_B\) receptor the only GPCR in which the agonist binding site and G-protein coupling domain are located in two distinct subunits. This also raises the interesting issue on how the signal is translocated from one subunit to the other.

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