GABA<sub>A</sub> receptors can be modulated by benzodiazepines, although these compounds do not directly activate or inhibit the receptors. The prototypic benzodiazepine, diazepam, potentiates responses to GABA in GABA<sub>A</sub> receptors that contain a γ subunit. Here we have used mutagenesis, radioligand binding, voltage clamp electrophysiology, and homology modeling to probe the role of the F-loop residues Asp<sup>192</sup>–Arg<sup>197</sup> in the GABA<sub>A</sub> receptor γ<sub>2</sub> subunit in diazepam potentiation of the GABA response. Substitution of all of these residues with Ala and/or a residue with similar chemical properties to the wild type residue decreased the level of diazepam potentiation, and one mutation (D192A) resulted in its complete ablation. None of the mutations changed the GABA EC<sub>50</sub> or the [<sup>3</sup>H]flumazenil binding affinity, suggesting they do not affect GABA or benzodiazepine binding characteristics; we therefore propose that they are involved in the diazepam-mediated conformational change that results in an increased response to GABA. Homology models of the receptor binding pocket in agonist-bound and unbound states suggest that the F-loop is flexible and has different orientations in the two states. Considering our data in relation to these models, we find that the F-loop residues could contribute to hydrogen bond networks and hydrophobic interactions with neighboring residues that change during receptor activation.

The GABA<sub>A</sub> receptor is a member of the Cys-loop ligand-gated ion channel family. Each receptor has a pentameric structure, often comprising 2α<sub>1</sub>, 2β<sub>2</sub>, and a γ<sub>2</sub> subunit, although many other subunit combinations are possible. GABA is the major inhibitory neurotransmitter in the adult mammalian brain, and it is therefore not surprising that its malfunction is implicated in a number of disorders in which normal brain inhibition is disrupted, including epilepsy, insomnia, and anxiety-related conditions. Many drugs used to treat these disorders target GABA<sub>A</sub> receptors, including anesthetics, barbiturates, and benzodiazepines (1).

Benzodiazepine agonists potentiate GABA<sub>A</sub> receptor-mediated GABA currents, whereas inverse agonists inhibit GABA responses, and antagonists competitively block the action of other benzodiazepines. Diazepam is the prototypical benzodiazepine agonist and potentiates the GABA response by increasing the channel opening frequency of the receptor (1). More recently, Rusch and Forman (2) used constitutively partially open channels to show that the classical benzodiazepine agonists diazepam and midazolam, and an inverse agonist (FG7142), altered currents through the receptors in the absence of GABA, supporting previous evidence that benzodiazepines contribute directly to channel gating by altering the open-close equilibrium of the receptor (3, 4). Numerous binding studies have shown that GABA can stimulate benzodiazepine binding and that in turn benzodiazepines can alter GABA binding, confirming the allosteric links between GABA and benzodiazepine-binding sites (1), and recently benzodiazepine-induced structural changes in the receptor have been identified (5). However, while details of the residues involved in the benzodiazepine binding area have been identified (e.g. see Ref. 6), the molecular mechanism by which benzodiazepines mediate their effects is not yet clear.

Some of the machinery involved in coupling ligand binding to channel gating in the Cys-loop receptors is located at the extracellular/transmembrane interface (7–11), and coupling of benzodiazepine agonist binding to allosteric modulation also appears to involve residues at this interface (12). The precise regions of the protein that may be involved in this modulation, however, remain unclear, although the flexible F-loop lies within a few angstroms of both the binding site and the extracellular/transmembrane interface and is therefore a good candidate. To probe the role of the F-loop, here we explore the role of six amino acids that constitute this loop in the γ<sub>2</sub> subunit; the data show that these residues play an important role in coupling binding of benzodiazepines to their allosteric effects.

**EXPERIMENTAL PROCEDURES**

**Materials**—All cell culture reagents were obtained from Invitrogen, except fetal calf serum, which was from Labtech International (Ringmer, UK). [<sup>3</sup>H]Flumazenil (87.0 Ci/mmol) was from PerkinElmer Life Sciences. All other reagents were from Sigma unless otherwise stated.

**Cell Culture**—Human embryonic kidney (HEK) 293 cells were maintained on 90-mm tissue culture plates at 37 °C and 7% CO<sub>2</sub> in a humidified atmosphere. They were cultured in Dulbecco’s modified Eagle’s medium/Nutrient Mix F-12 (1:1).

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3 The abbreviations used are: GABA<sub>A</sub>, γ-aminobutyric acid, type A; GABA<sub>B</sub>, γ-aminobutyric acid; ACh, acetylcholine; AChBP, acetylcholine-binding protein; 5-HT, 5-hydroxytryptamine; HEK, human embryonic kidney.
with GlutaMAX™ I and 10% fetal calf serum. For radioligand binding studies, cells in 90-mm dishes were transfected using calcium phosphate precipitation at 80–90% confluency and routinely incubated for 3 days before use (13).

Site-directed Mutagenesis—Mutagenesis reactions were performed using the method of Kunkel (14) using human GABA<sub>A</sub> receptor subunit DNA (the kind gift of Keith Wafford, Merck Sharp and Dohme) in pcDNA3.1 (Invitrogen). Oligonucleotide primers were designed according to the recommendations of Sambrook et al. (15) and some suggestions of the Primer Generator (16). A silent restriction site was incorporated into each primer to assist rapid identification.

Radioligand Binding—This was undertaken as described previously in HEK 293 cells, which provide an established and robust method of studying ligand binding (17, 18). Briefly, transfected HEK 293 cells were washed twice with phosphate-buffered saline and then scraped into 1 ml of ice-cold HEPES buffer (10 mM, pH 7.4) with proteinase inhibitors and frozen at −20 °C. After thawing, they were washed twice with HEPES buffer and resuspended, and 50 µg of cell membranes was incubated in 0.5 ml of HEPES buffer containing the benzodiazepine antagonist [3H]flumazenil. Saturation binding (eight point) assays were performed on at least three separate plates of transfected cells for each mutant using 0.1–40 nM [3H]flumazenil. Nonspecific binding was determined using 1 µM [3H]flumazenil. Non-specific binding was determined using 1 µM diazepam. Reactions were incubated for 1 h at 4 °C and terminated by rapid vacuum filtration using a Brandel cell harvester onto GF/B filters pre-soaked in 0.3% polyethyleneimine followed by two rapid washes with 4 ml of ice-cold HEPES buffer. Radioactivity was determined by scintillation counting (Beckman LS6000sc). Protein concentration was estimated using the Bio-Rad protein assay with bovine serum albumin standards. Data were analyzed by iterative curve fitting (Prism, GraphPad Software, San Diego) according to the equation, 

\[ B = (B_{\text{max}} \times [L]) / (K_d + [L]) \]

where \( B \) is bound radioligand; \( B_{\text{max}} \) is maximum binding at equilibrium; \( K_d \) is the equilibrium dissociation constant; \( [L] \) is the free concentration of radioligand. Values are presented as mean ± S.E. Statistical analysis was performed using analysis of variance in conjunction with a Dunnett’s post test.

**Xenopus Oocyte Isolation and mRNA Injection**—Harvested stage V–VI *Xenopus laevis* oocytes were prepared as described previously. Oocytes, which are well suited to the study of ligand-gated ion channels, especially those constituted from multiple subunits, were injected with 1:1:10 α:β:γ mRNA, generated by *in vitro* transcription using the mMESSAGE mMACHINE kit (Ambion) from DNA subcloned into pGEMHE (19). Oocytes were maintained in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES, pH 7.5) containing gentamicin (0.1 mg/ml), theophylline (0.12 mg/ml), and tetracycline (50 µg/ml). Electrophysiological measurements were routinely performed ~48 h post-injection, which yielded optimal diazepam potentiation.

**Electrophysiology**—Using a two electrode voltage clamp, *Xenopus* oocytes were routinely clamped at −60 mV using an OC-725 amplifier (Warner Instruments), and currents were digitized by the Digitizer132A interface (Axon Instruments). Micro-electrodes were constructed from borosilicate glass (GC120TF-10, Harvard Apparatus, Edenbridge, Kent, UK) using a two-stage horizontal pull (Sutter P-87, Novato, CA) and filled with 3 M KCl. Pipette resistances ranged from 0.3 to 3 mehmohms. Oocytes were perfused with ND96 buffer at a rate of 9 ml/min. Drug application was at 3–5 min intervals via a simple gravity-fed system calibrated to run at the same rate. For diazepam potentiation experiments, an EC<sub>10</sub> [GABA] was applied followed by EC<sub>10</sub> [GABA] with diazepam and then another EC<sub>10</sub> [GABA]. Responses were recorded using WinWCP freeware. Statistical comparison of EC<sub>50</sub> values was performed using a one-way analysis of variance with the Dunnett’s post test using Prism software.

**Modeling**—This was performed as described previously (20, 21). Briefly, the GABA<sub>A</sub> receptor subunit sequences were aligned to the AChBP sequence using FUGUE (22). The three-dimensional models of the extracellular region of the GABA<sub>A</sub> receptor were then built using MODELLER version 7.2 (23) by threading the aligned sequence over the crystal structure of AChBP in the unbound and agonist-bound states (PDB codes

### Table 1

**Parameters for wild type and mutant receptors**

| Receptor | logEC<sub>50</sub> ± S.E. | EC<sub>50</sub> µM | [3H]Flumazenil K<sub>d</sub> ± S.E. | Diazepam logEC<sub>50</sub> ± S.E. |
|----------|-----------------|----------------|----------------|-----------------|
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> | −4.49 ± 0.03 | 30 | 1.5 | 1.2 ± 0.1 | −6.62 ± 0.14 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>R197K | −4.83 ± 0.05 | 16 | 1.7 | 2.6 ± 0.5 | ND |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>R197E | −4.54 ± 0.03 | 28 | 2.0 | 1.8 ± 0.3 | −6.85 ± 0.65 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>R197N | −4.40 ± 0.06 | 40 | 1.1 | 0.8 ± 0.2 | ND |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>R197A | −4.48 ± 0.10 | 32 | 1.0 | 1.5 ± 0.7 | −7.22 ± 0.54 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>W196F | −4.71 ± 0.04 | 20 | 1.5 | 1.6 ± 0.3 | −7.22 ± 0.22 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>S195T | −4.68 ± 0.05 | 21 | 1.0 | 0.8 ± 0.1 | −6.56 ± 0.17 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>S195A | −4.45 ± 0.07 | 35 | 1.3 | 1.3 ± 0.4 | −7.44 ± 0.22 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>R194N | −4.62 ± 0.06 | 24 | 1.2 | 0.8 ± 0.1 | ND |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>R194K | −4.57 ± 0.02 | 27 | 1.2 | 1.2 ± 0.1 | ND |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>R194A | −4.43 ± 0.04 | 37 | 0.9 | 0.8 ± 0.1 | −6.84 ± 0.16 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>R194V | −4.57 ± 0.03 | 23 | 1.3 | 0.8 ± 0.1 | −7.44 ± 0.31 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>D192E | −4.73 ± 0.04 | 18 | 1.2 | 1.3 ± 0.4 | −6.93 ± 0.12 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>D192A | −4.43 ± 0.03 | 37 | 1.7 | 1.2 ± 0.1 | −7.36 ± 0.27 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>D192F | −4.63 ± 0.03 | 23 | 1.2 | 1.6 ± 0.4 | −6.81 ± 0.31 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>D192V | −4.76 ± 0.08 | 17 | 1.1 | 1.5 ± 0.1 | −6.65 ± 0.49 |
| α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>D192N | −4.54 ± 0.02 | 29 | 1.4 | 1.0 ± 0.1 | −6.79 ± 0.49 |

**FIGURE 1. Typical responses of wild type and Asp<sup>192</sup> GABA<sub>A</sub> mutant receptors to EC<sub>50</sub> [GABA] and EC<sub>10</sub> [GABA] with diazepam (1 µM).**

EC<sub>50</sub> GABA | EC<sub>50</sub> GABA + 1µM Diazepam
---|---
Wildtype | ![Wildtype](image1.png)
D192A | ![D192A](image2.png)
D192E | ![D192E](image3.png)
D192N | ![D192N](image4.png)
The most energetically favorable models were selected from the MODELLER output file using the Modelist program and checked for violations of the Ramachandran plot using RAMPAGE.

RESULTS

F-loop Mutations Do Not Affect GABA EC$_{50}$ Values, Diazepam EC$_{50}$ Values, or [3H]Flumazenil Kd Values—Wild type GABA$_A$ receptors ($\alpha_1\beta_2\gamma_2$) had a GABA EC$_{50}$ of 30 nM and a Hill coefficient of 1.5 (Table 1). Amino acid substitutions at the $\gamma_2$ F-loop resulted in no significant changes to GABA EC$_{50}$ values or Hill coefficients compared with wild type receptors. Amino acid substitutions also revealed no significant changes in [3H]flumazenil binding affinity at any of the six residues mutated (Table 1). $B_{\text{max}}$ values varied considerably (50–800 fmol/mg protein), probably because of differences in transfection efficiencies, but none were significantly different from wild type.

F-loop Mutations Modify the Potentiation of GABA Responses by Diazepam—Responses from wild type GABA$_A$ receptors elicited with EC$_{10}$ [GABA] increased with increasing [diazepam] from 1 nM to 1 M, with an EC$_{50}$ of 240 nM (Table 1 and Fig. 1). Responses above this concentration decreased as reported previously (24). EC$_{50}$ values for all F-loop mutant receptors tested were not significantly different to wild type, except for D192A (Table 1).

Asp$^{192}$ replacement by Ala completely ablated the response to diazepam, and replacement with the chemically similar Glu reduced the maximal potentiation to 20% of a wild type response (Fig. 2A and Table 2). This suggests a specific and critical role of this residue. Replacement of Thr$^{193}$ with either Ala or Ser reduced the maximal diazepam potentiation to ~50% that of wild type (Fig. 2B). Diazepam enhancement curves with both these mutant receptors were very similar, indicating that the hydroxyl group of Thr is probably not critical here, as replacement with Ser, which also has such a group, was no more efficacious than Ala.

Replacement of Arg$^{194}$ with Ala resulted in no change to the diazepam potentiation curves. However, replacement of this residue with Lys, which has similar chemical properties to Arg, decreased potentiation to ~20% that of wild type (Fig. 2C). Because this was an unexpected result, we also explored substitution with Asn and Gln. Potentiation curves from both these mutant receptors were very similar to Lys, although the maximum potentiation produced by Gln was slightly higher. These data suggest that Arg has a specific interaction here, although it can be replaced efficiently by a much smaller residue.

Replacement of Ser$^{193}$ with either Ala or Thr reduced the maximal diazepam potentiation to ~70 and ~20%, respectively. This suggests that the size of the residue located here may be more critical than its chemical properties, as replacement with Thr, which has a hydroxyl group like Ser, was more inhibitory than Ala, and even ablated diazepam potentiation below 300 nM (Fig. 2D).

Replacement of Trp$^{196}$ with Phe had no effect on the level of diazepam potentiation, but substitution of this residue by Tyr decreased the maximum potentiation to ~15% of WT. The effect of Ala substitution was intermediate, reducing the maximum potentiation to ~70% of WT (Fig. 2E).
Diazepam Acts via the GABA<sub>A</sub> Receptor F-loop

Replacing Arg<sup>197</sup> with either Ala or Lys reduced the maximal diazepam potentiation to ~30% that of wild type (Fig. 2F). Diazepam enhancement curves were very similar for both these mutant receptors, indicating that a positive charge is not critical here, and that Arg has a specific interaction that cannot be replaced by a residue with similar chemical properties.

Molecular Modeling—Homology modeling and ligand docking in Cys-loop receptors is being increasingly used to predict molecular interactions, but as there are as yet no high resolution structures of any complete Cys-loop receptors, the data obtained must be viewed with caution. This is particularly important for the F-loop region, which is largely flexible, making it difficult to assign residue locations with certainty, even in the different AChBPs whose structures have been resolved to atomic resolution. Such studies, however, can lead to useful testable hypotheses, and thus, as long as the limitations of such studies are appreciated, they can be extremely useful. Here F-loop modeling studies, using both agonist-free and agonist-bound <i>Aplysia</i> AChBP as templates, suggested some interesting potential interactions of some of the F-loop residues: Trp<sup>196</sup> makes a hydrophobic contribution to diazepam potentiation (Table 2), although only one mutation (D192A) resulted in a complete ablation of potentiation. These are discussed in more detail below. None of the mutations changed [<sup>3</sup>H]flumazenil binding affinity, suggesting they do not have a significant impact on benzodiazepine binding characteristics; we therefore propose that they are primarily involved in the conformational change that results in an increased GABA-mediated response. Homology models of the receptor binding pocket in agonist-bound and unbound states suggest that the F-loop is flexible, and that there are some differences in residue orientations in the two states. Considering our data in relation to these models show that the F-loop residues could contribute to hydrogen bond networks and hydrophobic interactions with neighboring residues that may change during receptor activation.

**Asp<sup>192</sup> Contributes to a Hydrogen Bond Network**—The data suggest a very specific role for Asp<sup>192</sup> in diazepam modulation of the GABA<sub>A</sub> receptor. Mutation of Asp<sup>192</sup> to Ala completely abolished diazepam enhancement of GABA currents, and replacement with the chemically similar Glu reduced receptor responsiveness to diazepam by 40%. However, replacement by Asn resulted in receptors that were similar to wild type. These data suggest that a negative charge is not required at this position for maximal potentiation but that size and/or hydrogen bonding ability are critical. Examination of the models reveals a possible explanation for these data, hydrogen bonds that contribute to a hydrogen bonding network. The unbound model shows the carbonyl side chain of Asp<sup>192</sup> has the potential to form two hydrogen bonds, one with Asn<sup>208</sup> and the other with Thr<sup>209</sup> (Fig 3A, panel i). Replacement of Asp<sup>192</sup> with Glu would extend the length of the side chain, which would allow only one of these hydrogen bonds to form (Fig 3A, panel ii), whereas replacement with Asn could replace both (Fig 3A, panel iii) and Ala neither (Fig 3A, panel iv). In the bound form Asp<sup>192</sup> is in a different orientation, facing the subunit interface. However, it still has the potential to hydrogen bond here, this time with Val<sup>190</sup> (data not shown). We suggest that these networks of hydrogen bonds are critical for benzodiazepine potentiation, and that their partial or complete removal compromises the response of the receptor to diazepam.

An aspartate residue may be important in the F-loop of all Cys-loop receptors. Ligand binding and/or function is disrupted by mutations to Asp residues in the F-loop of nicotinic AChγ (Asp<sup>203</sup>), δ (Asp<sup>208</sup>), and ε (Asp<sup>202</sup>) receptor subunits, and GABA<sub>A</sub> α1 (Asp<sup>189</sup>) and 5-HT<sub>3A</sub> (Asp<sup>204</sup>) receptor subunits (21, 25, 26). These residues may have equivalent roles to Asp<sup>192</sup>, although both Clustal and FUGUE alignments of complete receptor subunits suggest they are not in equivalent locations. This may, however, be due to poor alignment of the F-loop region due to poor conservation here. A Clustal alignment of the F-loop region alone supports this hypothesis by aligning these Asp residues (Fig. 4A).

**Trp<sup>196</sup> Makes a Hydrophobic Contribution to Diazepam Potentiation**—Hydrophobicity appears important at position 196. Phe, which has similar hydrophobicity to Trp, does not; it yields a maximal potentiation of only 20% of a wild type. However, Tyr is a polar residue; it is reasonably acidic and it can hydrogen bond; thus it appears that one or more of these characteristics is detrimental to receptor function.

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**TABLE 2**

Diazepam potentiation of F-loop mutants

| Receptor   | % Maximal Potentiation |
|------------|------------------------|
| αβ2γS     | 100 ± 18               |
| αβ2γ<sup>192</sup> | NP                     |
| αβ2γ<sup>192A</sup> | 60 ± 3           |
| αβ2γ<sup>192R</sup> | 70 ± 9               |
| αβ2γ<sup>192A</sup> | 40 ± 12              |
| αβ2γ<sup>192R</sup> | 50 ± 9               |
| αβ2γ<sup>192A</sup> | 100 ± 20              |
| αβ2γ<sup>192R</sup> | 25 ± 1               |
| αβ2γ<sup>192A</sup> | 40 ± 9               |
| αβ2γ<sup>192R</sup> | 75 ± 15              |
| αβ2γ<sup>192N</sup> | 60 ± 2               |
| αβ2γ<sup>192A</sup> | 20 ± 5               |
| αβ2γ<sup>192R</sup> | 50 ± 3               |
| αβ2γ<sup>192A</sup> | 100 ± 15             |
| αβ2γ<sup>192R</sup> | 20 ± 4               |
| αβ2γ<sup>192A</sup> | 20 ± 2               |
| αβ2γ<sup>192C</sup> | 30 ± 10              |

Data is normalized to wild type receptors (170% potentiation). Data = mean ± S.E., n = 3–9; * indicates statistically different from wild type, p < 0.05.
Investigation of homology models of the GABA<sub>α</sub> receptor γ<sub>2</sub> subunit, based on both the bound and unbound versions of AChBP, reveal that Trp<sup>196</sup> faces toward the principal subunit across the interface. This was unexpected considering the strong evidence that hydrophobicity is important at this position for diazepam modulation; we would expect to find the residue buried within the protein. Further examination of the model, however, reveals that Trp<sup>196</sup> is located in a groove in the neighboring α subunit, where it could participate in both hydrophobic and hydrogen bond interactions (Fig. 3B). Thus Trp<sup>196</sup> could as a pin to restrict the movement of this end of the loop, and perhaps may even serve as a rigid pivot point. Either or both of these possibilities would mean that replacement with a residue that has different chemical properties could be distinctly unfavorable.

**Other F Loop Residues**—Data from mutations of the other F-loop residues suggest specific interactions of Arg<sup>194</sup> and Arg<sup>197</sup>, as replacement of both with the chemically similar Lys reveal similar decreases in the levels of diazepam potentiation, although Ala can efficiently replace Arg<sup>194</sup> indicating a small residue is well tolerated here. The bound model reveals that these two arginines may form part of a critical hydrogen bond network required for effective F-loop function (Fig. 3C). Arg<sup>197</sup> appears to be central to this network, with the potential to contribute up to five hydrogen bonds to Thr<sup>193</sup>, Arg<sup>194</sup>, and Tyr<sup>199</sup>. An alanine substitution at position 194 would be well tolerated as the hydrogen bond involves the backbone carbonyl, although substitution with a more bulky residue such as lysine could disrupt the backbone architecture. Similarly substitution of Arg<sup>197</sup> would significantly disrupt the hydrogen bond network, and as our results show, neither Lys nor Ala mutations are well tolerated here. Interestingly, replacement of Thr<sup>193</sup> with Ala or Ser yielded almost identical decreases in the levels of potentiation (40–50% of WT), as would be expected by overreaching (in the case of Ser) or falling short (Ala) of the distance required to hydrogen bond. The data suggest that Thr<sup>193</sup> may also contribute to a different hydrogen bonding network in the unbound state, suggesting a change in orientation of this residue between the agonist-free and agonist-bound receptor binding states, as described for Asp<sup>192</sup> above. This “unbound state” network also appears to involve Ser<sup>195</sup>, which could explain why a small residue (Ala) was well tolerated at this position, although the
chemically similar Thr resulted in an 80% decrease in potentiation. Thus we propose that F-loop hydrogen bonding networks are important in both the unbound and bound states of the receptor for efficient benzodiazepine modulation.

F-loop Flexibility May Be Key to Allosteric Modulation—The models appear to be able to explain many of the results we have obtained, providing support for them. Of course, data from homology models must be viewed with some caution, and this is especially true in this region where there are few elements of secondary structure (7, 20, 27, 28). However, this lack of secondary structure suggests that this region is flexible, and we propose this could be the key to its function, allowing the F-loop to undergo structural rearrangements that result in or occur during channel gating. There is support for this hypothesis from other Cys-loop receptors; pentobarbital activation of GABA_A receptors resulted in methanethiosulfonate ethylammonium modification of Cys residues substituted for Val^{180} (aligns with Asp^{192}) and Ala^{181} (Thr^{193}) in the α1 GABA_A receptor subunit, suggesting that these regions undergo structural changes (exposing the residues to chemical modification) during ligand binding and/or channel gating (12), and movement in two regions of the F-loop has also been reported to occur in the 5-HT_3 receptor (21).

Interestingly our data suggest the F-loop needs to be precisely positioned in both bound and unbound versions of the receptor for efficient enhancement of GABA-mediated responses. If, as previously discussed, benzodiazepines act through modification of channel architecture and/or function (2, 12, 29, 30), the F-loop is ideally positioned to act as a conduit. It is located at the base of the binding site close to the Cys-loop, /H_9252^1-H_9252^2 loop, /H_9252^8-H_9252^9 loop, the /H_9252^10 region of the extracellular domain (Fig. 4B), and the M2-M3 loop and M1 region of the transmembrane domain, all of which are implicated in coupling ligand binding to channel gating. We hypothesize that the F-loop may directly contact and influence these interface residues.

In conclusion we have shown that residues in the GABA_A receptor γ_2 subunit F-loop affect diazepam modulation of GABA responses. We propose that Asp^{192}, Ser^{193}, Thr^{194}, and Arg^{197} make critical contributions to hydrogen bond networks, and a hydrophobic residue is necessary at position 196. Data from homology models indicate the F-loop is highly flexible, and that it is in distinct locations in bound and unbound states of the receptor; our data suggest that destabilizing the F-loop in either of these states decreases diazepam modulation. We pro-
pose that the flexibility of the F-loop may allow this region to modify interactions between the interface loops, and thereby enhance responses to GABA in the presence of diazepam.

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