Mapping the Agonist-binding Site of GABA<sub>B</sub> Type 1 Subunit Sheds Light on the Activation Process of GABA<sub>B</sub> Receptors

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The γ-amino-n-butyric acid type B (GABA<sub>B</sub>) receptor is composed of two subunits, GABA<sub>B1</sub> and GABA<sub>B2</sub>, belonging to the family 3 heptahelix receptors. These proteins possess two domains, a seven transmembrane core and an extracellular domain containing the agonist binding site. This binding domain is likely to fold like bacterial periplasmic binding proteins that are constituted of two lobes that close upon ligand binding. Here, using molecular modeling and site-directed mutagenesis, we have identified residues in the GABA<sub>B1</sub> subunit that are critical for agonist binding and activation of the heteromeric receptor. Our data suggest that two residues (Ser<sup>246</sup> and Asp<sup>471</sup>) located within lobe I form H bonds and a salt bridge with carboxylic and amino groups of GABA, respectively, demonstrating the pivotal role of lobe I in agonist binding. Interestingly, our data also suggest that a residue within lobe II (Tyr<sup>266</sup>) interacts with the agonists in a closed form model of the binding domain, and its mutation into Ala converts the agonist baclofen into an antagonist. These data demonstrate the pivotal role played by the GABA<sub>B1</sub> subunit in the activation of the heteromeric GABA<sub>B</sub> receptor and are consistent with the idea that a closed state of the binding domain of family 3 receptors is required for their activation.

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Experimental Procedures
Materials—GABA was obtained from Sigma (L’Isle d’Abeau, France). [3H]myoinositol (23.4 Ci/mol, PerkinElmer, Boston, MA) was used as a radiolabel. Triprolidine hydrochloride was purchased from Novartis Pharma in Basel (30). Serum, culture media, and other solutions used for cell culture were from Life Technologies Inc., Grand Island, NY.

Site-directed Mutagenesis—Single amino acid substitution was carried out by the Quick Change strategy (Stratagene, La Jolla, CA) according to the manufacturer’s instructions using pBSB5 as a template (15). 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. To allow a rapid screening of the mutated clones, 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 corresponding the mutation being cloned was amplified from the corresponding wild-type fragment of pPKR1a (15).

Cell Culture and Expression in HEK 293 Cells—Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies Inc.) supplemented with 10% fetal calf serum, penicillin, and streptomycin. Wild-type and mutated expression constructs were transfected in HEK 293 cells by electroporation as described previously (31). Electroploration was carried out in a total volume of 300 µl with 10 × 10⁶ cells. For membrane preparation, 2 µg of plasmid DNA containing either wild-type or the mutated GABA₆ₐ receptor coding sequences and 8 µg of carrier DNA were used. After electroporation, the cells were plated on polycarbonate-coated dishes.

Ligand Binding Assay—Membranes were prepared from HEK 293 cells expressing wild-type or mutated GABA₆ₐ receptors by brief homogenization in a buffer (Hepes 20 mM, pH 7.4, NaCl 146 mM, KCl 1.8 mM, MgCl₂ 0.5 mM, glucose 0.1%, Hepes 7.4), the incubation mixture was centrifuged at 105,000 x g for 1 h in a Beckman Ti75.5Ti rotor (EMS). The supernatant was used as a source of membrane proteins. Then, the supernatant was incubated at room temperature for 20 min with [3H]myoinositol (23.4 Ci/mol, PerkinElmer Life Sciences). After three washes with Krebs buffer (NaCl 146 mM, KCl 4.2 mM, MgCl₂ 0.5 mM, glucose 0.1%, Heps 20 mM, pH 7.4), the stimulation was conducted for 30 min in Krebs buffer containing 10 mM LiCl and the indicated concentration of agonist. The stimulation was stopped by removal of the incubation medium with perchloric acid (50% v/v) and, after the addition of NaOH (1 M), the radiolabeled substance was solubilized in the acid-precipitable fraction by heating at 80 °C. The labeled membranes were counted in a liquid-scintillation counter.

Determination of Inositol Phosphate (IP) Accumulation—Following the stimulation procedure, cell lysates were prepared as described above. IP accumulation was measured by the radiochemical assay. Membrane impermeant reagent Sulfo-NHS-Biotin (Pierce). Briefly, adherent cells were washed 3 times with ice on borettate buffer (10 mM HEPES, pH 8.8, 140 mM NaCl) and incubated with 0.2 mg/ml sulfo-NHS-biotin in borettate for 30 min. The reaction was stopped by following incubation in borettate buffer with 100 mM NH₄Cl for 10 min on ice. The cells were scraped in lysis buffer (Heps 20 mM, pH 7.4, NaCl 100 mM, EDTA 5 mM), and the membranes were pelleted and solubilized in lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS at a final concentration of 5 mg/ml. The lysate was centrifuged at 1 h at 100,000 × g. The soluble fraction was incubated with streptavidin-coated agarose beads, overnight at 4 °C. Bound proteins were eluted by boiling in Laemmli sample buffer. Phosphorylation and immunoblotting using the polyclonal anti-GABAB1 receptor antibody (33) and an ECL chemiluminescence system (Amersham Pharmacia Biotech). Analysis of wild-type or mutant receptor expression was conducted according to the same protocol.

Homology Modeling of Open and Closed Conformations and Docking of Baclofen—To generate the open form model of the GABA₆₁ receptor extracellular domain, we modified the multiple alignment proposed in previous work (15) in order to take into account the secondary structure prediction of the GABA₆₁ receptor extracellular domain, as determined using the PHD program, and the sequence of the recently cloned GABA₆₂ receptor. Compared with our previous alignment, this resulted in a change from residue 467 to 486 containing helix α₇ of LBPLVBP (15). The alignment of AmiC with LBPLVBP and LBVP was deduced from the structural superposition of each lobe of the closed form of AmiC on the corresponding lobe of the open form of LBVP (16).

The open form model of the GABA₆₁ receptor extracellular domain was generated by the automated homology modeling tool MODELER 5.00 (InsightII version 980, MS) (34) as described previously (15). The closed form model was constructed using the coordinates of the two most closely related structures, the dihydronicotinic acid receptors nicotinic acetylcholine receptor (2zyx) and Pro*Asp*; lobe II, Ser*Thr*Thr* and LBVP (Protein Data Bank code 2lvi: lobe I, Glu*Arg* and Pro*Asp*; lobe II, Ser*Thr*Thr*; lobe III, His*Thr* and Ser*Thr*) crystalline open forms, the coordinates of the two lobes of the open form model of GABA₆₁ receptor extracellular domain (lobe I, Ser*Thr* and Met*Ser*; lobe II, Thr*Thr* and Thr*) and the coordinates of the LBVP closed form model (gift from Drs. F. Quiocio and P. Ogden) were used as templates for the homology modeling. Similarly, AmiC crystalline closed form (Protein Data Bank code 1pea) was used as a template. The resulting closed form model of GABA₆₁ receptor was comparable to that obtained with the closed form model of LBVP. Only the latter has been used for the modeling experiments shown in this paper. The three-dimensional/one-dimensional compatibility scores for these models (35), as determined using the Profiles three-dimensional algorithm using a sequence window of 21 amino acids (InsightII version 980, MS), is always positive and similar to that previously published (15). The global score is in the range of those determined for the refined three-dimensional structures of LBVP, LBP, or AmiC determined from x-ray (data not shown). Most residues of our models were in allowed regions of the Ramachandran’s map.

For “docking” experiments, baclofen was designed with a deprotonated carboxylate group and a protonated amino group. Initially, baclofen (R-enantiomer) was manually docked in the closed form model of GABA₆₁ receptor, the carboxylate moiety closed to the side chain of Ser*Thr* and its γ-amino group facing the side chain of Asp*Thr*. In this position, the chlorophenyl group is pointing toward lobe II and is in the proximity of Tyr*Ser*. In order to suppress the steric hindrances and incompatibilities, the dihydronicotinic-acetylcholine receptor complex was submitted to molecular mechanics calculations using the Discover 3.0 calculation engine with the CFF force field (Insight II version 980, MS). The non-bond cut-off method and the dielectric constant were respectively set up as cell multipole and distance-dependent (ε = r). Initially, energy minimization were performed using a Steepest Descent algorithm (until the maximum derivative was less than 2 kcal/mol/A) followed by a...
conjugated gradient algorithm (until the maximum derivative was less than 0.01 kcal/mol/Å), whereas the Ce trace was tethered with a quadratic potential. Then molecular dynamics was applied to the minimized system at constant volume and temperature (298 K). The integration time step was set up to 1 fs. During the dynamics, the force constant value of the quadratic potential was reduced from 100 to 60, 30, 20, 10, and 0 every 40 ps. A snapshot of the system was saved every 400 fs. Once the system was equilibrated, the coordinates of 20 snapshots were averaged and resubmitted to the previously described minimization protocol with no Cα restraints. Contacts between the ligand and the protein were subsequently analyzed using the web interface of the WHATIF program.

RESULTS

Identification of Putative Structural Regions of the GABAB1 Receptor Involved in Ligand Binding—Several splice variants have been identified for the GABAB1 receptor which all possess an identical ligand binding domain but differ in their extreme N-terminal sequence (24) or in their heptahelix regions (36, 37). All our studies have been performed with the rat GABAB1a receptor (the first Met being residue number 1). A three-dimensional model of the GABAB1 binding domain has been constructed based on the structure of the open forms of LBP and LIVBP (Fig. 1a). According to this three-dimensional model and the known structural elements involved in leucine binding in LIVBP, and in amide binding in AmiC (see Fig. 1), the GABA-binding site would be formed by the following: (a) the loop between strand βC and helix αIII (loop βC-αIII according to the nomenclature of the secondary structural elements of LIVBP proposed by Sack et al. (18), residues 243–250), (b) the N-terminal portion of the loop βD–βE (residues 266–272), and (c) the N termini of helices α1 (residues 184–192) and αIX (residues 463–473) (Fig. 1). We therefore undertook a systematic analysis, by site-directed mutagenesis, of all residues that can interact with ligands within these regions (see Fig. 1b). Each mutant was analyzed for its ability to bind 125I-CGP64213 and to be activated by the agonists GABA and baclofen (Fig. 2).

Occupancy of the GABAB1 Subunit by GABA or Baclofen Plays a Pivotal Role in the Activation of the Heteromeric Receptor—We have previously reported that several mutations of the GABAB1 subunit either increase or decrease the potency of GABA and baclofen in displacing 125I-CGP64213 bound to the GABAB1 subunit expressed alone (see Table I). The effect of these mutations, as well as additional ones generated during this study, on the potency of GABA and baclofen at the heteromeric GABAβ receptor was analyzed after co-expression of the GABAB1 subunit, indicating a correct expression, folding, and Activity—

The mutation of a residue interacting with GABA in the GABAB1 subunit is expected 1) to either largely decrease the potency of GABA in displacing 125I-CGP64213 binding or to suppress 125I-CGP64213 binding and 2) to largely decrease the potency of agonists in activating the receptor. Within the loops βC-αIII and βD-βE, the mutations of Ser246, Ser265, and Tyr266 were found to largely decrease 125I-CGP64213 binding (Fig. 4), but only the mutation of Ser246 affected the potency of GABA and baclofen at the GABAB1 receptor (Fig. 1b and Table I). As shown in Fig. 4, 125I-CGP64213 did not bind to the S246A mutant although correctly expressed in HEK 293 cells. Both GABA and baclofen were able to activate the heteromeric receptor containing the S246A mutated GABAB1 subunit, indicating a correct expression, folding,
and dimerization of the receptor. However, the EC$_{50}$ values of both agonists were increased by a factor of 1000 compared with those determined with the wild-type receptor (Fig. 5). Interestingly, Ser$_{246}$ aligns with Ser$_{79}$ of LIVBP which interacts with ligands (Fig. 1b) (18) thus suggesting that the hydroxyl group of Ser$_{246}$ directly interacts with the agonists GABA and baclofen. In agreement with this proposal, mutation of this residue into the more bulky Asn residue, or into Pro which may affect the topology of this loop, suppressed both 125$I$-CGP64213 binding and agonist activation of the receptor (Table I and Fig. 5). Furthermore, even the replacement of Ser$_{246}$ by a Thr residue resulted in a large decrease in agonist potency (Table I and Fig. 5), suggesting that the orientation of the hydroxyl group is critical for a high agonist potency. All these latter mutants were found to be correctly expressed and targeted to the plasma membrane when co-expressed with the wild-type GABA$_B$2 subunit (data not shown).

**Exploring Helix I and IX, Asp$_{471}$ is a Critical Residue for Agonist Action**—We applied the same strategy to identify residues possibly interacting with agonists within helices I and IX. The mutations of Phe$_{463}$, Tyr$_{470}$, and Asp$_{471}$ were found to largely decrease 125$I$-CGP64213 binding (Fig. 4), but only the mutation of Asp$_{471}$ affected the potency of GABA and baclofen to activate the GABA$_B$ receptor (Fig. 1b and Table I). As shown in Fig. 4, no 125$I$-CGP64213 binding could be measured on membranes expressing the Asp$_{471}$ mutant receptor. Moreover, co-expression of the mutant D471A with the wild-type GABA$_B$2 receptor did not lead to a functional response upon application of GABA or baclofen (Fig. 6, a and b). Western blots clearly show that the lack of function of this mutant receptor was not due to a lack of expression (Fig. 3b). Moreover, the mutant D471A was correctly inserted in the plasma membrane when co-expressed with the GABA$_B$2 subunit (Fig. 6c), as shown by the large amount of biotinylated subunits after exposure of the intact cells to the non-permeant protein reagent, sulfo-NHS-biotin. Taken together, these data reveal that Asp$_{471}$ is a critical residue for agonist binding in GABA$_B$1 receptors. In agreement with this proposal, the conservative mutation of this Asp$_{471}$ residue into Glu was sufficient to dramatically reduce 125$I$-CGP64213 binding. However, a significant increase in IP formation could be detected upon application of either GABA or baclofen to cells co-expressing D471E and the wild-type GABA$_B$2 receptor, showing a very partial recovery of the agonist action at this mutant receptor (Fig. 6). This partial recovery is likely to have been underestimated since the expression level of the D471E mutant was lower than that of the wild-type or D471A mutant (Fig. 4b).

**Tyr$_{366}$ in Lobe II Decreased GABA and Baclofen Affinity and Converts Baclofen into an Antagonist**—In PBPs, or related proteins, which have been crystallized in a closed form complexed with a ligand, residues from both lobes contact the ligand (16, 19, 39–41). We therefore examined the role of residues within lobe II in ligand binding and agonist activity. We focused our efforts on residues within the loops βF–αV and βH–αVII because they both face the putative GABA-binding site, and a residue interacting with amide in AmIC is located within one of these loops (Fig. 1a and Fig. 8, a and c). None of the alanine substitutions generated in these loops prevent 125$I$-CGP64213 binding (Fig. 1b, Table I, and Fig. 3a). The IC$_{50}$ values of both GABA and baclofen for displacement of 125$I$-CGP64213 binding were similar to those of the wild-type receptor for all these mutants except Y366A. For this mutant, the IC$_{50}$ for values of these two agonists were increased by a factor 50–100 (Table I and Fig. 7a), whereas displacement by cold CGP64213 was not affected (1.1 ± 0.1 nM for the wild-type and 1.0 ± 0.4 nM for Y366A, n = 3). Although GABA and baclofen displayed a similar affinity for this mutant (Fig. 7a), the Y366A mutated subunit formed a functional receptor activated by GABA but not by baclofen (Fig. 7b). In cells co-expressing this receptor and GABA$_B$2, GABA stimulated IP formation by a factor 5–10, as observed with the wild-type receptor, indicating a correct expression, targeting to the plasma membrane, and association with the wild-type GABA$_B$2 subunit. However, no significant formation of IP could be measured on this mutant receptor with baclofen (Fig. 7b). Accordingly, if GABA and baclofen bind to the same site in GABA$_B$1, baclofen is expected to act as an antagonist at this mutant receptor (or as a very partial agonist). Indeed, as shown in Fig. 7c, 5 μM baclofen was found to inhibit the action of GABA at this mutant receptor in a competitive manner. Thus, the mutation Y366A converts baclofen into a competitive antagonist.

**Molecular Modeling of the GABA$_B$1 Extracellular Domain in a Closed Form and Docking of Baclofen**—In order to rationalize the effect of the mutations described above, the positions of the mutated residues were visualized in the three-dimensional model of the extracellular domain of the GABA$_B$1 receptor described above (Fig. 1a). As shown in Fig. 8, a and c, Ser$_{246}$ and Asp$_{471}$ are correctly located to interact with GABA in lobe I. The distance between the hydroxyl and carboxylic groups of these two residues (10 Å) is compatible with this possibility; however, the side chain of Tyr$_{366}$ in loop βH–αVII of lobe II, although facing the putative GABA-binding pocket in lobe I, is located more than 15 Å from these residues. As such, this open form model could not easily explain the binding and functional properties of the Y366A mutant.

We therefore undertook the construction of a three-dimensional model for a closed form of the GABA$_B$1 binding domain using the coordinates of the three-dimensional model of the GABA$_B$2 receptor described above (Fig. 1a). As shown in Fig. 8, a and c, Ser$_{246}$ and Asp$_{471}$ are correctly located to interact with GABA in lobe I. The distance between the hydroxyl and carboxylic groups of these two residues (10 Å) is compatible with this possibility; however, the side chain of Tyr$_{366}$ in loop βH–αVII of lobe II, although facing the putative GABA-binding pocket in lobe I, is located more than 15 Å from these residues. As such, this open form model could not easily explain the binding and functional properties of the Y366A mutant.

In this model, the carboxyl group of baclofen forms H bonds with the hydroxyl group of Ser$_{246}$ (from
Agonist Binding Pocket of the GABAB1 Subunit

TABLE I

Effect of various mutations in GABA_B1 receptor on the affinity of both GABA and baclofen as determined by displacement of [125I]-CGP64213 binding (IC_{50} in mM) and receptor activation (EC_{50} in mM).

| Mutation, WT | CGP binding IC_{50} (mM) | IP assay EC_{50} (mM) | Baclofen |
|--------------|---------------------------|-----------------------|--------|
|              |                           |                       | GABA   |
| Lobe-I       |                           |                       |        |
| Loops βC-αIII and βD-βE | S246A | NB* | >1000 | NB* |
|               | S246P | NB | NE | NB |
|               | S246T | NB | >1000 | NB |
|               | S246N | NB | NE | NB |
|               | S247A | 5.32 ± 0.88* | 0.37 ± 0.09b | 11.3 ± 0.9b | 0.38 ± 0.04b |
|               | T250A | 36.4 ± 12.8* |                |            |            |
|               | S265A | NB | 1.9 ± 0.4 | NB |
|               | Y266F | NB | 3.9 ± 1.3 | NB |
|               | S268A | 27.8 ± 3.4* | 1.02 ± 0.15b | 43.0 ± 4.1b | 0.91 ± 0.41b |
|               | S269A | 238 ± 62* | 14.9 ± 0.8 | 28.8 ± 5.4 | 1.78 ± 0.22 |
|               | S270A | 406 ± 26* | 24.5 ± 8.1 | 322 ± 1 | 51 ± 11 |
| Helices αI and αIX | C187A | 36.8 ± 3.6* | 1.8 ± 0.3 |        |
|               | E192A | 22.3 ± 6.6 |        |        |
|               | E458A | 16.5 ± 0.8* |        |        |
|               | E459A | 35.0 ± 0.1* |        |        |
|               | T460A | 421 ± 4.7* |        |        |
|               | F463A | NB | 3.23 ± 0.67 | NB |
|               | Q464A | 29.3 ± 7.6* |        |        |
|               | E465A | 63 ± 28 | 114 ± 30 | 78.0 ± 5.5 |
|               | Y470A | NB* | 1.74 ± 0.69 | NB |
|               | Y470F | NB | 0.96 ± 0.47 | NB |
|               | D471A | NB | NE | NE |
|               | D471E | NB | NE | NE |
| Lobe-II       |                           |                       |        |
| Loop βF-αVI  | Q312A | 4.1 ± 0.9* | 0.51 ± 0.21 | 8.0 ± 1.4a | 1.04 ± 0.16 |
|               | Q313A | 13.4 ± 0.8* |        |        |
|               | T314A | 9.9 ± 3.6* |        |        |
|               | T315A | 43.6 ± 15* |        |        |
|               | E316A | 61.1 ± 17.3* |        |        |
| Loop βH-αVII | F365A | 17.6 ± 0.1 | 8.2 ± 6.1 | 30.8 ± 4.5 | 2.6 ± 0.7 |
|               | Y366A | 3420 ± 522 | 97.1 ± 5.2 | 1559 ± 28 | 28.0 ± 0.8 |
|               | E367A | 35.4 ± 8.2 | 6.35 ± 4.25 | 14.9 ± 3.2 |        |

* Values are from Ref. 15.

a Values are from Ref. 43.

lobo I) and those of Tyr^{366} (from lobe II) (distance O–O <3 Å), and its amino group forms a salt bridge with the carboxylate of Asp^{471} (distance N–O <3 Å) (Fig. 8d). This latter interaction is stabilized by the aromatic ring of Tyr^{366} and an H bond with the backbone (carbonyl of Pro^{467}). The chlorophenyl group of baclofen was found to fit into a pocket lined by Tyr^{366} and Trp^{394} from lobe II and Leu^{265} from lobe I.

DISCUSSION

Our mutagenesis and modeling study has identified a set of residues in the GABA_B1 subunit that are critical for agonist binding and activation of the heteromeric GABA_B receptor. Our data suggest that two residues in lobe I, Ser^{246} and Asp^{471}, and one residue in lobe II interact with GABA and baclofen. Interestingly, the mutation of Tyr^{366} into Ala not only decreases GABA and baclofen affinity but also converts baclofen into an antagonist.

We have previously reported that Ser^{246} is critical for [125I]-CGP64213 binding at GABA_B1a receptors (15). By using a functional assay, we show here that this residue plays a critical role in both GABA and baclofen action, their apparent affinity being decreased by a factor 1000 when Ser^{246} is mutated into Ala or even Thr. In accordance with the possible importance of this residue, a search within the Drosophila and Caenorhabdito
**tis elegans** genomes for GABAB1-like receptors shows that this residue is conserved through evolution (43). Moreover, this residue aligns with Ser 79 of LBP and LIVBP, the hydroxyl group of which forms H bonds with the $\alpha$-carboxylic group of leucine (18) (Fig. 9). Ser246 also aligns with Ser85 of AmiC which interacts with acetamide (Fig. 1) (16). This Ser residue is also conserved in all mGlu receptors and has been proposed to form H bonds with the $\alpha$-carboxylic group of glutamate (Fig. 9) (13, 14). Accordingly we propose that Ser246 of GABAB1a also contacts via an H bond the carboxylic group of GABA and baclofen. This proposal is consistent with both our modeling studies and the identification of Asp471 as possibly forming an ionic interaction with the amino group of these GABAB agonists (see below).

In addition to this Ser residue, the hydroxyl group of a Thr102...
Indeed we previously reported that Ser\textsuperscript{269} was involved in the effect of Ca\textsuperscript{2+} on this receptor subtype (43).

Among all other mutated residues from lobe I, only Asp\textsuperscript{471} was found to play a critical role in the action of GABA and baclofen. Mutation of this residue into Ala suppresses antagonist binding as well as GABA- and baclofen-induced activation of the receptor. This total loss of function did not result from a lack of expression of this mutant protein. Moreover, the D471A mutant was found to be inserted correctly into the plasma membrane when co-expressed with the GABAB\textsubscript{2} receptor.

Since GABAB\textsubscript{2} is required for the plasma membrane localization of the GABAB\textsubscript{1} receptors (28, 44), these data also indicate that the mutation of Asp\textsuperscript{471} does not affect the formation of the heterodimer. In agreement with such an important role of Asp\textsuperscript{471} in agonist binding, this residue is conserved in Drosophila and C. elegans GABAB\textsubscript{1}-like receptors (data not shown).

Moreover, our modeling studies revealed that the position of this residue allows its side chain to interact correctly with the amino group of GABA. Indeed, this residue is located at the bottom of the binding pocket and points toward a hydrophobic environment that lacks any polar residue in LIVBP and constitutes the binding pocket for the side chain of leucine (18). In this region in mGlu receptors, the side chain of an Arg (Arg\textsubscript{78}) points toward the binding pocket (Fig. 9) and has been proposed to interact with the γ-carboxyl group of glutamate in mGlu\textsubscript{1a} (14) and mGlu\textsubscript{1b} (45) receptors.

Taken together, our data show that leucine, glutamate, and GABA interact in a similar binding pocket in lobe I of LIVBP, mGlu, and GABAB\textsubscript{1} receptors, respectively (Fig. 9). They also highlight how the same binding pocket in lobe I has evolved within these LIVBP-like proteins to specifically recognize different but related molecules (Fig. 9).

Our study also identified 4 residues within lobe I, Ser\textsuperscript{265}, Tyr\textsuperscript{366}, Phe\textsuperscript{463}, and Tyr\textsuperscript{470}, the mutation of which dramatically decreases \textsuperscript{125}I-CGP64213 binding but does not change the properties and apparent affinity of both GABA and baclofen. Although the lack of significant binding to the S265A, Y266F, and Y470F mutants may result from their low expression level, this cannot be the case for the F463A and Y470A mutants (data not shown and Fig. 4). One may therefore propose that these two residues are required for the binding of this large GABAB antagonist that possesses two aromatic moieties (Fig. 2). However, additional experiments are required to characterize better the role of these residues in GABAB antagonists binding and action.

The data discussed above demonstrate the pivotal role of lobe I in ligand binding to the GABAB\textsubscript{1} receptor and in the agonist activation of the heteromeric receptor. However, our data also revealed that the mutation of Tyr\textsuperscript{366} from lobe II largely decreases the affinity of both GABA and baclofen. This mutant subunit is correctly expressed and targeted to the plasma membrane when associated with the wild-type GABAB\textsubscript{2} subunit, as shown by its activation by GABA which is to an extent similar to that obtained with the wild-type receptor. This large decrease in affinity therefore suggests that Tyr\textsuperscript{366} has either direct or indirect contact with these agonists. Such an interaction does not appear to be possible in our three-dimensional model for an open form of the GABAB\textsubscript{1} binding domain. However, in a closed form model, the hydroxophenyl of Tyr\textsuperscript{366} points toward the GABA-binding site in lobe I, and its oxygen forms an H bond with the carboxyl group of baclofen. Further experiments are required to validate this possibility. However, these data already show that residues in lobe II affect the affinity of agonists, in agreement with the observation that residues from both lobes contact the ligands in many PB-like proteins (16, 19, 39–41). Interestingly, this mutation also prevents baclofen from activating the heteromeric receptor and...
converts it into a competitive antagonist. In our three-dimension-
ational model, the chlorophenyl group of baclofen points toward
a pocket lined by the hydroxyphenyl group of Tyr366. It is
therefore possible that the replacement of this Tyr by an Ala
changes the position of surrounding residues such that the
chlorophenyl group in no longer accepted in a closed/active
conformation of the binding domain, therefore converting ba-
clofen into an antagonist. Although additional work is required
for the correct insertion of GABA1 in the plasma membrane (28, 44), other roles in receptor functioning
have not yet been characterized. Our data show a correlation
between the affinity of both GABA and baclofen measured in
binding studies on wild-type or mutant GABA1 receptors, and
their EC50 values were measured in a functional assay after
co-expression with GABA2 receptor. Moreover, the mutation of
Asp471, a residue that is likely to play a critical role in GABA
binding according to our modeling studies, suppresses the func-
tion of the heteromer. Finally, a single mutation in the GABA1
subunit is sufficient to convert the selective heteromeric GABA
receptor agonist baclofen into an antagonist. Taken together, and
in agreement with recent additional data (47), these results add
further strength to the proposal that GABA binding on the
GABA1 subunit is required and is the main determinant for the
activation of the heteromer.

The GABA1 receptor requires the presence of two subunits,
GABA1 and GABA2, for its full activity. Although one role of
GABA2 is to allow the correct insertion of GABA1 in the
plasma membrane (28, 44), other roles in receptor functioning
have not yet been characterized. Our data show a correlation
between the affinity of both GABA and baclofen measured in
binding studies on wild-type or mutant GABA1 receptors, and
their EC50 values were measured in a functional assay after
co-expression with GABA2 receptor. Moreover, the mutation of
Asp471, a residue that is likely to play a critical role in GABA
binding according to our modeling studies, suppresses the func-
tion of the heteromer. Finally, a single mutation in the GABA1
subunit is sufficient to convert the selective heteromeric GABA
receptor agonist baclofen into an antagonist. Taken together, and
in agreement with recent additional data (47), these results add
further strength to the proposal that GABA binding on the
GABA1 subunit is required and is the main determinant for the
activation of the heteromer.

Comparison of the putative binding
pocket of GABA1 reveals that although Asp246 of
GABA1 is conserved in GABA2, Tyr366 and Ser246 are replaced
by Asp and Pro in GABA2, respectively (see Fig. 1 b). The mu-
tation of Ser246 into Pro in GABA1 is sufficient to suppress the
action of GABA on the heteromer. However, GABA and baclofen
have been shown to activate occasionally the GABA2 receptor

![Fig. 8. Ribbon views of the three-
dimensional models of putative open
(a) and closed (b) conformations of
the GABA1 receptor binding do-
main (the insertion comprising resi-
dues 488–502 is not shown in a). Close
view of the cleft that separates the two
lobes in the open (c) and closed (d) con-
formation. c and d, the residues identified as
being important for GABA and baclofen
action (Ser246, Asp471, and Tyr366) are
shown. Baclofen docked on the closed con-
formation is shown in d. A closer view of
baclofen in its binding pocket in the model
of the closed conformation of the GABA1
receptor, showing the ionic interaction be-
tween the amino group of baclofen with
the side chain of Asp471, and the H bonds
of the carboxylic group of baclofen with
the side chain of Ser246.

![Fig. 9. Differences and similarities in the ligand-binding
pocket of LIVBP, GABA1 receptor, and mGlu1 receptor.](image-url)
expressed alone (25, 48), suggesting that the GABAB2 receptor is able to bind these two agonists. Our data indicate that if GABA and baclofen bind to the GABAB2 subunit, they have to do it in a different way from GABA1.

In conclusion, our data strongly support the importance of lobe I for binding properties of agonists in family 3 heptahelix receptors. They also reveal that residues in lobe II can be critical for the agonist property of family 3 receptor ligands. This is consistent with the proposal that the closure of the two lobes (the so-called Venus fly-trap mechanism of action (19)) constitutes a key step in family 3 receptor activation.

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