Two invariant tryptophan residues on the α1 subunit define domains necessary for GABA<sub>A</sub> receptor assembly*

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Two invariant tryptophan residues on the N-terminal extracellular region of the rat α1 subunit, Trp-69 and Trp-94, are critical for the assembly of the GABA<sub>A</sub> (γ-aminobutyric acid, type A) receptor into a pentamer. These tryptophans are common not only to all GABA<sub>A</sub> receptor subunits, but also to all ligand-gated ion channel subunits. Converting each Trp residue to Phe and Gly by site-directed mutagenesis allowed us to study the role of these invariant tryptophan residues. Mutant α1 subunits, coexpressed with β2 subunits in baculovirus-infected SF9 cells, displayed high affinity binding to [3H]muscimol, a GABA site ligand, but no binding to [3H]-butyl bicyclophosphorothionate, a ligand for the receptor-associated ion channel. Neither [3H]muscimol binding to intact cells nor immunostaining of nonpermeabilized cells gave evidence of surface expression of the receptor. When expressed with β2 and γ2 polypeptides, the mutant α1 polypeptides did not form [3H]flunitrazepam binding sites. The distribution of the mutant receptors on sucrose gradients suggests that the effects on ligand binding result from the inability of the mutant α1 subunits to form pentamers. We conclude that Trp-69 and Trp-94 participate in the formation of the interface between α and β subunits, but not of the GABA binding site.

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** The γ-aminobutyric acid, type A receptor (GABA<sub>A</sub> receptor) is the postsynaptic target for GABA, the major inhibitory neurotransmitter of the mammalian central nervous system. Along with the nicotinic acetylcholine, glycine, and serotonin (5HT3) receptors, the GABA<sub>A</sub> receptor belongs to the superfamily of ligand-gated ion channels, all of which share significant sequence and structural features (1–3). Molecular cloning has led to the identification of nine genes that encode distinct GABA<sub>A</sub> receptor polypeptides, including six α, four β, three γ, one δ, three ρ, one ε, and one π polypeptides. Several splice variants are also known, suggesting that a large number of heteropentameric isomers could form (4–8). Most studies so far suggest that functional receptors consist of two α, two β, and one γ subunits (9–12).

The actual diversity of GABA<sub>A</sub> receptors in the brain depends on the precise processes of receptor assembly. The N-terminal segments of the receptor subunits contain signals for subunit oligomerization (13), but the specific domains responsible for receptor assembly are not yet known. Extensive mutagenesis studies performed on GABA<sub>A</sub> receptors have led to the identification of specific residues responsible for GABA binding on the interface of the α and β subunits and for benzodiazepine binding on the interface of the γ and α subunits (14–16). Two of these residues, Phe-64 and His-101 on the α1 subunit of the GABA<sub>A</sub> receptor, were also identified by photoaffinity labeling studies using [3H]muscimol and [3H]flunitrazepam to contribute to the GABA and benzodiazepine binding sites, respectively (15, 17, 18). Although these residues apparently contribute to separate binding pockets for the receptor agonist and allosteric modulator respectively, they are separated by only 37 amino acid residues, and as indicated in Fig. 1, four of these residues are identical not only among all GABA<sub>A</sub> receptor subunits, but also among all ligand-gated ion channel subunits. This constancy suggests selective value, and we reasoned that these residues are likely to play a critical role in GABA<sub>A</sub> receptor function. In particular, Smith and Olsen (15) have hypothesized that the two tryptophan residues, Trp-69 and Trp-94, because of their bulky and unique structure, may define a functional domain that is critical for channel opening. Galzi and Changeux (16) have proposed that both these invariant Trp residues behave as “structural canonical residues” that constrain the folding of the loops. If indeed this is the case, mutating the Trp residues could affect the folding of the loops and alter the conformational characteristics of the receptor. We have tested this hypothesis by mutating Trp-69 and Trp-94 into Phe and Gly and then studying the effects of each of these mutations on the binding characteristics and assembly of the GABA<sub>A</sub> receptor.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The epitope tag and point mutations were introduced in the cDNA for the α1 subunit of the GABA<sub>A</sub> receptor using the Altered Sites<sup>®</sup> In <i>vitro</i> Mutagenesis Systems (Promega, Madison, WI). First, the entire coding region of the α1 subunit was subcloned into pALTER and a 10-amino acid 9E10 epitope (EQKLL-SEEDL) from c-Myc was introduced between residues 4 and 5 of the mature peptide as described in the manual using the mutagenic oligonucleotides: GTCCCTAAGTCATCGATCCTTCTCTGATATTAGC- TTGTTCTGGAGGAGGCTGTC. Specific point mutations were incorporated in a second round of mutagenesis performed on the epitope-tagged α1 subunit construct using the following mutagenic oligonucleotides: W94F, AATAAGTCATCGATCCTTCTCTGATATTAGC; W94G, AATAAGTCATCGATCCGAGATTGTTACTGCGCAT; W96F, TAATCTTCTATGAGTTGTTGCGGCAGAAA; W96G, TAATCTTCTATGAGTTGTTGCGGCAGAAA. Positive clones were identified
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by restriction digests and DNA sequencing.

Recombinant Protein Expression—Sf9 cells (Pharmpingen, San Diego CA) were maintained in serum-free XL-400 medium (JRH Biosciences, Lenexa, KS) in shaking culture at 28 °C as described previously (19). cDNAs for the β2 and either wild-type or mutant α1 subunits were subcloned into BaculoGold transfer vector and linearized with HindIII, pAcBac1, to control the p10 and p3 promoters, respectively. Recombinant baculoviruses were constructed by homologous recombination between the transfer vector and linearized BaculoGold viral DNA (Pharmpingen) using a liposome-mediated transfection protocol. After 5 days, the supernatant was collected to harvest recombinant viruses, which were then subjected to a high titer stock (0.5–1 million plaque-forming units/ml). GABAA receptors were produced by infecting Sf9 cells with recombinant baculovirus (containing cDNAs for both subunits) at a multiplicity of infection of 5. In experiments testing for [3H]flunitrazepam binding, cells were infected with a recombinant γ2 virus in addition to the virus encoding the wild-type or mutant α1 and β2 subunits. Infected cells were harvested at 60 h post-infection by pelleting the cells at 10000 × g for 10 min. Cell pellets were stored at −20 °C until ready to use.

Western Blots—Membranes of Sf9 cells expressing either wild-type or mutant α1 along with β2 subunits were analyzed by SDS-PAGE, and proteins were transferred on to nitrocellulose membranes. The blots were blocked in phosphate-buffered saline with 0.1% Tween 20 and 10% nonfat dry milk for 1 h at room temperature and incubated with the polyclonal anti-myc tag antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at a dilution of 1:1000 for 1 h at room temperature. After extensive washing, the blots were incubated with anti-rabbit horseradish peroxidase-linked secondary antibodies (Amersham Pharmacia BioTech) at a dilution of 1:1000 for 1 h at room temperature. After extensive washing, immunoreactivity was detected by chemiluminescence (ECL, Amersham Pharmacia BioTech).

Radioligand Binding Assays—Radioligand binding assays were performed either on lysed cell membranes or on intact cells. Cell membranes were prepared by homogenizing the cell pellet at 10 000 × g for 20 min in membrane wash buffer (20 mM K2HPO4/KH2PO4 buffer with 50 mM KCl, pH 7.5, 0.5 mM EDTA, 2 mM benzamidine chloride, 0.1 mM benzethonium chloride, 0.5 units/ml bacitracin, 0.3 mM phenylmethylsulfonyl fluoride, 10 mM/g liquid ovo-mucoid trypsin inhibitor, 10 mg/g liquid soya trypsin inhibitor). The membranes were recovered by centrifugation at 150,000 × g for 20 min, resuspended to a final concentration of 0.5 mg/ml, and used for the binding assays. For whole cell binding, the frozen cell pellet was thawed, resuspended in 10 mM phosphate buffer, pH 7.5, to a concentration of 0.5 mg/ml, and used for the binding assays. Binding assays were carried out using an appropriate concentration of the radioligand in 10 mM phosphate buffer with 100 mM KCl, pH 7.5, and 100 μg of total membrane protein in a total volume of 250 μl. Triplicate samples were incubated with either 20 nM [3H]muscimol, 10 nM [3H]flunitrazepam, or 5 nM [35S]TBPS at 4 °C for 20 min ([3H]muscimol) or 60 min ([3H]flunitrazepam) or at room temperature ([35S]TBPS). The reaction mixtures were diluted with 4 ml of assay buffer and filtered under vacuum over Whatman GF/B filters using a Brandel cell harvester. The filters were washed twice with 4 ml of assay buffer and counted for radioactivity. Undisplaceable background was estimated in the presence of 100 μM GABA, 10 μM diazepam, or 100 μM picrotoxin, respectively, and was subtracted from total binding to compute specific binding. GABA displacement assays were conducted using 20 nM [3H]muscimol and non-radioactive GABA concentrations ranging from 1 nM to 0.1 mM. Dose-response profiles were generated by fitting the data to a three-parameter Hill equation.

Cell Surface Localization—Sf9 cells were seeded on each well of poly-L-lysine-treated eight-well chamber slides. The cells were infected with appropriate baculovirus constructs and incubated at 28 °C for 60 h. The cells were fixed in 4% paraformaldehyde and washed three times with 0.1% phosphate-buffered saline. Cells were permeabilized with 1% Triton X-100 in 0.1% phosphate-buffered saline for 30 min and blocked using 3% fetal calf serum. Incubations with the polyclonal anti-myc tag antibodies at a dilution of 1:250 were carried out overnight at 4 °C in the presence of 1% fetal calf serum. The cells were washed extensively with goat anti-rabbit fluorescent isothiocyanate (Vector Laboratories, Burlingame, CA) at a dilution of 1:200 for 2 h at room temperature. The cells were then washed extensively and examined using a confocal microscope (Carl Zeiss LSM 310).

Sucrose Gradients—Membranes were prepared from infected cells as described for the binding assays. An equal volume of 2X solubilization buffer (20 mM K2HPO4/KH2PO4, pH 7.5, 2% Triton X-100, 200 mM KCl, 50 mM sodium phosphate, 0.1% Triton X-100, 2% sodium deoxycholate) and 0.1% sodium deoxycholate was added, and the homogenate was solubilized during magnetic stirring at 4 °C for 1 h. The supernatant was collected and concentrated to about one-fifth of its original volume in Centricon-30 concentrators. The concentrated solubilized protein solution was layered on top of a 5–30% sucrose gradient made of solubilization buffer containing only 0.1% Triton X-100. Gradients were centrifuged in a Beckman SW40 rotor for 40 h at 34,500 rpm. Fractions of 500 μl were collected from the top of the tube, precipitated following a chloroform-methanol protocol (20), and analyzed by Western blots using the anti-myc tag antibody as described earlier. The intensity of the bands was quantitated by densitometry. Cytochrome c (1.9 S), bovine serum albumin (4.3 S), alcohol dehydrogenase (7.4 S), and catalase (11.4 S) were run as standards, in parallel in a separate tube. The positions of the standards in the gradient were determined by SDS-PAGE.

RESULTS

FIG. 1. Sequence alignment of GABAA receptor polypeptides and other members of the ligand-gated ion channel superfamily shows high sequence identity in the region corresponding to Tyr-59 and Asn-102 on the rat α1 GABAA receptor subunit. The residues that were covalently modified by [3H]muscimol (α1P64) and [3H]flunitrazepam (α1H101) are indicated by arrows. The invariant residues are indicated by +, and the conservatively replaced residues by dots. The two absolutely conserved tryptophan residues, α1W69 and α1W94, are shown in bold font.

containing 0.02% NaNO3, 0.5 mM dithiothreitol, and various protease inhibitors (as described for the membrane preparation) was added, and the homogenate was solubilized during magnetic stirring at 4 °C for 1 h. The supernatant was collected and concentrated to about one-fifth of its original volume in Centriprep-30 concentrators. The concentrated solubilized protein solution was layered on top of a 5–30% sucrose gradient made of solubilization buffer containing only 0.1% Triton X-100. Gradients were centrifuged in a Beckman SW40 rotor for 40 h at 34,500 rpm. Fractions of 500 μl were collected from the top of the tube, precipitated following a chloroform-methanol protocol (20), and analyzed by Western blots using the anti-myc tag antibody as described earlier. The intensity of the bands was quantitated by densitometry. Cytochrome c (1.9 S), bovine serum albumin (4.3 S), alcohol dehydrogenase (7.4 S), and catalase (11.4 S) were run as standards, in parallel in a separate tube. The positions of the standards in the gradient were determined by SDS-PAGE.

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Epitope Tagging of α1 Does Not Affect the Function or the Subcellular Location of GABAA Receptors in Sf9 Cells—GABAA receptors expressed in Sf9 and in mammalian cells showed appropriate binding and functional characteristics. Co-expression of α1 and β2 polypeptides produced GABA A receptors that bound [3H]muscimol and [35S]TBPS and that also showed GABA-activated chloride currents. Individual α1 or β2 polypeptides did not bind either ligand and did not show any GABA-activated currents. Co-expression of α1, β2, and γ2 subunits formed functional receptors, which also showed [3H]flunitrazepam binding and GABA-activated currents that were enhanced by benzodiazepines (21, 22). To aid in the biochemical analyses and subcellular localization of the α1 subunits of the GABAA receptor, we introduced a 9E10 epitope tag at the N-terminal of the mature wild-type α1 peptide, and we ex-
pressed this construct along with the β2 subunit in Sf9 cells. 

The epitope tag had no effect on either GABA affinity in Sf9 cells (data not shown) or on the functional characteristics of the receptor expressed in A293 cells (23).

Epitope-tagged Mutant Polypeptides Are Synthesized in Sf9 Cells—We incorporated all four mutations into the epitope-tagged α1 polypeptide to facilitate detection by Western blots and subcellular localization. All mutant α1 subunits were expressed and could be detected by antibodies that recognized the myc epitope tag on Western blots (Fig. 2). The antibody did not recognize the untagged α1 subunit or the β2 subunit.

Trp-69 and Trp-94 Mutants Bind Muscimol but Not TBPS—Homogenates of cells expressing the four mutant α1 subunits (Trp-69 and Trp-94 each converted to Phe and Gly) along with β2 subunits showed binding to [3H]muscimol, a GABA site ligand (Fig. 3A). This result was reproducible in many experiments, with different batches of viruses. The density of [3H]muscimol binding sites varied between batches of cells and viruses and reflects differences in the $B_{\text{max}}$ values of the expressed receptor.

We determined the affinity of the mutant receptors for GABA by GABA displacement of [3H]muscimol binding (Table I). There was no significant change in the GABA affinity of the [3H]muscimol binding sites formed by the Trp-94 mutants. The Trp-69 mutants, however, showed a slight increase in GABA affinity. Taken together, these data suggest the formation of a relatively normal GABA binding site despite the mutation of α1 and β2 subunits.

Interestingly, the α1W69 and α1W94 mutants behaved differently from the wild-type α1 subunits with respect to three other binding characteristics. When expressed with β2 subunits, all α1 mutants could not bind [35S]TBPS, a ligand that recognizes the chloride channel (Fig. 3A). Furthermore, no significant [3H]muscimol binding was detected on the surfaces of intact cells coexpressing mutant α1 and β2 subunits (Fig. 3B). Finally, expression of the Trp-69 and Trp-94 mutant α1 subunits with β2 and γ2 subunits yielded no [3H]flunitrazepam binding (Fig. 3C).

Trp-69 and Trp-94 Mutations Affect Intracellular Targeting—When α1 and β2 subunits are expressed independently in cells, they are retained in the endoplasmic reticulum, but when they are expressed simultaneously, both subunits are targeted to the cell surface (23). We tested the ability of mutant α1 subunits to target to the cell surface with an antibody that recognizes the myc epitope tag. 9E10. Surface expression was detected by immunostaining nonpermeabilized cells (Fig. 4, panels A1–H1). We confirmed that the Sf9 cells were expressing the appropriate peptide by processing permeabilized cells in parallel (Fig. 4, panels A2–H2).

In cells that express only epitope-tagged α1 (α19E10), we found epitope-tagged peptide only in permeabilized cells (Fig. 4, panel A1) and not in the nonpermeabilized sample (Fig. 4, panel A2). This result confirmed that expression of the α1 polypeptide alone resulted in an intracellular localization of the α1 subunit. As expected, the anti-myc antibody did not recognize the β2 subunit (Fig. 4, panels B1 and B2) or the untagged α1 subunit (Fig. 4, panels C1 and C2).

Cells that simultaneously expressed wild-type α19E10 and β2 subunits showed antibody staining even in nonpermeabilized cells (Fig. 4, panels D1 and D2), confirming surface expression. Similar results were observed using bd17, a monoclonal antibody that recognizes the β2 subunit (data not shown).

Fig. 4, panels E1 through H1 and E2 through H2, show the localization of the α1W69 and α1W94 mutants expressed with the β2 subunits. In all cases, no immunostaining was observed in nonpermeabilized cells indicating that the mutant α1 subunits were deficient in their targeting to the cell surface. Peptide expression, as well as antibody reaction, was confirmed by positive staining in permeabilized cells (Fig. 4, panels E1–H1). Similar results were observed with bd17, which recognizes the β2 subunit (data not shown).

α1W69 and α1W94 Mutant Polypeptides Fail to Assemble Normally into Pentamers—To identify the oligomeric species formed by the mutant α1 polypeptides, we solubilized Sf9 cells that produced α19E10β2, α19E10(W69G)β2 and α19E10(W94G)β2 receptors and loaded the solubilized extracts on to sucrose gradients. We analyzed sucrose gradient fractions collected by immunoblotting with anti-myc antibody (Fig. 5). The α1W94G and α1W69G polypeptides formed oligomers that were most concentrated in fraction 8, while wild-type α19E10β2 receptor was most concentrated in fraction 13. Based on the sedimentation velocity of known markers, the sedimentation coefficients of polypeptides in these fractions were 5.3 and 8.7 S. Similar results were obtained by monitoring the migration of the β2 subunits using the monoclonal antibody, bd17 (data not shown). Knight et al. (24) have identified a pentameric species of GABA$\text{A}$ receptors expressed in Sf9 cells that migrates at about 8.8 S. Thus, the α19E10β2 receptors probably migrated as pentamers. The receptors that contain the α1W69 and α1W94 mutations did not have the same sedimentation coefficient as the pentameric α19E10β2 receptor. Instead, they formed smaller oligomers, probably dimers, which may be the [3H]muscimol binding species. [3H]Muscimol binding was lost on solubilization of cells coexpressing the mutant α1 and β2 subunits, preventing us from monitoring the [3H]muscimol binding species on the sucrose gradient.

DISCUSSION

The GABA$\text{A}$ receptor gene family forms a subset of the ligand-gated ion channel superfamilies. At least 19 genes encode distinct GABA$\text{A}$ receptor subunits, which can form a large number of heteropentamers (4). Since the channel characteristics and pharmacology of the receptor depend on its subunit composition (25–27), a major challenge to understanding the physiological and pharmacological diversity of GABA$\text{A}$ receptors is determining the control of receptor assembly. Our data suggest the identity of a domain on the α1 subunit that is necessary for the formation of an interface between the α1 and the β2 subunits.

Pentameric Receptors Are Required for [3H]Flunitrazepam and [35S]TBPS Binding as Well as for Cell Surface Expression—We designed mutations to probe the role of two invariant tryptophan residues, Trp-69 and Trp-94, on the α1 subunit of the GABA$\text{A}$ receptor. These residues are close to α1F64 and α1H101, the residues photolabeled by [3H]muscimol and [3H]flunitrazepam, respectively. To facilitate immunodetection, we introduced all these mutations into an epitope-tagged α1 subunit, α19E10.
When expressed with β2 subunits, the Trp-69 and Trp-94 mutant α1 subunits formed a normal GABA binding site, which bound [3H]muscimol with normal affinity (Table I). But not [35S]TBPS, and when expressed with β2 and γ2 subunits, this species cannot bind [3H]flunitrazepam, suggesting that [35S]TBPS and [3H]flunitrazepam only recognize pentameric species, while [3H]muscimol will bind to smaller intermediates. These results are consistent with the studies of Im et al. (12), which showed that a tandem construct of α6 and β2 subunits expressed in HEK293 cells could not by itself form pentamers (channels), although it retained the ability to bind muscimol. The Trp-69 and Trp-94 mutations compromised the ability of the α1 polypeptides to assemble with β2 or β2 and γ2 polypeptides to form a pentamer.

For GABA\textsubscript{A} receptors, only the α1β2 and α1β2γ2 combinations were detected on the surface of cells. Monomers, or α1γ2 and β2γ2 combinations remained in the endoplasmic reticulum (23). Furthermore, these were the only combinations that could form pentamers (9), suggesting that only fully assembled pentameric GABA\textsubscript{A} receptors are capable of surface expression. The α1W69 and α1W94 mutants showed no cell surface binding (Fig. 3C) or immunostaining (Fig. 4) when expressed with β2 subunits.

How Can the Two Trp Mutations Interfere with the Assembly of the Receptor into a Pentamer?—Since the GABA binding site lies on the interface of the α and β subunits, and since the mutant α1 subunits expressed with the β2 subunits bind [3H]muscimol, the GABA binding interface is unaffected by the Trp-69 and Trp-94 mutations. The failure of these subunits to assemble into pentamers suggests that the other interface involving β-α interactions must not have formed. If we call the

![Image](248x344 to 554x729)

**Fig. 3.** α1W69 and α1W94 mutants coexpressed with β2 subunits bind [3H]muscimol but not [35S]TBPS. Membrane homogenates of Sf9 cells expressing the wild-type or mutant α1 subunit (as shown) with β2 subunits were tested for their ability to bind [3H]muscimol (diagonal stripes) and [35S]TBPS (solid columns) (A). Intact Sf9 cells expressing the same combinations were also tested for [3H]flunitrazepam binding (B). The same combinations were expressed with γ2 subunits in Sf9 cells and membrane homogenates from these cells were analyzed for [3H]flunitrazepam binding sites (C). Error bars represent the mean ± S.E. of three experiments in triplicates.

### Table I

| IC\textsubscript{50} for GABA inhibition of [3H]muscimol binding to α1W69 and α1W94 mutants when expressed with β2 subunits |
|---|
| IC\textsubscript{50} (mM) |
| α1 | 93.2 ± 25 |
| α1W10 | 64.8 ± 8.5 |
| W94 | 77.9 ± 6.4 |
| W94F | 60.3 ± 8 |
| W94F | 14.0 ± 1.2 |
| W94G | 36.6 ± 11 |

but not [35S]TBPS, and when expressed with β2 and γ2 subunits, this species cannot bind [3H]flunitrazepam, suggesting that [35S]TBPS and [3H]flunitrazepam only recognize pentameric species, while [3H]muscimol will bind to smaller intermediates. These results are consistent with the studies of Im et al. (12), which showed that a tandem construct of α6 and β2 subunits expressed in HEK293 cells could not by itself form pentamers (channels), although it retained the ability to bind muscimol. The Trp-69 and Trp-94 mutations compromised the ability of the α1 polypeptides to assemble with β2 or β2 and γ2 polypeptides to form a pentamer.

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interface on which the GABA binding site lies the "α → β interface," the α1W69 and α1W94 mutations prevent the assembly of the subunits into pentamers by interfering with the formation of the "β → α interface" (Fig. 6A). Fig. 6B shows one possible scheme for the assembly of a receptor composed of two α and three β subunits and the point at which assembly of the receptor into a pentamer can be blocked by the mutant α1 subunits. A receptor composed of three α and two β subunits would not assemble into pentamers by a similar mechanism.

The γ subunit is unable to rescue the altered α1 subunits created by the Trp-94 mutations, since mutant α1 subunits are unable to form [3H]flunitrazepam binding sites when expressed with β2 and γ2 subunits. This can be attributed to one of two
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reasons: (i) they are unable to form a γ → α interface, or (ii) the α1 mutants are unable to form pentamers due to a compromised β → α interface. In either case, no [3H]flunitrazepam binding can be detected due to the absence of a pentamer.

Why Should the Mutation of Trp-69 or Trp-94 Residues on the α1 Subunit of the GABA<sub>A</sub> Receptor Affect Subunit Interactions?—Several recent reports in the literature have identified critical Trp residues involved in protein-protein interactions (28). Our inference that mutations in α1W69 and α1W94 disrupt subunit interactions in the GABA<sub>A</sub> receptor is consistent with the known roles of tryptophan in protein-protein interactions. Trp-69 and Trp-94 may both lie at the β → α interface, although the Trp-69 residue more probably lies in close proximity to the α → β interface. Alternately, these residues may demarcate a region necessary for appropriate interactions of the α1 subunit at the β → α interface. These residues could play a structural role and the lack of assembly of the subunits could be the result of misfolding of the protein in this region flanked by Trp-69 and Trp-94. If the mutant α1 peptides are folded improperly, however, the disruption is likely to be local, since the α1 peptide is still able to form a normal [3H]muscimol binding site.

The only detectable difference in the effect of the mutagenesis of the two W residues was in the affinity of the [3H]muscimol binding species for GABA (Table I). The α1W69 mutants expressed with β2 had a higher affinity for GABA than the wild-type receptors, as well as the α1W94 mutants. This change in affinity may be evidence of the role of Trp-69 in conformational coupling, an effect that is masked by the more dramatic effect of preventing the assembly of the subunits into pentamers.

Are Trp-69 and Trp-94 Critical for the Assembly of all GABA<sub>A</sub> Receptor Subunits and all Ligand-gated Ion Channels?—Residues Trp-69 and Trp-94 are invariant, not only in all GABA<sub>A</sub> receptor subunits, but also among all ligand-gated ion channel subunits (Fig. 1). If the two Trp residues perform the same function in all these proteins, as is suggested by their constancy, then this region may contribute to the assembly of all members of the ligand-gated ion channel superfamily. This model is consistent with the proposal of Galzi and Changeux (16) that these two tryptophan residues are structural canonical residues, suggesting that they play the same role in all ligand-gated ion channel receptor subunits.

In conclusion, α1W69 and α1W94 either directly contribute to or demarcate a region of the α1 subunit forming the β → α interface and possibly also the γ → α interface. Mutagenesis of these residues destroys the β → α or γ → α interface and prevents the assembly of the mutant α1 subunit into pentamers. Homologous regions on other GABA<sub>A</sub> receptor subunits are also likely to perform the same function.

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