Mutagenesis and Modeling of the GABA_B Receptor Extracellular Domain Support a Venus Flytrap Mechanism for Ligand Binding*

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The γ-aminobutyric acid type B (GABA_B) receptor is distantly related to the metabotropic glutamate receptor-like family of G-protein-coupled receptors (family 3). Sequence comparison revealed that, like metabotropic glutamate receptors, the extracellular domain of the two GABA_B receptor splice variants possesses an identical region homologous to the bacterial periplasmic leucine-binding protein (LBP), but lacks the cysteine-rich region common to all other family 3 receptors. A three-dimensional model of the LBP-like domain of the GABA_B receptor was constructed based on the known structure of LBP. This model predicts that four of the five cysteine residues found in this GABA_B receptor domain are important for its correct folding. This conclusion is supported by analysis of mutations of these Cys residues and a decrease in the thermostability of the binding site after dithiothreitol treatment. Additionally, Ser-246 was found to be critical for CGP64213 binding. Interestingly, this residue aligns with Ser-79 of LBP, which forms a hydrogen bond with the ligand. The mutation of Ser-269 was found to differently affect the affinity of various ligands, indicating that this residue is involved in the selectivity of recognition of GABA_B receptor ligands. Finally, the mutation of two residues, Ser-247 and Gln-312, was found to increase the affinity for agonists and to decrease the affinity for antagonists. Such an effect of point mutations can be explained by the Venus flytrap model for receptor activation. This model proposes that the initial step in the activation of the receptor by agonist results from the closure of the two lobes of the binding domain.

A very large number of membrane receptors modulate the activity of intracellular effectors by activating heterotrimeric GTP-binding proteins (G-proteins). All G-protein-coupled receptors characterized so far possess seven putative transmembrane domains that define intracellular loops critical for the recognition and activation of G-proteins. Sequence comparison revealed the existence of at least three major families of G-protein-coupled receptors. The members from different families share no sequence similarity. Receptors homologous to rhodopsin (receptors for catecholamines, acetylcholine, certain peptides, glycoproteins, etc.) constitute the first family (family 1), which is to date the best characterized one. Family 2 receptors are those homologous to the vasoactive intestinal peptide and the glucagon receptors. Family 3 receptors comprise the metabotropic glutamate receptors (mGluRs) (1, 2), the Ca2+-sensing receptor (3), and a recently discovered new family of putative pheromone receptors (4–6).

Family 3 G-protein-coupled receptors possess several unique features. They all have a surprisingly large extracellular N terminus that shares some sequence similarity with bacterial periplasmic amino acid-binding proteins (PBPs) such as the leucine-binding protein (LBP) and the leucine/isoleucine/valine-binding protein (LIVBP) (7). In agreement, this portion of the receptor has been shown to play a critical role in ligand recognition in mGluRs (7–10). Recently, the production of the entire extracellular domain of mGluR1 in insect cells revealed that this domain produced as a protein is able to fold correctly, is soluble, and is sufficient to bind glutamate and its analogues in a very similar manner as the wild-type receptor does (11). However, further evidence that this domain shares a similar three-dimensional structure with PBPs is still missing due to the lack of good radioligands. The family 3 receptors also contain 20 conserved cysteine residues; 17 are located in the N-terminal extracellular domain, 9 of which are concentrated among the 100 residues that separate the LBP-like domain and the first transmembrane domain. The functional importance of this Cys-rich region has not yet been elucidated. However, this region has been shown to be necessary for the LBP-like domain of mGluR1 to bind glutamate (11). Other characteristic features of family 3 G-protein-coupled receptors are a highly conserved and short third intracellular loop critical for G-protein activation (12) and a variable second intracellular loop important for G-protein coupling selectivity (13, 14).

The original structure of the binding domain of the family 3 receptors led to a hypothesis for their mechanism of activation. PBPs are known to be constituted of two lobes that close upon binding of the ligand, like a Venus flytrap when touched by an insect (15, 16). It has therefore been proposed that the two lobes of the LBP-like domain of family 3 receptors also close upon binding of the agonist and that this change in conformation is transduced to the transmembrane region to activate the G-protein (7, 17). The agonist-binding domains of the ionotropic

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† The abbreviations used are: G-proteins, GTP-binding proteins; mGluR, metabotropic glutamate receptor; PBPs, periplasmic amino acid-binding proteins; LBP, leucine-binding protein; LIVBP, leucine/isoleucine/valine-binding protein; GABA_B, γ-aminobutyric acid type B; GABA_AR1, GABA_AR receptor 1; DTT, dithiothreitol; APPA, 3-aminopropylphosphinic acid.
glutamate receptor subunits are also homologous to PBPs (7, 18–21), and the closure of this domain upon agonist binding has also been proposed to be responsible for receptor activation (22).

Recently, the cloning of the GABAB receptor revealed a protein that is distantly related to family 3 receptors. Indeed the GABAB receptor does not share all of the characteristic features of family 3 receptors described above (23). Like other family 3 receptors, the GABAB receptor possesses a large extracellular domain that shares some limited but significant similarity with PBPs such as LBP. However, the intracellular loops of the GABAB receptor are not as well conserved as in the other family 3 receptors. Moreover, the cysteines in the LBP-like domain, highly conserved in the other family 3 receptors, are not present in the GABAB receptor, and no Cys-rich region is found in this receptor. Because high affinity and specific radioligands are available for the GABAB receptor and because this receptor is related to the family 3 G-protein-coupled receptors, it appears to be a good model for a further analysis of the possible structure of the extracellular domain of this new receptor family. This would help identify the possible role of this domain in ligand recognition and receptor activation.

Here, we report the generation of a model for the GABAB-binding domain and its assessment by site-directed mutagenesis. This study shows that the extracellular domain of the GABAB receptor likely folds into two lobes separated by a hinge region like the PBPs and identifies specific residues within this domain important for ligand recognition.

**EXPERIMENTAL PROCEDURES**

**Materials—γ-Amino-n-butyric acid (GABA) was obtained from Sigma (L’Isle d’Abeau, France).** 1125-I-CGP46213 was synthesized from ethyl (1,1-diethoxyethyl)phosphinate as described (24) and labeled to a specific radioactivity of >2000 Ci/mmol (ANAWA AG, Wangen, Switzerland). All other ligands were synthesized in-house (24, 25). Dithiothreitol (DTT) was from Roche Molecular Biochemicals (Mannheim, Germany). Serum, culture media, and other solutions used for cell culture were from Life Technologies, Inc. (Cergy Pontoise, France). The polyclonal anti-GABA-R1 antibody was raised against the C-terminal intracellular part of the receptor as described previously (26). A secondary fluorescein-labeled anti-rabbit antibody (Sigma) was used for immunostaining. The ECL chemiluminescence system (Amersham Pharmacia Biotech) was used to detect the secondary antibody on Western blots.

**Sequence Alignment—** A multiple alignment of the metabotropic glutamate receptor rat mGlurR1–8 plus the Drosophila receptor DmGlurA and Ca2+-sensing receptor LBP-like domains according to O’Hara et al. (7) and of the bacterial periplasmic proteins that bind leucine/isoleucine/valine (LIVBP) and leucine (LBP) was generated using the ClustalW Version 1.60 program (27). A gap penalty of 30, an extension gap penalty of 0.5, and the Blosum62 matrix were used. The alignment was then manually modified in order not to introduce gaps into the sequence that aligned with the known secondary structure elements of LIVBP and LBP. On top of the multiple alignment, part of the extracellular domain of GABA-B-R1a (residues 109–558) was then aligned using the profile alignment command of the ClustalW Version 1.60 program and a gap penalty ranging from 2 to 50. These alignments indicated that the LBP-like domain of GABA-B-R1a maps from residues 184 to 550. This later segment of the GABA-B-R1a sequence was therefore used to perform additional alignments. These were further analyzed and manually modified using the hydrophobic cluster analysis (28). The resulting alignments of the extracellular GABA-B-binding site, LIVBP, and LBP were then used to generate three-dimensional models using the program Modeller (see below). The coordinates of these models were then subjected to the Verify3D algorithm (29) using the Verify3D Structure Evaluation Server to identify regions of improper folding. After further manual modifications, the alignment giving rise to models with better Verify3D scores was selected.

**Molecular Modeling—** The three-dimensional model of the GABA-B-binding domain was constructed by homology using the coordinates of both LIVBP and LBP from Escherichia coli (Protein Data Bank accession numbers 2LVJ and 2LBQ, respectively) obtained by x-ray crystallography. The sequence alignment used was obtained as described above. Several models were generated using the program Modeler (30) in the Insight-II environment (Molecular Simulation Inc., San Diego, CA) on a Silicon Graphics O2 workstation. The hydrophobic cluster analysis suggested that part of the insertion found just after α-helix 9 (see Fig. 1) also likely folds into an α-helix. Accordingly, new models were generated in which parts of the insertion (residues 398–406 and 412–418) were folded into an α-helix. Accordingly, the side chains of Cys-375 and Cys-409 were close in proximity, making them likely to form a disulfide bond. This was further imposed during the energy minimization procedure. These modifications improved the scores obtained in this area of the sequence with the Verify3D algorithm (29). The model giving the best scores was selected and subjected to energy minimization using the program Discover Version 2.9.7 (Molecular Simulation Inc.) and the CVFF force field. A steepest descent followed by a conjugate gradient method was applied (without taking into account the electrostatic term) until the maximum energy derivative was <1.0 kcal A⁻¹ mol⁻¹.

**Site-directed Mutagenesis and Expression in HEK-293 Cells—** Single amino acid replacement was carried out by the Quick change method (Stratagene, La Jolla, CA) according to the manufacturer’s instructions using pBSB5 as a template. This vector was constructed by subcloning the coding sequence of GABA-B-R1a into the EcoRI and NolI restriction sites of the plasmid (Stratagene). For each mutation, a complementary 30-mer oligonucleotides (sense and antisense) were designed to contain the desired mutation in their middle. To allow a rapid screening of the mutated primers, the primers carried an additional silent mutation introducing (or removing) a restriction site. The presence of each mutation of interest and the absence of undesired ones were confirmed by DNA sequencing. Subsequently, a short fragment surrounding the mutation had been subcloned in place of the corresponding wild-type fragment of pRKBR1a (an expression vector constructed by inserting the open reading frame of GABA-B-R1a downstream of the cytomegalovirus promoter of pRK5).

**Wild-type and mutated expression constructs were transfected into HEK-293 cells by electroporation as described previously (31). Electroporation was carried out in a modified Eurospin Transfection Device (Life Sciences). The primary polyclonal antibody used for the immunodetection of the GABAB-R1a protein was described previously (26).** Protein concentrations were measured with the Bradford method using a bovine serum albumin (BSA) standard.

**Ligand Binding Assay—** Ligand competition experiments were performed on membranes of HEK-293 cells prepared as followed. 24 h after transfection, the cells were solubilized with 10 µl of carrier DNA, 2 µg of plasmid DNA containing the wild-type or mutated GABA-B-R1a coding sequences, and 10 × 10⁶ cells. The cells were cultured in Dulbecco’s modified Eagle’s medium (Life Sciences, Inc.) supplemented with 10% fetal calf serum and antibiotics.

**Identification and Molecular Modeling of the LBP-like Domain of the GABA-B Receptor—** Sequence comparison between glutamate receptor subunits and the PBPs such as LBP has also been proposed to be responsible for receptor activation (22).
the extracellular domain of the GABAB receptor, the metabotropic glutamate receptors, and PBPs reveals that the sequence between residues 167 and 550 of GABAB-R1a shares sequence similarity with the LBP-like domain of mGluRs (18.9% with mGluR1 and 15.8% with the Ca\textsuperscript{2+}-sensing receptor) and LBP and LIVBP (38.1 and 37.1%, respectively) (Fig. 1a). GABA-B-R1a and GABA-B-R1b are two splice variants that differ in their N-terminal sequence. N-terminal residues 1–163 of GABA-B-R1a are replaced by 47 different residues in GABA-B-R1b (Fig. 1b) (23). Interestingly, the similarity to the LBP and LIVBP proteins starts just after the splice junction site, suggesting that the LBP-like domain of GABAB receptors starts at the beginning of the first exon common to both GABA-B-R1a and GABA-B-R1b. In agreement with this proposal, the specific sequence of GABA-B-R1a folds in a tandem pair of complement protein modules (also known as sushi repeats) (33–35), the first corresponding to Thr-25–Arg-97 and the second to Ile-84–Asn-159. The second sushi-like domain of GABA-B-R1a ends almost exactly before the splice site. This reveals that GABA-B-R1a and GABA-B-R1b share an identical LBP-like domain (Fig. 1b). Accordingly and in agreement with the hypothesis that the LBP-like domain constitutes the ligand recognition domain of family 3 receptors (7–11), the two GABAB receptor variants have identical pharmacological profiles (23). Sequence comparison reveals that the large insertions found in the LBP-like domain of mGluRs (\textit{I1}, \textit{I2}, and \textit{I3} in Fig. 1a) are not found in the GABAB receptor, further indicating that the GABA-B receptor is most distantly related to the other family 3 receptors. Our sequence alignment also confirms that most of the extracellular domain of GABA-B-R1b is homologous to LBP (Fig. 1b). A short se-
sequence of only 39 residues links this domain to the first transmembrane domain.

A three-dimensional model was generated using the known three-dimensional structure of the open state of LIVBP and LBP (Fig. 2). This model was assessed using the Verify 3D algorithm according to Luthy et al. (29). As shown in Fig. 3, the three/one-dimensional scores of our model are always positive and are similar to those obtained with the template structures of LBP and LIVBP. The scores obtained are in the range of scores for highly refined correct X-ray structure determinations. This indicates that this domain of the GABA B receptor can fold like the LBP protein. As a further validation of the model, all Asn residues that are part of a consensus glycosylation site (Fig. 1a) were found to be at the surface of the protein (brown residues in Fig. 2b). This model reveals that the LBP-like domain of the GABA B receptor is composed of two lobes linked by three short linkers (Fig. 2a). Both lobes comprise an alternation of α-helices and β-sheets, one lobe having three additional helices. The latter will be referred to as lobe I, and the other as lobe II (Fig. 2).

Importance of Cysteine Residues for 125I-CGP64213 Binding—Among the five cysteine residues found in the extracellular domain, the model predicts that Cys-219 and Cys-245 as well as Cys-375 and Cys-409 are in close proximity and likely form disulfide bonds (Fig. 2a) (the residue numbers correspond to those of the GABA B-R1α sequence, with the first Met being 1; this numbering will be used throughout this work). Cys-187 is buried inside lobe I and cannot be involved in a disulfide bond (Fig. 2a). Cys-219 and Cys-245 correspond to Cys-53 and Cys-78 of LIVBP and LBP, which are disulfide-bonded (36, 37). This putative disulfide bridge found in the GABA B receptor is not found in the other family 3 receptors since only one of the Cys residues is conserved (see Fig. 1a for the mGluR1 sequence).

To examine if these Cys residues are important for the correct folding of the protein and/or for the binding of GABA B receptor ligands, they were changed into either Ala or Ser by in vitro mutagenesis. The mutation of Cys-187 did not prevent the binding of the GABA B receptor antagonist 125I-CGP64213 (Ta-
correspond to the insertions found in the GABAB receptor domain. The lack of binding observed with the Cys mutants, one may propose that these disulfide bonds are required for the correct folding of the protein during synthesis. After the protein has been synthesized and correctly folded. To explain the loss of binding observed with the Cys mutants, one may propose that these disulfide bonds are required for the correct folding of the protein during synthesis. After the protein is fully matured, these covalent links may only increase the stability of the binding site in the presence or absence of 10 mM DTT treatment (Fig. 5). These results are consistent with the hypothesis that disulfide bonds are involved in the stability of the binding domain of the GABA\textsubscript{B} receptor. However, we cannot exclude the possibility that the DTT effect results from its action on another protein stabilizing the GABA\textsubscript{B} receptor-binding site. Selection and Mutagenesis of Additional Residues Possibly Involved in \textsuperscript{125}I-CGP64213 Binding—The amino acid residues of LIVBP (open state) involved in the binding of leucine have been identified by x-ray crystallography and were found in lobe I (36). The side chain of Ser-79 and Thr-102 have been shown to form hydrogen bonds with the \textsuperscript{125}I-CGP64213 binding site. The vertical gray bars in the plot for LBP and LIVBP correspond to the insertions found in the GABA\textsubscript{B} receptor domain. The x axis numbering corresponds to the amino acid numbering of GABA\textsubscript{B}-R1a. The y axis gives the average three/one-dimensional scores for residues in a 21-residue sliding window.

![Image](https://example.com/figure3.png)

**Fig. 3.** Plots obtained using the Verify3D program with a window of 21 residues using the coordinates of the LBP and LIVBP crystal structures (Protein Data Bank accession numbers 2LBP and 2LIV, respectively) (top) and the coordinates of the three-dimensional model of the LBP-like domain of the GABA\textsubscript{B} receptor (bottom). The vertical gray bars in the plot for LBP and LIVBP correspond to the insertions found in the GABA\textsubscript{B} receptor domain. The x axis numbering corresponds to the amino acid numbering of GABA\textsubscript{B}-R1a. The y axis gives the average three/one-dimensional scores for residues in a 21-residue sliding window.

![Image](https://example.com/figure4.png)

**Fig. 4.** Expression of the Cys mutants of GABA\textsubscript{B}-R1a. Membranes (1 \mu g of total protein) prepared from mock-transfected HEK-293 cells or cells expressing wild-type (W) GABA\textsubscript{B}-R1a or the indicated mutated receptors were subjected to SDS-polyacrylamide gel electrophoresis and blotted as described under "Experimental Procedures." Concentrations giving rise to 50% inhibition of specific binding (IC\textsubscript{50}) and Hill coefficients (n\textsubscript{H}) were determined. Values are means \pm S.E. of at least three experiments performed in triplicates.

| GABA\textsubscript{B} receptor | IC\textsubscript{50} (M) | n\textsubscript{H} | IC\textsubscript{50} (M) | n\textsubscript{H} |
|-------------------------------|-----------------|-------------|-----------------|-------------|
| R1a (wild type)               | 20.8 \pm 0.7    | 0.90 \pm 0.03| 1.90 \pm 0.22    | 0.90 \pm 0.12|
| C187A                         | 49.3 \pm 3.1    | 0.80 \pm 0.01| 2.12 \pm 0.13    | 0.81 \pm 0.14|
| C187S                         | 36.8 \pm 2.1    | 0.84 \pm 0.01| 3.05 \pm 0.32    | 0.89 \pm 0.04|

TABLE I

Effect of the C187A and C187S mutations on the binding affinities of GABA\textsubscript{B} and CGP54626A

Binding experiments were performed using 0.1 \textsuperscript{125}I-CGP64213 as described under "Experimental Procedures." Displacement curves performed with 11 different concentrations of GABA or CGP54626A were fitted as described under "Experimental Procedures." Concentrations giving rise to 50% inhibition of specific binding (IC\textsubscript{50}) and Hill coefficients (n\textsubscript{H}) were determined. Values are means \pm S.E. of at least three experiments performed in triplicates.
Among these 20 different mutations, only six were found to change the binding properties of the GABA<sub>B</sub> receptor (Fig. 2 and Table II). These include S246A, S247A, S269A, S270A, Q312A, and Y470A. All these mutated receptors were detected on Western blots as proteins with the correct molecular mass (Fig. 6) and had the same subcellular distribution as the wild-type receptor (Fig. 7), indicating that the changes in their binding properties were not due to a loss of correct expression or trafficking.

**Mutations That Suppress Binding**—Two of these mutations, S246A and Y470A, resulted in a total loss of the antagonist <sup>125</sup>I-CGP64213 binding, even when a 10-fold higher concentration of the radioligand (1 nM) was used (data not shown). Ser-246 aligns with Ser-79 of LIVBP and Ser-165 of mGluR1 (Fig. 1), which have been shown and proposed to form a hydrogen bond with the α-carboxylic group of leucine and glutamate in LIVBP and mGluR1, respectively (7, 36). It is therefore possible that Ser-246 is directly involved in the binding of agonists and antagonists. Tyr-470 is conserved in all family 3 receptors, LIVBP, and LBP (Fig. 1a). As the side chain of Tyr-470 is located inside lobe I (Fig. 2a), it is unlikely directly interacting with the ligand. Instead, the removal of the phenyl ring may simply affect the conformation of lobe I. Assuming that this lobe contains the binding site, this would affect ligand binding. In agreement with this proposal, mutation of Tyr-470 into phenylalanine restored binding and affinity for <sup>125</sup>I-CGP64213 and GABA, although binding was difficult to detect due to a very low expression of the mutant protein (data not shown).

**Mutations That Differently Affect Binding Affinities of Various Ligands**—The mutations S269A and S270A affected the affinities of some but not all ligands (residues shown in violet in Fig. 2b). In mutant S269A, the affinity of the antagonist CGP54626A was decreased by a factor of 50, whereas that of the agonists CGP64213 and CGP56999A was affected by a factor of only 5 (Fig. 8 and Table II). Similarly, the affinities of the three agonists GABA, APPA, and CGP47656 were decreased by 11-, 30-, and 10-fold, respectively, whereas the affinity of baclofen possessing an additional chlorophenyl group was not affected (Fig. 8 and Table II). In mutant S270A, the affinities of the agonists GABA, APPA, and baclofen were decreased by a factor of >10, whereas those of the antagonists CGP54626A, CGP64213, and CGP56999A were not affected significantly (Fig. 8 and Table II). Due to the relatively small changes in affinity observed, it is unlikely that these two residues are directly involved in an interaction with the ligands. Ser-269 aligns with Thr-102 of LIVBP and Thr-188 of mGluR1 (Fig. 1a). These residues have been shown and proposed to form a hydrogen bond with the α-amino group of leucine and glutamate in LIVBP and mGluR1, respectively (7, 36). Accordingly, mutation of Thr-188 into Ala in mGluR1 resulted in a decrease in the quisqualate potency by a factor of 10,000. GABA lacks the typical amino acid moiety found on the α-carbon of both

![Fig. 5. Effect of DTT treatment on the thermostability of the CGP64213-binding site.](image)

**TABLE II**

| GABA<sub>B</sub> receptor | Comment | IC<sub>50</sub> (nM) | n<sub>H</sub> |
|--------------------------|---------|----------------|--------|
| R1a (wild type)          | wt      | 20.8 ± 0.7     | 0.90 ± 0.03 |
| Q188A                    | wt      | 25.5 ± 2.7     | 0.77 ± 0.01 |
| E192A                    | wt      | 22.3 ± 4.6     | 0.94 ± 0.11 |
| S246A                    | No binding | 5.3 ± 0.4     | 0.81 ± 0.02 |
| S247A                    | +       | 26.2 ± 7.7     | 0.97 ± 0.09 |
| T250A                    | wt      | 36.4 ± 7.4     | 0.90 ± 0.11 |
| S269A                    | wt      | 27.8 ± 1.9     | 0.82 ± 0.02 |
| S269A                    | −       | 238.8 ± 28.4   | 0.78 ± 0.03 |
| S270A                    | −       | 405.7 ± 15.1   | 0.79 ± 0.02 |
| T310A                    | wt      | 19.4 ± 0.7     | 0.73 ± 0.02 |
| Q312A                    | +       | 3.2 ± 0.2      | 0.74 ± 0.02 |
| Q313A                    | wt      | 16.4 ± 0.5     | 0.86 ± 0.03 |
| T314A                    | wt      | 9.9 ± 2.5      | 0.63 ± 0.03 |
| T315A                    | wt      | 43.6 ± 1.1     | 1.08 ± 0.09 |
| E316A                    | wt      | 61.1 ± 12.2    | 0.61 ± 0.03 |
| E458A                    | wt      | 18.5 ± 0.6     | 0.85 ± 0.11 |
| E459A                    | wt      | 35.1 ± 0.1     | 0.84 ± 0.01 |
| T460A                    | wt      | 42.1 ± 3.3     | 0.84 ± 0.03 |
| Q464A                    | No binding | 29.3 ± 5.4     | 0.84 ± 0.07 |

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**Effect of various mutations of GABA<sub>B</sub>-R1a on the binding affinities of GABA and CGP54626A**

Binding experiments were performed using 0.1 nM <sup>125</sup>I-CGP64213 as described under "Experimental Procedures." Displacement curves performed with 11 different concentrations of GABA or CGP54626A were fitted as described under "Experimental Procedures." Concentrations giving rise to 50% inhibition of specific binding (IC<sub>50</sub>) and Hill coefficients (n<sub>H</sub>) were determined. Values are means ± S.E. of at least three experiments performed in triplicates. wt, mutants in which the IC<sub>50</sub> values of GABA and CGP54626A were similar to those of the wild-type receptor; −, mutants in which the affinity of some ligands was decreased (see Fig. 8); +, mutants in which the affinity of agonists was increased.
leucine and glutamate. It is therefore not surprising that Ser-269 of the GABAB receptor does not play a role similar to that of Thr-102 and Thr-188 of LIVBP and mGluR1, respectively.

Mutations That Increase Agonist Affinities—Finally, both mutations S247A (lobe I) and Q312A (lobe II) (blue residues in Fig. 2b) resulted in an increase in agonist affinity (GABA, APPA, and baclofen) by a factor of >3 and in a decrease in antagonist affinity (CGP54626A, CGP64213, and CGP56999A) by a factor of 2 (Fig. 8 and Table II). The changes in affinity were too small to assume that Ser-247 and Gln-312 directly interact with these ligands. How can one explain the opposite effect of these two mutations on the binding affinities of agonists and antagonists? One possible explanation, as detailed below, is that these mutations affect the closure of the LBP-like domain of the GABAB receptor. As mentioned in the Introduction, PBPs such as the histidine-binding protein have been shown to stabilize the closed state. In contrast, mutations in PBPs such as the histidine-binding protein result in a decrease in the affinity constant of a ligand (Kd) as follows: Kd = ([R] + [L])/([R] + [L]) and is therefore equal to Kd = (1 + αKd).

According to the similarity between the GABAB receptor-binding domain and PBPs, the same model can be proposed for the GABAB receptor. If we consider that the closure of the LBP-like domain is a necessary step for receptor activation, the factor α must be lower than 1 for agonists (the agonist stabilizes the closed state). In contrast, α may be lower or equal to 1 in the case of antagonists (they do not favor or even prevent the formation of the closed state). According to the above equation, an increase in Kd due to a mutation in the receptor will result in a decrease in the Kd for an agonist and an increase in Kd for an antagonist, as observed for the S247A and Q312A mutations.

As shown in the three-dimensional model of the open state of the GABAB receptor (Fig. 2a and b), Ser-247 and Gln-312 are located in such a position that they can be in close proximity to the other lobe in the closed state (43). Accordingly, mutation of these residues may affect Kd and therefore have opposite effects on the affinities of agonists and antagonists. In agreement with this proposal, amino acids at comparable positions in PBPs such as the histidine-binding protein have been

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**Fig. 6.** Expression of some mutants of GABAB-R1a. Membranes (1 μg) prepared from mock-transfected HEK-293 cells or cells expressing wild-type (Wt) GABAB-R1a or the S246A, S247A, S270A, S269A, Q312A, or Y470A mutated receptor where subjected to SDS-polyacrylamide gel electrophoresis as described under “Experimental Procedures.” The receptor proteins were detected using a polyclonal antibody directed against the carboxyl-terminal region of the protein. The specific binding of 125I-CGP64213 was determined on the same membranes and is indicated at the top. 0, no specific binding was detected.

**Fig. 7.** Fluorescence immunostaining of wild-type GABAB-R1a and mutated receptors in HEK-293 cells. Cells expressing the wild-type receptor (wt; a), mock-transfected cells (b), and cells expressing the S246A (c) or Y470A (d) mutated receptor were immunostained with the same polyclonal antibody used for Western blotting, followed by a fluorescein-coupled goat anti-rabbit secondary antibody.

**Fig. 8.** Displacement curves for the S269A, S270A, S247A, and Q312A mutants with the agonists GABA, APPA, and baclofen and the antagonists CGP54626A, CGP64213, and CGP56999A. All experiments were carried out with 0.1 nM 125I-CGP64213 displaced by unlabeled ligands at the concentrations indicated on the graph. Closed circles, GABA; open squares, APPA; closed triangles, baclofen; open circles, CGP54626A; closed squares, CGP64213; open triangles, CGP56999A. The dashed lines correspond to the positions of the IC50 values of each drug (represented by the appropriate symbol at the bottom of each graph) obtained with the wild-type receptor. Each curve is the mean of three independent experiments performed in triplicates.

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\[ \frac{[R]}{[L]} = \frac{K_d}{K_a} \]

where \( R \) is the closed state of the protein, \( R \) is the open state, \( K_2 \) is the equilibrium constant between the closed and open states (\( K_2 = [R]/[R] \)), \( K_1 \) is the binding affinity of the ligand (L) on the open state, and \( α \) is the change in the equilibrium constant between \( R \) and \( R \) when the ligand is bound on the protein.
shown to affect the equilibrium constant between the closed and open states (40, 41, 44). It is interesting to note here that Ser-247 aligns with Ser-73 in LBP and Ser-166 in mGluR1. This latter residue in mGluR1 is responsible for the calcium-induced activation of mGluR1 (38).

Conclusion—Taken together, our study shows that the structure of the GABA<sub>B</sub> receptor-binding site is likely to be similar to that of PBPs. Our three-dimensional model predicts that four Cys residues are important for the structural stability of this domain in the GABA<sub>B</sub> receptor and is confirmed by mutations of these residues and by the decrease in the thermostability of the binding site resulting from DTT treatment. In agreement with the three-dimensional model, 14 different mutations do not affect the binding properties of the receptor, and one residue (Ser-246) that is likely to be directly involved in the binding of GABA<sub>B</sub> receptor ligands has been identified. These data support a Venus flytrap model for the GABA<sub>B</sub> receptor activation, as previously proposed for the other family 3 receptors.

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REFERENCES

1. Nakanishi, S. (1992) Science 258, 597–603
2. Conn, P., and Pin, J.-P. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 205–237
3. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, S. C. (1995) Nature 376, 575–580
4. Herrada, G., and Dulac, C. (1997) Cell 90, 763–773
5. Ryba, N., and Tirindelli, R. (1997) Neuron 19, 371–379
6. Matsunami, H., and Buck, L. B. (1997) Cell 90, 775–784
7. O’Hara, P. J., Sheppard, P. O., Thägersen, H., Venezia, D., Haldeman, B. A., McGrane, V., Houamed, K. M., Thomsen, C., Gilbert, T. L., and Mulvihill, E. R. (1993) Neuron 11, 41–52
8. Takahashi, K., Tsuchida, K., Tanabe, Y., Masu, M., and Nakanishi, S. (1993) J. Biol. Chem. 268, 19341–19345
9. Tones, M. A., Bendali, H., Fler, P. J., Knopfle, T., and Kuhn, R. (1995) Neuropeptides 27, 117–120
10. Parmentier, M.-L., Joly, C., Restituito, S., Bockaert, J., Grau, Y., and Pin, J.-P. (1996) Mol. Pharmacol. 53, 778–786
11. Okamoto, T., Sekiyama, N., Otsu, M., Shimada, Y., Sato, A., Nakanishi, S., and Jingami, H. (1998) J. Biol. Chem. 273, 13089–13096
12. Francesconi, A., and Duvoisin, R. M. (1998) J. Biol. Chem. 273, 5615–5624
13. Pin, J.-P., Joly, C., Heinemann, S. F., and Bockaert, J. (1994) EMBO J. 13, 342–348
14. Gomez, J., Joly, C., Kuhn, R., Knopfle, T., Bockaert, J., and Pin, J.-P. (1996) J. Biol. Chem. 271, 2189–2205
15. Mao, B., Pearson, M. R., McCammon, J. A., and Quiocho, F. A. (1982) J. Biol. Chem. 257, 1131–1133
16. Quiocho, F. A. (1990) Philos. Trans. R. Soc. Lond. B Biol. Sci. 336, 341–351
17. Pin, J.-P., and Bockaert, J. (1995) Curr. Opin. Neuro. 5, 342–349
18. Kuryatov, A., Laube, B., Betz, H., and Kuhse, J. (1994) Neuron 12, 1291–1300
19. Stern-Bach, Y., Bittiger, H., Hartley, M., Sheppard, P. O., O’Hara, P. J., and Heinemann, S. F. (1984) Neuron 13, 1345–1357
20. Mano, I., Lamed, Y., and Teichberg, V. I. (1996) J. Biol. Chem. 271, 15299–15302
21. Kuusinen, A., Arvola, M., and Keinanen, K. (1995) EMBO J. 14, 6327–6332
22. Miao, L., Lamed, Y., and Teichberg, V. I. (1996) J. Biol. Chem. 271, 15299–15302
23. Kaufmann, K., Huggel, K., Hein, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, O., Angel, C., Bittiger, H., Froestl, W., and Bittiger, H. (1997) Nature 386, 239–246
24. Froestl, W., Mickel, S. J., Hall, R. G., von Sprecher, G., Struh, D., Baumann, P. A., Brugger, F., Gentsch, C., Jaeckel, J., Olpe, H.-R., Rih, G., Vassout, A., Walmeier, P. C., and Bittiger, H. (1995) J. Mol. Chem. 39, 3297–3312
25. Bittiger, H., Froestl, W., Mickel, S. J., and Olpe, H.-R. (1993) Trends Pharmacol. Sci. 14, 391–394
26. Malitschek, B., Ruegg, D., Heid, J., Kaufmann, K., Bittiger, H., Froestl, W., Bittiger, H., and Ruhn, R. (1998) Mol. Cell. Neurosci. 12, 56–64
27. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
28. Callebaut, I., Labesse, G., Durand, P., Poupon, A., Canard, L., Chemlizer, J., Henrisrat, B., and Morris, J. (1997) Cell Mol. Life Sci. 53, 621–645
29. Luthy, R., Bowie, J. U., and Eisenberg, D. (1992) Nature 356, 83–85
30. Sali, A., and Blundell, T. (1993) J. Mol. Biol. 234, 779–815
31. Gomez, J., Mary, S., Ibraeht, F., Parmentier, M.-L., Restituito, S., Bockaert, J., and Pin, J.-P. (1996) Mol. Pharmacol. 50, 923–930
32. Mary, S., Gomez, J., Prezeau, L., Bockaert, J., and Pin, J.-P. (1998) J. Biol. Chem. 273, 425–432
33. Wiles, A. P., Shaw, G., Bright, J., Perezel, A., Campbell, I. D., and Barlow, P. N. (1997) J. Mol. Biol. 272, 253–265
34. Rank, K. B., and Day, A. J. (1989) Immunol. Today 10, 177–180
35. Hawser, E., Xiao, Y., Shi, Q.-I., Norman, D., Kirtikadze, M., and Barlow, P. N. (1999) FEBS Lett. 432, 103–108
36. Sack, J. S., Saper, M. A., and Quiocho, F. A. (1989) J. Mol. Biol. 206, 171–191
37. Sack, J. S., Tr kissing, S., Teigens, K. H., and Quiocho, F. A. (1989) J. Mol. Biol. 206, 193–207
38. Kubo, Y., Miyahita, T., and Murata, Y. (1998) Science 279, 1722–1725
39. Oh, B.-H., Kang, C.-H., De Bondt, H., Kim, S.-H., Nikonos, K., Jouhi, A. K., and Ames, G. F.-L. (1994) J. Biol. Chem. 269, 4135–4143
40. Wolf, A., Shaw, E. W., Nikonos, K., and Ames, G. F.-L. (1994) J. Biol. Chem. 269, 23051–23058
41. Wolf, A., Shaw, E. W., Oh, B.-H., De Bondt, H., Jouhi, A. K., and Ames, G. F.-L. (1995) J. Biol. Chem. 270, 16097–16106
42. Walmsley, A. R., Shaw, J. G., and Kelly, D. J. (1992) Biochemistry 31, 11175–11181
43. Ohl, G. A., Trakhannov, S. D., Trewhella, J., and Quiocho, F. A. (1993) J. Biol. Chem. 268, 16241–16247
44. Wolf, A., Lee, K. C., Kirsch, J. F., and Ames, G. F.-L. (1996) J. Biol. Chem. 271, 21245–21250