GABA_A Receptor Assembly

IDENTIFICATION AND STRUCTURE OF \( \gamma_2 \) SEQUENCES FORMING THE INTERSUBUNIT CONTACTS WITH \( \alpha_1 \) AND \( \beta_3 \) SUBUNITS

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GABA_A receptors are ligand-gated chloride channels composed of five homologous subunits that specifically recognize one another and assemble around an aqueous pore. To identify domains responsible for the specificity of subunit association, we constructed C-terminal truncated \( \gamma_2 \) subunits, as well as mutated and chimeric fragments. From their ability to interfere with \( \alpha_1 \beta_3 \gamma_2 \) receptor assembly and to associate with full-length subunits, we concluded that amino acid sequences \( \gamma_2 \)p(91–104) and \( \gamma_2 \)m(83–90) form the sites mediating assembly with \( \alpha_1 \) and \( \beta_3 \) subunits, respectively. Neural network-based secondary structure prediction, Monte Carlo optimization, and hydrophobicity analysis led to the conclusion that these sites also form the intersubunit contacts in the completely assembled receptor and provided important information on the benzodiazepine-binding site and structure of GABA_A receptors.

\( \gamma \)-Aminobutyric acid (GABA), the quantitatively most important inhibitory neurotransmitter in the central nervous system, mediates fast synaptic inhibition by opening the chloride ion channel intrinsic to the GABA_A receptor (1). This receptor is the site of action of various pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, steroids, anesthetics, and convulsants. These drugs modulate GABA-induced chloride ion flux by interacting with separate and distinct allosteric binding sites (2).

The GABA_A receptor is a hetero-oligomeric protein consisting of five subunits (3, 4). So far at least 20 GABA_A receptor subunits belonging to several subunit classes (six \( \alpha \), four \( \beta \), four \( \gamma \), one \( \delta \), one \( \epsilon \), one \( \pi \), and three \( \rho \)) have been identified (5). In situ hybridization and immunocytochemical studies indicate a distinct but overlapping temporal and regional expression of these subunits. The finding that multiple receptor subunits are expressed within single neurons (6, 7), raises the possibility for these subunits. The finding that multiple receptor subunits are distinct but overlapping temporal and regional expression of situ hybridization and immunocytochemical studies indicate a distinct but overlapping temporal and regional expression of these subunits. The finding that multiple receptor subunits are expressed within single neurons (6, 7), raises the possibility for these subunits. The finding that multiple receptor subunits are distinct but overlapping temporal and regional expression of these subunits.
μg of subunit cDNAs via the calcium phosphate precipitation method (18). The cells were harvested 44 h after transfection.

Immunoprecipitation of Receptors Expressed on the Cell Surface and Receptor Binding Studies—The culture medium was removed from HEK 293 cells transfected with α1, β2, and γ2 subunits together with a truncated γ2 construct (cDNA ratio 1:1:1:1) and the cells were washed once with phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH2PO4, 140 mM NaCl, and 4.3 mM Na2HPO4, pH 7.3). Cells were then detached from the culture dishes by incubating with 2.5 ml of 5 mM EDTA in phosphate-buffered saline for 5 min at room temperature. The resulting cell suspension was diluted in 6.5 ml of cold Dulbecco’s modified Eagle’s medium and centrifuged for 5 min at 1000 × g.

The pellet from two dishes was incubated with 30 μg of α1-(1–9) antibodies in 3 ml of the same medium for 30 min at 37 °C. Cells were then pelleted and free antibodies were removed by washing twice with 10 ml of phosphate-buffered saline buffer. Then receptors were extracted with low IP buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) containing 1% Triton X-100 for 1 h under gentle shaking. Cell debris was removed by centrifugation (30 min; 150,000 × g; 4 °C). After addition of Immunoprecipitin (Life Technologies, Gaithersburg, MD; see Ref. 4) and 0.5% nonfat dry milk powder and shaking for 3 h at 4 °C, the precipitate was centrifuged for 10 min at 10,000 × g and washed three times with low IP buffer. The precipitated receptors were then suspended in 1 ml of a solution containing 0.1% Triton X-100, 50 mM Tris citrate buffer, pH 7.1, 150 mM NaCl, and 20 mM [3H]Ro 15-1788 (20.9 Ci/mmol; NEW Life Science Products, Dreisch, Germany). After incubation for 90 min at 4 °C, the suspensions were filtered through Whatman GF/B filters. The filters were washed twice with 5 ml of a 50 mM Tris citrate buffer, pH 7.1, and the amount of [3H]Ro 15-1788 bound to receptors was measured by liquid scintillation counting.

To verify that only receptors on the cell surface were labeled by the antibodies, parallel samples were incubated with antibodies directed against the intracellular loop of GABA<sub>A</sub> receptor subunits (experiments not shown). These antibodies could not precipitate any GABA<sub>A</sub> receptor subunits.

To investigate a possible redistribution of the antibodies during the extraction procedure, in other experiments the extract containing the cell surface-labeled receptors was divided into two fractions. One fraction was kept at 4 °C for 2 h, whereas the other fraction was incubated with additional α1-(1–9) antibodies at 4 °C for the same time period. Immunoprecipitation was added to both fractions, the resulting precipitates were centrifuged, washed, dissolved in sample buffer (108 mM Tris sulfate, pH 8.2, 10 mM EDTA, 25% (w/v) glycerol, 2% SDS, and 3% diethiothreitol) and were subjected to SDS-PAGE and Western blot analysis (4) using digoxigenized γ2-(1–33) antibodies. In both α1-(1–9) precipitates full-length γ2 subunits could be detected. The truncated γ2 subunit, however, could only be detected in the fraction where additional α1-(1–9) antibodies had been added after cell lysis (experiments not shown). Since aggregates consisting of full-length α1 and truncated γ2 subunits are not transported to the cell surface and are present only intracellularly (11), this experiment demonstrated that there was no redistribution of antibodies during extraction and that only receptors present on the cell surface were detected by this procedure.

In other experiments membranes from HEK cells transfected with full-length α1 and truncated γ2 subunits were incubated with 10 nM [3H]flunitrazepam (83.0 Ci/mmol; Amersham Pharmacia Biotech) in the absence or presence of 10 μM diazepam. After incubation for 90 min at 4 °C, the suspensions were filtered through Whatman GF/B filters and subjected to scintillation counting.

**RESULTS**

Truncated γ2 Subunits Reduce the Expression of Recombinant α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> Receptors on the Cell Surface—Experiments investigating the assembly of the nicotinic acetylcholine receptor have indicated that truncated subunits bearing assembly signals form unproductive complexes with full-length subunits and thus inhibit receptor assembly and its subsequent expression on the cell surface (11).

In the present study, HEK cells were transfected with full-length α1, β3, and γ2 subunits together with a truncated γ2 subunit. The truncated γ2 subunits used are shown in Fig. 2A. GABA<sub>A</sub> receptors expressed on the surface of transfected HEK cells were labeled with subunit-specific antibodies. Antibody-labeled receptors were extracted and precipitated by addition of Immunoprecipitin. The amount of receptors precipitated was quantified using a [3H]Ro 15-1788 binding assay and was compared with that precipitated from cells transfected in the absence of truncated subunits. As shown in Fig. 2B the construct γ2-(1–234) consisting of the complete extracellular N-terminal domain was able to reduce the expression of α1β2γ2 receptors on the cell surface by 50%. When higher amounts of γ2-(1–234) cDNA were co-transfected with full-length α1, β3, and γ2 subunits (cDNA ratio 2:1:1) the amount of receptors expressed on the cell surface was reduced to 40% (data not shown). A subsequent reduction in the size of the truncated γ2 subunit indicated that γ2-(1–113) was the smallest N-terminal fragment that could reduce the expression of α1β2γ2 receptors on the cell surface. Control experiments indicated that the failure of γ2-(1–110) to reduce the expression of α1β2γ2 receptors on the cell surface was not due to a weak expression of this construct (Fig. 2C).

Truncated γ2 Subunits Are Able to Bind to Full-length α<sub>1</sub> or β<sub>2</sub> Subunits—In order to investigate whether the reduction in the surface expression of α1β2γ2 receptors was due to an interaction of the γ2 fragments with α1 subunits, HEK cells were transfected with γ2-(1–234), γ2-(1–110), γ2-(1–117), γ2-(1–113), or γ2-(1–113) constructs together with full-length α1 subunits. Expressed subunits were detected from these cells, and were immunoprecipitated with α1-(1–9) antibodies. The precipitate was subjected to SDS-PAGE and Western blot analysis using digoxigenized γ2-(1–33) antibodies. Results obtained indicated that the fragments γ2-(1–234), γ2-(1–110), γ2-(1–117) (experiments not shown) as well as γ2-(1–113) (Fig. 3A) could be co-precipitated with α1 subunits from appropriately trans-
The values represent the amount of receptors expressed on the cell surface was determined as β-galactosidase (pg), from those obtained after co-transfection with sequences of the C-terminal truncated (domain with the typical cystein-loop, of four transmembrane domains (TM1-4)) and the large cytoplasmic loop between TM3 and TM4. The constructs (cDNA ratio 1:1:1:1) or β-galactosidase, a protein, that does not interfere with GABAA receptor assembly (100% value). The relative cell extracts were immunoprecipitated with: α1-(9) (A), γ2-(33) (B), or β2-(345–408) (C) antibodies. γ2-(1–113) or γ2-(1–110) fragments precipitated were identified by SDS-PAGE and Western blot analysis using digoxigenin γ2-(1–33) antibodies. The experiment was performed four times with comparable results.

As shown for cells co-transfected with γ2-(1–113) and with α1 subunits, three protein bands (apparent molecular mass 14, 17, and 20 kDa) were precipitated by antibodies (Fig. 3A). The molecular mass of the smallest protein band (14 kDa) corresponds with that expected for the unglycosylated γ2-(1–113) fragment. Since two glycosylation sites are present in γ2-(1–113) (28), the 17- and 20-kDa bands presumably represent partially and fully glycosylated γ2-(1–113) fragments, respectively. The fragment γ2-(1–110), in contrast to γ2-(1–113), could not be co-precipitated with α1 subunits from appropriately co-transfected HEK cells (Fig. 3A), although the actual expression of both truncated constructs could be confirmed by immunoprecipitation and subsequent detection of the proteins with γ2-(1–33) antibodies (Fig. 3B).

The inability to co-precipitate the fragment γ2-(1–110) indicated that α1-(1-9) antibodies did not cross-react with truncated γ2 subunits and support the conclusion of an interaction between γ2 fragments and α1 subunits. As with γ2-(1–113), three protein bands were observed for γ2-(1–110) fragments corresponding to differentially glycosylated proteins. The slight differences in the apparent molecular mass of the unglycosylated and partially glycosylated γ2-(1–113) and γ2-(1–110) frag-
ments (Fig. 3D) might have been due to incomplete denaturation by SDS and a corresponding atypical migration of these proteins with low molecular mass and distinct secondary structure (see “Discussion”).

In other experiments it was investigated whether γ2 fragments could also interact with β3 subunits. For this, HEK cells were co-transfected with truncated γ2 constructs together with full-length β3 subunits. Subunits expressed were precipitated from cell extracts with β2-(345–408) antibodies, and the precipitates were subjected to SDS-PAGE and Western blot analysis using digoxygenized γ2-(1–33) antibodies. The truncated γ2-(1–234), γ2-(1–130), and γ2-(1–117) subunits (experiments not shown), as well as the γ2-(1–113) fragment (Fig. 3C) could be co-precipitated with β3 subunits. A co-precipitation of γ2-(1–110) with β3 subunits, however, was not observed, again excluding a possible cross-reactivity of the β3-(345–408) antibodies with truncated γ2 subunits. The actual expression of the truncated subunits in HEK cells co-transfected with β3 subunits was confirmed by precipitation and detection of the fragments with γ2-(1–33) antibodies as shown for the fragments γ2-(1–113) and γ2-(1–110) in Fig. 3D. The amount of γ2-(1–110) precipitated with γ2-(1–33) antibodies was smaller than that of γ2-(1–113) (Fig. 3, B and D). This might have been due to a stabilization of the γ2-(1–113) fragment by binding to α1 or β3 subunits.

Amino Acids 111–113 of the γ2 Subunit Do Not Form the Binding Site for α1 or β3 Subunits—The co-precipitation of γ2-(1–113) with α1 or β3 subunits indicated that this fragment is able to directly bind to these subunits. Since γ2-(1–110) could not be co-precipitated with these subunits, it was possible that amino acids 111–113 form the binding site responsible for interaction with α1 and β3 subunits. In order to investigate this possibility, amino acids 111–113 of the γ2 subunit were replaced by amino acids present at homologous positions in subunits of the same receptor superfamily. As shown in Fig. 4A, the β1 subunit of the GABA A receptor contains a hydrophobic methionine at a position homologous to the hydrophilic threonine 111 of the γ2 subunit. Since it has been demonstrated that the β1 subunit is unable to assemble with α1 or β3 subunits (29), the γ2-(1–113)mut1 fragment was cloned, containing the T111M mutation (Fig. 4A). This construct was transfected into HEK cells together with α1 or β3 subunits. Cell extracts were precipitated with α1-(1–9) or β3-(345–408) antibodies, respectively, and the precipitate was subjected to SDS-PAGE and Western blot analysis using digoxygenized γ2-(1–33) antibodies. As shown in Fig. 4B, γ2-(1–113)mut1, similar to γ2-(1–113), was able to bind to α1 as well as to β3 subunits. In order to investigate whether the two phenylalanines at position 112 and 113 of the γ2 subunit were responsible for binding to α1 and β3 subunits in the γ2-(1–113) construct (Fig. 4A), γ2-(1–113)mut2 was cloned in which amino acids 111–113 of the γ2 subunit were replaced by the corresponding amino acids of the δ subunit of the acetylcholine receptor (Ile, Val, and Leu), that are completely different from those of the γ2 subunit (Thr, Phe, and Phe). As shown in Fig. 4B, γ2-(1–113)mut2 was also able to bind to α1 and to β3 subunits in appropriately transfected HEK cells.

The observation, that binding of the γ2-(1–113) fragment to α1 or β3 subunits was not dependent on the structure of the amino acid residues 111–113, indicated that amino acids 111–113 do not form the site(s) responsible for specific interaction with α1 or β3 subunits. Since the fragment γ2-(1–110) did not bind to α1 or β3 subunits, the presence of the amino acids 111–113 might have been necessary for stabilizing the conformation of the actual binding sites in the γ2-(1–113) construct.

Identification of Amino Acid Sequences of the γ2 Subunit That Are Important for Binding to α1 and β3 Subunits—In order to identify amino acid sequences of the γ2 subunit that are essential for the binding to α1 or β3 subunits, it would have been possible to replace additional amino acids of the γ2-(1–113) fragment by mutagenesis until its ability to bind to α1 or β3 subunits was lost. The replacement of a single amino acid, however, could change the conformation of the sites responsible for binding to α1 or β3 subunits even if the replaced amino acid is not located in the respective binding site. In order to avoid this possibility, a different strategy was used: instead of aiming to eliminate the binding sites by mutagenesis, it was investigated which γ2 amino acid sequences could induce binding to α1 or β3 subunits after incorporation into a fragment that originally could not bind to these subunits.

A comparison of the N-terminal sequences of the α1- and γ2 subunits indicated that the first 100 amino acids of the α1 subunit are homologous to γ2-(1–113) (Fig. 5). But in contrast to γ2-(1–113), α1-(1–100) could not be co-precipitated with α1 or β3 subunits after co-expression in HEK cells. In order to incorporate binding sites of the γ2 subunit, several chimeras were constructed by replacing the C-terminal part of the α1-(1–100) fragment with the corresponding γ2 sequences (Fig. 5). These chimeras were transfected into HEK cells together with full-length α1 or β3 subunits. Expressional subunits were precipitated from cell extracts with α1-(328–382) or β3-(345–408) antibodies. The epitopes recognized by these antibodies were present on the full-length α1 or β3 subunits, respectively, but not on the truncated chimeras. The precipitate was subjected to SDS-PAGE and the proteins were detected with digoxygenized α1-(1–9) antibodies in Western blots. The actual expression of the
chimeras was confirmed by precipitation and detection with α1-(1–9) antibodies (data not shown).

Although in Chim1 the nine C-terminal amino acids of the α1-(1–100) fragment were replaced by amino acids 105–113 of the γ2 subunit, due to the homology of sequences this chimera differed from α1-(1–100) in one amino acid (Thr95) only. As indicated in Fig. 5, this chimera could not be co-precipitated with full-length α1 or β3 subunits from appropriately co-transfected HEK cells, demonstrating the specificity of the α1-(328–382) and β3-(345–408) antibodies used and indicating that amino acids γ2-(105–113) are not able to induce the formation of binding sites for α1 or β3 subunits in α1-(1–100). In Chim2, the amino acid sequence α1-(78–100) was replaced by γ2-(91–113). This construct was able to bind to full-length α1, but not to full-length β3 subunits in appropriately co-transfected cells (Fig. 5). Since amino acids γ2-(105–113) were not sufficient to induce binding as discussed above, this indicated that amino acids 91–104 of the γ2 subunit are important for binding to α1 subunits. Finally Chim3 was constructed, in which the amino acid sequence α1-(70–100) was replaced by γ2-(83–113) (Fig. 5). Chim3 not only was able to bind to full-length α1, but also to full-length β3 subunits in appropriately transfected HEK cells. These results indicated that the additionally incorporated amino acids 83–90 of the γ2 subunit are able to induce the formation of the site responsible for binding to full-length β3 subunits.

Interestingly, three protein bands representing differentially glycosylated constructs were observed in the experiments with Chim3. This was similar to previous experiments (Fig. 3) performed with γ2-(1–113), but was in contrast to experiments with α1-(1–100), Chim1, or Chim2, where only two protein bands were observed. The only amino acid that could be glycosylated and differed between Chim3 and Chim2 was the newly introduced Asn90 of the γ2 subunit. These results suggest that Asn90 of the γ2 subunit is actually glycosylated, confirming a previous prediction on glycosylation of this amino acid (28).

To directly confirm that amino acids γ2-(91–104) mediate binding to α1 subunits, an α1-(1–100) fragment was constructed (Chim4, Fig. 5), in which amino acids γ2-(91–104) were incorporated, replacing amino acids α1-(78–91). This chimera, as expected, was able to bind to α1, but not to β3 subunits. In an approach to confirm that the sequence γ2-(83–90) was responsible for binding to β3 subunits, another α1-(1–100) fragment was constructed (Chim5), in which amino acids γ2-(83–90) replaced amino acids α1-(70–77). Chim5, however, could neither bind to α1 nor to β3 subunits (Fig. 5). Since Western blot analysis indicated that the newly introduced Asn90 was not glycosylated in Chim5, it was investigated whether glycosylation of Asn90 was essential for the formation of the β3-binding site. Glycosylation of asparagines requires the sequence N-X-S or N-X-T (where X is any amino acid except proline). Therefore Chim6 was cloned in which amino acids α1-(70–79) were replaced by amino acids γ2-(83–92). In this chimera Asn90 was glycosylated. Surprisingly, however, Chim6 was able to bind to β3 as well as to α1 subunits.

To further demonstrate the importance of the sequences γ2-(83–92) and γ2-(91–104) for the assembly of α1β3γ2 receptors, it was investigated whether Chim4 or Chim6 could inhibit the expression of α1β3γ2 receptors on the cell surface. For this Chim4 or Chim6 were transfected into HEK cells together with full-length α1, β3, and γ2 subunits and the amount of receptors on the cell surface was determined. In contrast to α1-(1–100), Chim4 and Chim6 were able to significantly reduce expression of α1β3γ2 receptors on the cell surface and the percent reduction was similar to that of the γ2-(1–113) construct (Fig. 5).

Secondary Structure Prediction for Amino Acids 71–113 of the γ2 Subunit—To investigate the secondary structure of the amino acid sequence γ2-(71–113), a neural network-based prediction using PHDsec (20, 21) was performed. As shown in Fig. 6A, the probability for the formation of an α-helix (solid line) was relatively high for amino acids 83–88. The probability for the formation of an extended structure (dashed line) was highest for amino acids 72–77 and 93–98, whereas the probability for the formation of a loop (dotted line) was highest for amino acids 80–83, 89–93, 98–103, and 108–113. Overall, from this prediction the probability for the formation of long range α-helices or β-sheet structures is low. Interestingly, however, the predicted short α-helix for amino acids γ2-(83–88) is located within the experimentally identified region important for binding to both β3 and α1 subunits. To evaluate the significance of the prediction in this region, force field calculations within a DMC optimization scheme were performed. A pool of structurally equivalent low energy conformations could be calculated that were separated by 8–12.5 kJ/mol from other local minimum structures and represented an unconstrained α-helix (backbone dihedral angles in the range of −60°) comprising one and a half turns. The α-helical conformation in this region was defined by electro-
Interestingly, two of these regions are identical with the \( \gamma_2 \) (83–90) or \( \gamma_2 \) (91–104) sequences identified to be important for binding to both \( \beta_3 \) and \( \alpha_1 \) or \( \alpha_2 \) subunits, respectively. Thus, amino acid residues 83–90 form a hydrophilic structure and amino acid residues 91–104 form a structure with intermediate hydrophobicity. In contrast, amino acid residues 105–113 that have been demonstrated to stabilize the conformation of the binding sites, are highly hydrophobic.

**DISCUSSION**

The N-terminal 113 Amino Acids of the \( \gamma_2 \) Subunit Contain Recognition Sites Important for Assembly with \( \alpha_1 \) and \( \beta_3 \) Subunits—The present study demonstrated that the N-terminal domain of the \( \gamma_2 \) subunit (\( \gamma_2 \) (1–234)) could be co-precipitated with full-length \( \alpha_1 \) or \( \beta_3 \) subunits after co-expression in HEK cells. This indicated that \( \gamma_2 \) (1–234) was able to bind to \( \alpha_1 \) as well as \( \beta_3 \) subunits. These results are consistent with \textit{in vitro} translation experiments, demonstrating that N-terminal sequences without transmembrane domains of \( \gamma_2 \) receptor \( \rho \) subunits (30) or of \( \kappa \) channel subunits (31) can bind to and be co-precipitated with full-length subunits. Binding between \( \gamma_2 \) (1–234) fragments and full-length \( \alpha_1 \) or \( \beta_3 \) subunits seemed to be fairly stable, because it survived extraction by detergent, immunoprecipitation, and several washing steps. In addition, heterodimers consisting of \( \gamma_2 \) (1–234) fragments and full-length \( \alpha_1 \) subunits were able to form specific high affinity benzodiazepine-binding sites assumed to be formed at the interface of \( \alpha_1 \) and \( \gamma_2 \) subunits in \( \gamma_2 \) receptors (26). This indicated that binding between truncated \( \gamma_2 \) constructs and the full-length subunits was not caused by unspecific interaction, but by a specific assembly process. This conclusion was strengthened by the observation that \( \gamma_2 \) (1–234) on co-transfection with \( \alpha_1 \), \( \beta_3 \), and \( \gamma_2 \) subunits was able to decrease the amount of \( \alpha_1 \beta_3 \gamma_2 \) receptors expressed on the cell surface, suggesting an interference of the truncated subunit with receptor assembly (11).

A subsequent reduction in the size of the truncated \( \gamma_2 \) subunit indicated, that \( \gamma_2 \) (1–113) was the smallest N-terminal fragment that could be co-precipitated with \( \alpha_1 \) or \( \beta_3 \) subunits and could interfere with \( \alpha_1 \beta_3 \gamma_2 \) receptor assembly. These results demonstrated that the N-terminal 113 amino acids of the \( \gamma_2 \) subunit contain binding sites for \( \alpha_1 \) as well as for \( \beta_3 \) subunits and that these sites are important for \( \gamma_2 \) receptor assembly.

The observation that the longest N-terminal fragment exhibits the strongest interference with \( \gamma_2 \) receptor assembly (Fig. 2B), can be explained by the possibility that longer fragments are more able to stabilize their binding sites for \( \alpha_1 \) or \( \beta_3 \) subunits. Alternatively, additional contact sites for these subunits could be located between \( \gamma_2 \) (1–234). Aromatic Acid Sequences \( \gamma_2 \) (91–104) and \( \gamma_2 \) (83–90) Mediate Assembly with \( \alpha_1 \) and \( \beta_3 \) Subunits—In contrast to \( \gamma_2 \) (1–113), the fragment \( \gamma_2 \) (1–110) could not interfere with \( \alpha_1 \beta_3 \gamma_2 \) receptor assembly and this fragment could not be co-precipitated with full-length \( \alpha_1 \) or \( \beta_3 \) subunits, indicating that the presence of amino acids 111–113 is necessary for assembly with \( \alpha_1 \) and \( \beta_3 \) subunits. After replacement of amino acid residues 111–113 in the \( \gamma_2 \) (1–113) fragment by site-directed mutagenesis, the resulting fragments were still able to bind to \( \alpha_1 \) and \( \beta_3 \) subunits. These results indicate that the structure of amino acid residues 111–113 of the \( \gamma_2 \) subunit is not essential for binding to \( \alpha_1 \) and \( \beta_3 \) subunits. Aromatic acid residues therefore do not directly form these binding sites. A hydrophobicity analysis for amino acid residues 70–135 (Fig. 6B) indicated that aromatic acid residues 105–113 form a highly hydrophobic structure. Presumably, an appropriate length of this sequence is required for an efficient interaction with other hydrophobic regions in the protein.
order to stabilize the conformation of the actual binding sites located more N-terminal.

These binding sites were then identified by incorporating various γ2 sequences into the α1(1–100) fragment. This fragment is homologous to γ2(1–113) but could not bind to full-length α1 or β3 subunits after co-expression in HEK cells. Replacement of amino acids α1(78–91) by γ2(91–104) in the α1(1–100) fragment (Chim4) was sufficient to induce binding to α1, but not to β3 subunits and this chimera was able to interfere with the assembly of α1β3γ2 receptors. This indicated that the sequence γ2(91–104) forms the binding site to α1 subunits. On replacement of amino acids α1(70–79) by γ2(83–92) in the α1(1–100) fragment, the resulting chimera (Chim6) was able to bind to β3 as well as to α1 subunits and could also interfere with the assembly of α1β3γ2 receptors. The importance of both γ2 regions for structure and function of GABA_A receptors is supported by the observation that the amino acid sequence γ2(83–104) is conserved during evolution and is identical in rat, mouse, chicken, bovine, and human γ2 subunits (19).

Predictions on the Structure of Amino Acid Sequences γ2(83–90) and γ2(91–104)—Hydropathy analysis, a neural network-based secondary structure prediction (PHDsec), as well as force field optimization within a Dynamic Monte Carlo scheme, indicated that the sequence γ2(83–90), that induces binding to β3 and α1 subunits, forms a hydrophilic structure that contains a short α-helix. Since electrostatic interactions and a hydrogen bond stabilize the α-helix as a structural element, this helix is ideally suited as a specific recognition site and presumably interacts with another hydrophilic structure on the β3 or α1 subunit. The α-helix is terminated by an aromatic phenyalanine and the asparagine 90. Since glycosylation of Asn90 is essential for the formation of the benzodiazepine-binding site of GABA_A receptors that is assumed to be located at the interface of γ2 and α1 subunits (26). In order to contribute to this site, these residues not only should be located close to the α1/γ2 interface, but should also be located in close proximity to each other. Interestingly, the amino acid sequence γ2(91–104) that is sufficient to induce binding to the α1 subunit is located between these two amino acid residues in the primary structure of this subunit. Since amino acid Phe77 is located in the center of a highly hydrophobic region (Fig. 6B), it is reasonable to assume that Phe77 is not located on the surface but in the interior of the γ2 subunit. The suggested interaction of Phe77 with the phenyl substituent of diazepam during binding to the benzodiazepine site of GABA_A receptors (32) will therefore strongly affect the conformation of the γ2 subunit.

In contrast, Met130 is located at the end of the sequence γ2(121–130) that forms a structure with a hydrophobicity comparable to that of γ2(91–104) (Fig. 6B). It is tempting to speculate that amino acids γ2(121–130) form another part of the α1/γ2 contact site, the core of which is formed by γ2(91–104). Interaction of diazepam with Met130 might then increase the cleft between α1 and γ2 subunits and thus cause an additional change in the conformation of the GABA_A receptor.

In addition to Phe77 and Met130 of the γ2 subunit, amino acid residues His101, Tyr159, Thr206, and Tyr209 of the α1 subunit also contribute to the formation of the benzodiazepine binding pocket (26). The observation that these α1 amino acid residues (data not shown), as well as Phe77 and Met130 of the γ2 subunit are located in hydrophobic regions of the respective subunits, further supports the conclusion that the whole interface between α1 and γ2 subunits is formed by hydrophobic interactions and that the sequence γ2(91–104) contributes to this interface. In the completely assembled receptor, Phe77 and Met130 of the γ2 subunit must be in a position close to His101, Tyr159, Thr206, and Tyr209 of the α1 subunit to form the benzodiazepine binding pocket. The additional observation that a benzodiazepine-binding site is formed by heterodimers consisting of full-length α1 and truncated γ2(1–234) constructs, thus, supports the conclusion that amino acid residues forming this site exhibit the same relative position in heterodimers as in intact α1β3γ2 receptors. This indicates that the sequence γ2(91–104) identified to form the primary contact between γ2 and α1 subunits also forms part of the interface between these subunits in the completely assembled receptor (Fig. 7).

Amino Acid Sequence γ2(83–90) Forms Part of the Interface to β3 Subunits—The observation that amino acids γ2(83–92) not only mediate binding to β3 but also to α1 subunits could be explained by the possibility that this sequence forms the primary binding site to α1 and β3 subunits and that further assembly steps and conformational changes are necessary for the formation of the final interface between subunits. The robust binding between the truncated γ2 subunits and β3 or α1 subunits, however, indicates that a shift of contact sites would only be possible by a change in the conformation of the γ2(83–90) sequence mediating this binding. This is highly improbable. Force field calculations within a Dynamic Monte Carlo optimization scheme indicated that the α-helix formed by γ2(83–90) is stable enough to be retained in the tertiary and quaternary structure of the receptor. In addition, the hydrophilic sequence γ2(83–90) is surrounded by the strongly hydrophobic sequences γ2(70–82) and γ2(105–113), as well as by the sequence γ2(91–104) with intermediate hydrophobicity, indicating that a change in the interactions between γ2(83–90) and
neighboring sequences and thus, a conformational change of γ2(83–90) is not easily possible. Therefore it can be assumed that the sequence γ2(83–90) not only forms the primary contact site but also the final interface between γ2 and β3 or γ2 and α1 subunits in the completely assembled receptor.

Evidence discussed above indicates that the α1/γ2 contact is formed by hydrophobic interactions partially mediated by the sequence γ2(91–104). The hydrophilic sequence γ2(83–90) then presumably forms the interface between γ2 and β3 subunits in completely assembled α1β3γ2 receptors (Fig. 7). In the absence of β3 subunits, however, the sequence γ2(83–90) possibly could be used to accommodate a second α1 subunit. Assembly of a γ2 with two α1 subunits is consistent with the observation that α1 and γ2 subunits under certain conditions are able to form chloride ion channels that can be opened by GABA (2, 33). Conflicting results on the existence of pentameric α1γ2 receptors (2, 33), however, and recent studies indicating that the α1γ2 subunit combination predominantly forms subunit dimers (4) that could not be identified on the surface of transfected cells (10, 15), indicate that α1γ2 receptors form less readily than α1β3γ2 receptors. As with the nicotinic acetylcholine receptor (34), additional sequences C-terminal to γ2(1–113) might exist that could contribute to a selective assembly with α1 or β3 subunits. Further experiments will have to identify these sequences as well as the respective counterparts of the γ2-binding site on α1 and β3 subunits.

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GABA<sub>A</sub> Receptor Assembly: IDENTIFICATION AND STRUCTURE OF γ<sub>2</sub> SEQUENCES FORMING THE INTERSUBUNIT CONTACTS WITH α1 AND β3 SUBUNITS

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