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Alternate Use of Distinct Intersubunit Contacts Controls $GABA_A$ Receptor Assembly and Stoichiometry

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$GABA_A$ receptors are the major inhibitory transmitter receptors in the CNS. Recombinant $GABA_A$ receptors composed of $\alpha_1\beta_3\gamma_2$ subunits have been demonstrated to assemble as pentamers consisting of two α_1, two β_3, and one γ_2 subunit. Using truncated and chimeric α_1 subunits, we identified the α_1(80–100) sequence as a major binding site for γ_2 subunits. In addition, we demonstrated its direct interaction with γ_2(91–104), a sequence that previously has been identified to form the contact to α_1 subunits. The observation that the amino acid residues α_1P96 and α_1H101, which can be photolabeled by [^{3}H]flunitrazepam, are located within or adjacent to the α_1(80–100) sequence, indicates that the benzodiazepine binding site of $GABA_A$ receptors is located close to this intersubunit contact. The observation that α_1(80–100) interacts with γ_2 but not with β_3 subunits indicates the existence of an additional β_3 binding site on α_1 subunits. The preferred alternate use of the γ_2 and β_3 binding sites in two different α_1 subunits of the same receptor ensures the incorporation of only a single γ_2 subunit and thus, determines subunit stoichiometry of $\alpha_1\beta_3\gamma_2$ receptors. Distinct binding sites and their alternate use can therefore explain how subunits of hetero-oligomeric transmembrane proteins assemble into a defined protein complex.

Key words: $GABA_A$ receptor; assembly; subunit interface; structure; subunit stoichiometry; benzodiazepine binding pocket

Members of the ligand-gated ion channel family, such as the nicotinic acetylcholine receptor (nAChR), the $GABA_A$ receptor, the glycine receptor, or the 5-HT_3 receptor, are heteromeric proteins composed of five subunits (Bertrand and Changeux, 1995). The subunits of these proteins are cotranslationally inserted into the membrane, lumen, or both, of the endoplasmic reticulum, after which the subunits fold and oligomerize (Verrall and Hall, 1992; Green and Claudio, 1993; Connolly et al., 1996a; Griffon et al., 1999). During these folding and oligomerization events, each subunit must recognize its neighbors by specific high-affinity interactions. To achieve the correct order of subunits around the pore, in addition selective discriminations must be made between different subunits. So far, little is known about the molecular structures involved in these mechanisms.

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the CNS. These receptors are chloride ion channels that can be opened by GABA (Macdonald and Olsen, 1994) and are 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 (Sieghart, 1995).

$GABA_A$ receptors are hetero-oligomeric proteins consisting of five subunits (Nayeem et al., 1994; Tretter et al., 1997). So far at least 19 $GABA_A$ receptor subunits belonging to several subunit classes (six α, three β, three γ, one δ, one ϵ, one π, one θ, and three ρ) have been identified in mammalian brain (Barnard et al., 1998; Sieghart et al., 1999). *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 (Fritschy et al., 1992; Pirker et al., 2000) raises the possibility for the formation of an extremely large variety of $GABA_A$ receptor subtypes. However, not all receptors that can be formed theoretically are formed in the cells (Sieghart et al., 1999). Thus, $GABA_A$ receptor heterogeneity is limited by the temporal and spatial pattern of subunit expression and by the selective oligomerization mediated by receptor assembly.

Recently, the subunit stoichiometry and arrangement of recombinant $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors have been determined (Tretter et al., 1997). In this receptor only a single γ_2 subunit is present and is situated between an α_1 and a β_3 subunit. In addition, the amino acid sequence γ_2(91–104) was identified to form the binding site to α_1 subunits (Klausberger et al., 2000).

In the present study truncated and chimeric α_1 subunits were used to identify the α_1 sequence mediating assembly with γ_2 subunits. It was demonstrated that the sequence α_1(80–100) directly interacts with γ_2(91–104) and forms part of the α_1–γ_2 interface. The observation that α_1(80–100) mediates assembly with γ_2 but not with β_3 subunits suggests the existence of an additional binding site for β_3 subunits. The preferred alternate use of the γ_2 and β_3 binding sites in different α_1 subunits of the same receptor indicates that the α_1–γ_2 intersubunit contact controls assembly and subunit stoichiometry of $GABA_A$ receptors.

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MATERIALS AND METHODS

Antibodies. The antibodies anti-peptide α_1(1–9), anti-peptide β_3(1–13), anti-peptide γ_2(319–366), and anti-peptide γ_2(1–33) were generated and affinity purified as described previously (Tretter et al., 1997; Jechlinger et al., 1998; Klausberger et al., 2000).

Generation of cDNA constructs. For the generation of recombinant receptors, α_1, β_3, and γ_2 subunits of $GABA_A$ receptors from rat brain were cloned and subcloned into pCDM8 expression vectors (Invitrogen, San Diego, CA) as described previously (Tretter et al., 1997). Truncated subunits were constructed by PCR amplification using the full-length subunit as a template. The PCR primers contained *Eco*RI and *Hin*dIII restriction sites, which were used to clone the fragments into pCDNAI-Amp vectors (Invitrogen). The truncated subunits were confirmed by sequencing. Chimeras were constructed using the "gene splicing by overlap extension" technique (Horton, 1993) and were cloned into pCDNAIAmp vectors using the *Eco*RI and *Hin*dIII restriction sites of the primers.

Culture and transfection of human embryonic kidney 293 cells. Transformed human embryonic kidney (HEK 293) cells (CRL 1573; American Type Culture Collection, Rockville, MD) were cultured as described in Tretter et al. (1997). We transfected 3×10^6 cells with 20 μg of subunit cDNA for single subunit transfection using the calcium phosphate precipitation method (Chen and Okayama, 1988). After cotransfection with two different subunits, for each subunit 10 μg of cDNA was used. When cells were cotransfected with three different subunits, 7 μg of cDNA was used per subunit. A total of ~20 μg of cDNA per transfection and a cDNA ratio of 1:1:1 seemed to be optimal for the expression of $GABA_A$ receptors under the conditions used, as judged by receptor binding studies in cells transfected with α_1, β_3, and γ_2 subunits. Changing the subunit ratio by doubling the amount of a single subunit at the cost of other subunits did not significantly change the number of [^{3}H]Ro 15–1788 binding sites detected.

The cells were then harvested 36 hr after transfection. At this time point the number of [^{3}H]Ro 15–1788 binding sites formed per milligram of protein was at its maximum for cells transfected with α_1, β_3, and γ_2 subunits. Results obtained, however, did not change when cells were harvested 34–48 hr after transfection. In addition, judged by Western blot analysis, expression levels of full-length, truncated, or chimeric subunits were comparable (see Figs. 1, 6) at all harvesting times.

Purification and immunoprecipitation of complete, truncated, and chimeric subunits. The culture medium was removed from transfected HEK 293 cells, and cells from four culture dishes were extracted with 800 μl of a Lubrol extraction buffer (1% Lubrol PX, 0.18% phosphatidylcholine, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, containing 0.3 mM PMSF, 1 mM benzamidine, and 100 μg/ml bacitracin) for 8 hr at 4°C. The extract was centrifuged for 40 min at 150,000 $\times$ *g* at 4°C, and the clear supernatant was incubated overnight at 4°C under gentle shaking with 15 μg antibodies directed against the full-length subunit. After addition of Immunoprecipitin (Life Technologies, Gaithersburg, MD; for preparation, see Tretter et al., 1997) and 0.5% nonfat dry milk powder and shaking for additional 3 hr at 4°C, the precipitate was washed three times with a low-salt buffer for immunoprecipitation (IP low buffer) (50 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl, and 1 mM EDTA, pH 8.0). The precipitated proteins were dissolved in sample buffer [108 mM Tris-sulfate, pH 8.2, 10 mM EDTA, 25% (w/v) glycerol, 2% SDS, and 3% dithiothreitol]. SDS-PAGE and Western blot analysis with digoxygenized antibodies was performed as described in Tretter et al. (1997).

All truncated or chimeric constructs used in this study could be expressed to a comparable extent after single transfection into HEK cells. After cotransfection of different constructs, however, the stability of fragments that could not bind stably to each other was reduced. This might have been caused by proteolytic degradation because of an unstable or unproductive interaction of the fragments. In all control experiments the extent of expression of fragments was therefore determined in singly transfected HEK cells.

Immunoprecipitation of receptors expressed on the cell surface. The culture medium was removed from HEK 293 cells transfected with cDNA (21 μg per 3×10^6 cells) of $GABA_A$ receptor subunits (cDNA ratio 1:1:1), and the cells were washed once with PBS (in mM: 2.7 KCl, 1.5 KH_2PO_4, 140 NaCl, and 4.3 Na_2HPO_4, pH 7.3). Cells were then detached from the culture dishes by incubating with 2.5 ml of 5 mM EDTA in PBS for 5 min at room temperature. The resulting cell suspension was diluted in 6.5 ml of cold DMEM and centrifuged for 5 min at 1000 $\times$ *g*. The pellet from two dishes was incubated with 30 μg of α_1(1–9) antibodies in 3 ml of the same medium for 30 min at 37°C. Cells were again pelleted, and free antibodies were removed by washing twice with 10 ml of PBS buffer. Then receptors were extracted with IP low buffer containing 1% Triton X-100 for 1 hr under gentle shaking. Cell debris was removed by centrifugation (30 min; 150,000 $\times$ *g*; 4°C). After addition of Immunoprecipitin and 0.5% nonfat dry milk powder and shaking for 3 hr at 4°C, the precipitate was centrifuged for 10 min at 10,000 $\times$ *g* and washed three times with IP low buffer. The precipitated proteins were dissolved in sample buffer and subjected to SDS-PAGE and Western blot analysis using digoxygenized antibodies. Secondary antibodies (anti-digoxygenin-AP, Fab fragments; Roche Diagnostics GmbH, Mannheim, Germany) were visualized by the reaction of alkaline phosphatase with CSPD (Tropix, Bedford, MA). Protein bands were quantified by densitometry of Kodak X-Omat S films with the Docu Gel 2000i gel documentation system using restriction fragment length polymorphism scan software (MWG Biotech, Ebersberg, Germany). The linear range of the detection system was established by determining the antibody response to a range of antigen concentrations after immunoblotting. The experimental conditions were designed such that immunoreactivities obtained in the assay were within this linear range, thus permitting a direct comparison of the amount of antigen applied per gel lane between samples. Different exposures of the same membrane were used to ensure that the measured signal was in the linear range of the x-ray film.

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 $GABA_A$ receptor subunits (data not shown). These antibodies could not precipitate any $GABA_A$ receptor subunits under the conditions used. A possible redistribution of the antibodies during the extraction procedure could be excluded by an experiment performed analogous to that described in Klausberger et al. (2000).

Immunofluorescence. HEK cells were fixed with 2% paraformaldehyde in PBS 30–35 hr after transfection, followed by a 10 min wash in 50 mM NH_4Cl in PBS. Washes between incubation steps were performed in PBS. For detection of intracellular receptors, cells were permeabilized with 0.1% Triton X-100 for 5 min. Blocking was performed in 5% bovine serum albumin (BSA) in PBS for 10 min, followed by an incubation with primary antibody in 1% BSA in PBS. Primary antibodies were detected with goat anti-rabbit $IgG_{(H+L)}$ bodipy FL (Molecular Probes, Eugene, OR) in 1% BSA in PBS. Labeling was visualized using a Zeiss Axiovert 135 M microscope attached to a confocal laser system (Carl Zeiss LSM 410, BRD), equipped with an argon laser and a helium–neon laser and suitable filter sets. To verify that labeling of cells without permeabilization was restricted to the cell surface, parallel samples were stained with antibodies directed against the intracellular loop of $GABA_A$ receptor subunits (data not shown). These antibodies detected $GABA_A$ receptor subunits only after permeabilization of transfected cells.

Electrophysiological investigations. HEK cells were cotransfected with $GABA_A$ receptor subunits together with pEGFP-N1 (Clontech, Palo Alto, CA) as a transfection marker. Electrophysiologic recordings were performed at room temperature 1–2 d after transfection using the perforated patch technique (Rae et al., 1991). GABA and $ZnCl_2$ were applied using a DAD-12 superfusion system (Adams and List Associates Ltd., Westbury, NY). Extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES, pH 7.4. The pipette solution contained (in mM): 140 KCl, 11 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 0.2 amphotericin B, pH 7.2. The cells were clamped at −60 mV, and currents were filtered at 1 kHz, recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and analyzed with Clampfit software (Axon Instruments).

RESULTS

Truncated α_1 constructs are able to assemble with full-length γ_2 subunits

In the present study C-terminally truncated α_1 subunits (Fig. 1*A*) were cloned, and it was investigated which of these fragments could assemble with full-length γ_2 subunits. For this, HEK cells were cotransfected with γ_2 subunits and either full-length or truncated α_1 subunits. Expressed subunits were extracted from these cells and were immunoprecipitated with γ_2(319–366) antibodies. The precipitate was subjected to SDS-PAGE and Western blot analysis using digoxygenized α_1(1–9) antibodies. As shown in Figure 1*B*, full-length α_1 subunits (protein band of 51 kDa), as

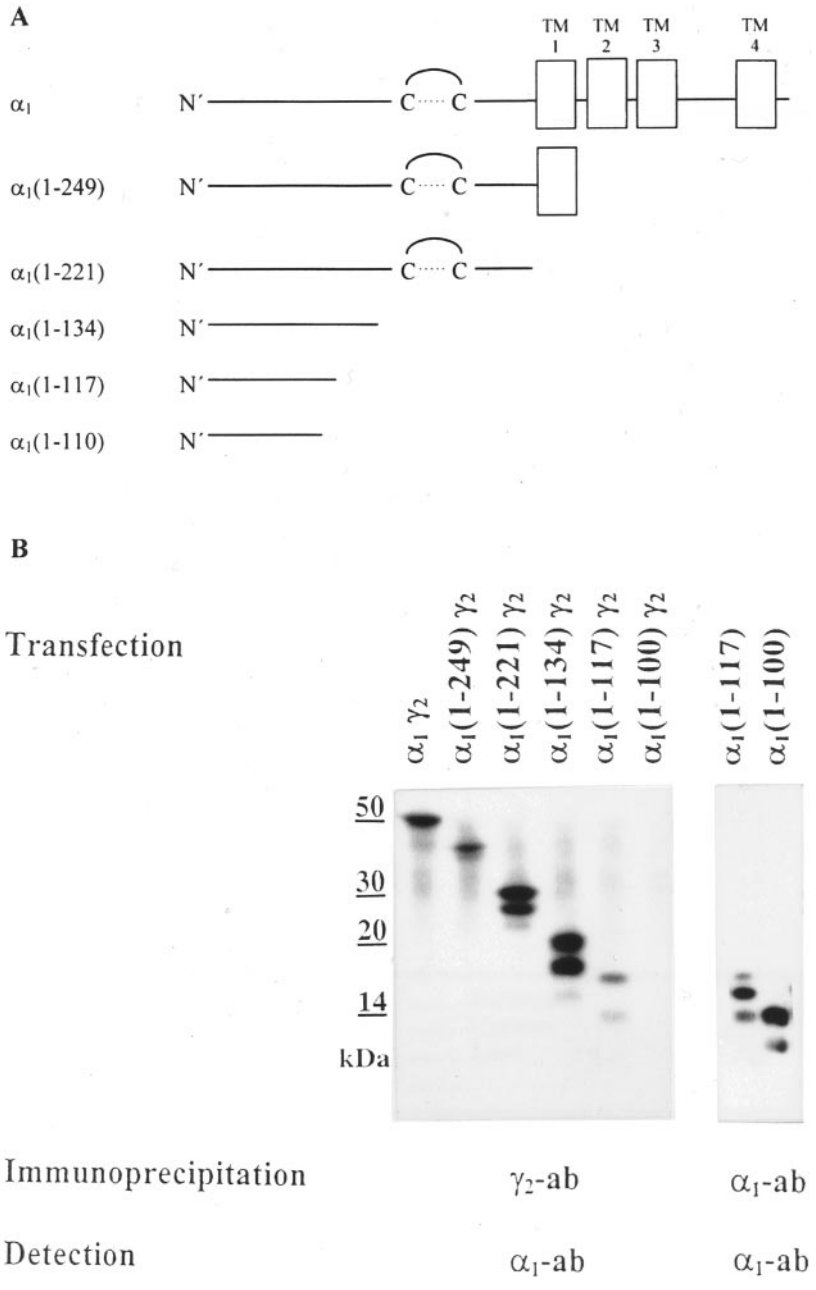

Figure 1. Coimmunoprecipitation of truncated α_1 with full-length γ_2 subunits. *A,* Schematic drawing of the α_1 subunit and of C-terminally truncated α_1 constructs. The α_1 subunit consists of the N-terminal extracellular domain with the typical cysteine loop, of four transmembrane domains (TM1–4), and the large cytoplasmic loop between TM3 and TM4. The sequences of the C-terminally truncated α_1 constructs are indicated by the amino acid numbers given in *parentheses*. A *1* represents the first amino acid of the mature subunit. *B,* HEK cells were transfected with truncated α_1 constructs together with full-length γ_2 subunits, as indicated. Cell extracts were immunoprecipitated with γ_2(319–366) antibodies. α_1 fragments coprecipitated were identified by SDS-PAGE and Western blot analysis using digoxygenized α_1(1–9) antibodies (*lanes 1–6*). In control experiments (*lanes 7, 8*), truncated α_1(1–117) and α_1(1–100) constructs were transfected separately into HEK cells, and the fragments formed were precipitated with α_1(1–9) antibodies and subjected to SDS-PAGE and Western blot analysis using digoxygenized α_1(1–9) antibodies. The protein fragments formed from these constructs [apparent molecular mass: 14, 16, and 18 kDa for α_1(1–117) and apparent molecular mass: 12 and 14 kDa for α_1(1–100)] were expressed to a similar extent. Interestingly, the relative abundance of the unglycosylated, monoglycosylated, and diglycosylated α_1(1–117) fragments differed when these fragments were expressed in the absence or presence of γ_2 subunits, possibly suggesting that γ_2 subunits preferentially assemble with fully glycosylated α_1(1–117) fragments. All experiments were performed three times with comparable results.

well as the fragments α_1(1–249) (two bands of 39 and 41 kDa), α_1(1–221) (three bands of 26, 28, 30 kDa), α_1(1–134) (three bands of 17, 19, 21 kDa), and α_1(1–117) [three bands of 14, 16 (very weak), and 18 kDa] could be coimmunoprecipitated with full-length γ_2 subunits from appropriately transfected HEK cells. Because all α_1 fragments investigated contained two glycosylation sites, the three bands presumably represented unglycosylated, partially, and fully glycosylated fragments. The observation that only one or two protein bands could be observed for the full-length α_1 subunit or the α_1(1–249) construct might indicate that these subunits predominantly occur in the double-glycosylated or double- and mono-glycosylated state, respectively. This conclusion is supported by the apparent molecular mass of these proteins that amounted to 51 kDa or 41 kDa for the full-length α_1 subunit or the α_1(1–249) construct, respectively, although the ungycosylated mass of these proteins can be calculated to be 47 or 37 kDa. Alternatively, the differentially glycosylated protein bands with higher molecular mass might not have been resolved by the 15% polyacrylamide gel used in this investigation.

Binding between γ_2 subunits and these fragments seemed to be the result of a specific assembly process because after cotransfection of HEK cells with full-length γ_2 subunits and the fragment α_1(1–221), high-affinity binding sites for the benzodiazepine [^{3}H]Ro15–1788 were formed. These sites are assumed to be located at the interface of α_1 and γ_2 subunits in $GABA_A$ receptors (Sigel and Buhr, 1997). The number of [^{3}H]Ro15–1788 binding sites formed (16.8 ± 2.3 fmol/mg protein) was comparable with that observed after transfection of HEK cells with full-length α_1 and γ_2 subunits (17.6 ± 1.2 fmol/mg protein) but was significantly smaller ($p < 0.001$; unpaired Student's t test) than that of HEK cells transfected with α_1, β_3, and γ_2 subunits in parallel experiments (874 ± 19 fmol/mg of protein). Data given

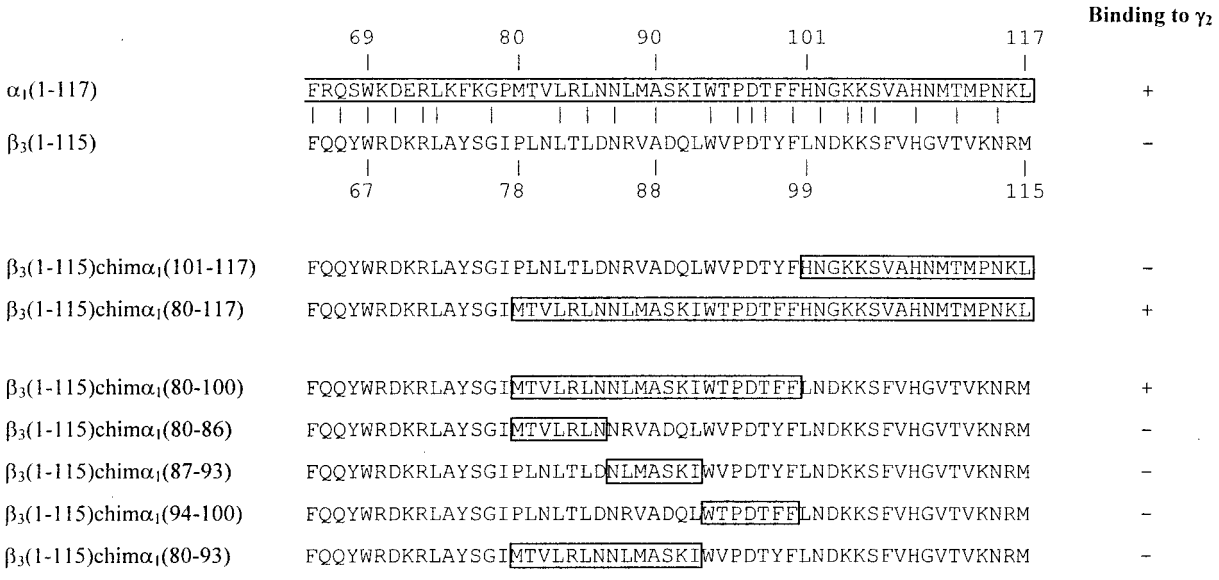

Figure 2. α_1(80–100) forms the contact site to γ_2 subunits. C-terminal sequences of the fragments α_1(1–117), β_3(1–115), and of different chimeras are shown. Amino acid sequences of the α_1 subunit are *boxed*. HEK cells were cotransfected with these constructs together with γ_2 subunits, and a possible coimmunoprecipitation was investigated, as described in Results. + indicates binding, and − indicates absence of binding between these constructs and full-length γ_2 subunits. The experiments were performed three times with similar results.

are mean values ± SEM from three different experiments performed in triplicate.

In contrast to the protein fragments formed from α_1(1–117), the fragments formed from α_1(1–100) could not be coprecipitated with γ_2 subunits from appropriately cotransfected HEK cells (Fig. 1*B*), although the extent of expression of these fragments (12 and 14 kDa) was similar to that of the fragments derived from the α_1(1–117) construct (Fig. 1*B*, *lanes 7, 8*). The inability of γ_2(319–366) antibodies to coprecipitate the fragment α_1(1–100) confirmed previous conclusions (Jechlinger et al., 1998) that these antibodies did not cross-react with α_1 subunits. These results indicate that the amino acid sequence of the α_1 subunit that is responsible for binding to γ_2 subunits is located in the N-terminal 117 amino acids of the α_1 subunit.

Amino acid sequence α_1(80–100) mediates binding to γ_2 subunits

To identify this contact site, it was investigated which α_1 amino acid sequence could induce binding to γ_2 subunits after incorporation into a fragment that originally could not bind to these subunits. The fragment β_3(1–115) seemed to be suitable for this purpose because it is homologous to α_1(1–117) but could not be coprecipitated with γ_2 subunits (or β3 subunits) after coexpression in HEK cells (Fig. 2). To incorporate binding sites of the α_1 subunit, several chimeras were constructed by replacing the C-terminal part of the β_3(1–115) fragment with the corresponding α_1 sequences (Fig. 2). These chimeras were transfected into HEK cells together with full-length γ_2 subunits. Expressed subunits were precipitated from cell extracts with γ_2(319–366) antibodies. The precipitate was subjected to SDS-PAGE, and the proteins were detected with digoxygenized β_3(1–13) antibodies in Western blots. The actual expression of the chimeras was confirmed by precipitation and detection with β_3(1–13) antibodies (data not shown).

In β_3(1–115)chimα_1(101–117) the 17 C-terminal amino acids of the β_3(1–115) fragment were replaced by amino acids 101–117 of the α_1 subunit. As indicated in Figure 2, this chimera could not be coprecipitated with full-length γ_2 subunits from appropriately cotransfected HEK cells, demonstrating the specificity of the γ_2(319–366) antibodies used and indicating that amino acids α_1(101–117) are not able to induce binding to γ_2 subunits. In β_3(1–115)chimα_1(80–117), the amino acid sequence β_3(78–115) was replaced by α_1(80–117). This construct was able to bind to full-length γ_2 subunits (Fig. 2), but not to full-length β_3 subunits (data not shown). Because amino acids α_1(101–117) were not sufficient to induce binding to γ_2 subunits as discussed above, this indicated that amino acids 80–100 of the α_1 subunit are important for binding to γ_2 subunits. To directly confirm this conclusion, the construct β_3(1–115)chimα_1(80–100) was generated (Fig. 2), in which amino acids α_1(80–100) were incorporated into β_3(1–115), replacing amino acids β_3(78–98). As expected, this chimera was able to bind to γ_2 subunits.

To investigate which part of the α_1(80–100) sequence is responsible for binding to γ_2 subunits, four additional chimeras were constructed. In β_3(1–115)chimα_1(80–86), amino acids β_3(78–84) were replaced by the amino acids α_1(80–86), in β_3(1–115)chimα_1(87–93) the sequence β_3(85–91) was replaced by α_1(87–93), in β_3(1–115)chimα_1(94–100) the sequence β_3(92–98) was replaced by amino acids α_1(94–100), and in β_3(1–115)chimα_1(80–93) the sequence β_3(78–91) was replaced by α_1(80–93) in the β_3(1–115) fragment. None of these chimeras was able to bind to γ_2 subunits. These results indicate that the whole α_1(80–100) sequence is necessary for binding to γ_2 subunits.

The sequence α_1(80–100) is important for the assembly of GABA$_A$ receptors composed of $\alpha_1\beta_3\gamma_2$ subunits

To investigate the importance of the α_1(80–100) sequence not only for the assembly of truncated subunits and dimers, but also for assembly of full-length subunits and pentameric receptors, a full-length α_1 chimera (α_1*) was constructed in which the sequence α_1(79–100) was replaced by the sequence β_3(77–98). The additional exchange of the amino acid 79 of the α_1 subunit in α_1* was necessary to avoid the generation of two adjacent prolines that could have destroyed the conformation of the resulting chi-

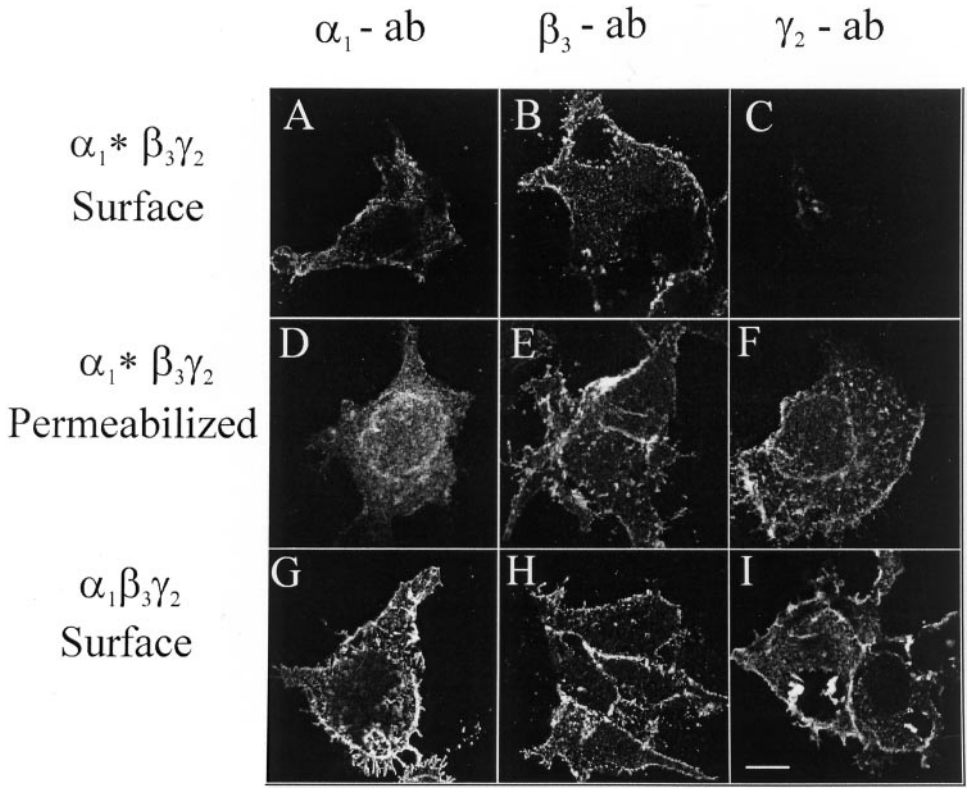

Figure 3. Immunofluorescence of HEK cells cotransfected with $GABA_A$ receptor subunits. HEK cells were cotransfected with α_{1*}, β_3, and γ_2 subunits *(A–F)* or with α_1, β_3, and γ_2 subunits *(G–I)*. Immunofluorescence was performed using α_1(1–9) antibodies *(A, D, G)*, β_3(1–13) antibodies *(B, E, H)*, or γ_2(1–33) antibodies *(C, F, I)* on the cell surface *(A–C, G–I)* or in permeabilized cells *(D–F)* by confocal laser microscopy (single sections). Scale bar, 10 μm. The experiment was performed four times with similar results.

mera (Fig. 2). In control experiments, it was demonstrated that the extent of expression of the α_1* chimera was similar to that of the α_1 subunit in HEK cells (data not shown).

HEK cells were then cotransfected with α1*, β_3, and γ_2 subunits and subunits expressed were investigated by immunofluorescence and confocal laser microscopy. As shown in Figure 3, α_1* (Fig. 3*A*) and β_3 subunits (Fig. 3*B*) could be easily detected on the surface of intact cells, but for the γ_2 subunit only a weak labeling was observed (Fig. 3*C*), although the labeling of the γ_2 subunit in permeabilized cells (Fig. 3*F*) was comparable with that of α_1 (Fig. 3*D*) and β_3 (Fig. 3*E*) subunits. In HEK cells cotransfected with α_1, β_3, and γ_2 subunits, all three subunits could be detected on the cell surface (Fig. 3*G–I*). Because previous results have indicated that α_1 subunits alone in contrast to $\alpha_1\beta_3$ subunit combinations do not form receptors that are incorporated into the plasma membrane to a significant extent, these results suggested that α_1* predominantly formed receptors with β_3 subunits that are expressed on the cell surface, but the ability to form receptors containing γ_2 subunits was significantly reduced.

To quantify this phenomenon, HEK cells were cotransfected with α_1, β_3, and γ_2 subunits or with α_1*, β_3, and γ_2 subunits. $GABA_A$ receptors expressed on the surface of the cells were labeled by an incubation of intact cells with α_1(1–9) antibodies. Antibody labeled receptors were extracted and precipitated by addition of Immunoprecipitin. The precipitate was subjected to SDS-PAGE and Western blot analysis using digoxygenized α_1(1–9) antibodies (Fig. 4). In contrast to α_1 subunits (51 kDa, the weak 46 kDa band presumably represents a degradation product), the protein band of α_1* exhibited an apparent molecular mass of 53 kDa because of an additional glycosylated asparagine at position 80 of the newly introduced β_3 subunit insert. The protein bands were quantified, and results obtained indicated that α_1* and α_1 subunits were expressed to a similar extent on the surface of transfected cells. Then, the Western blot was stripped and analyzed using digoxygenized β_3(1–13) antibodies (Fig. 4). Finally, blots were again stripped and were probed with γ_2(1–33) antibodies. Whereas similar amounts of β_3 subunits (54 kDa) were coprecipitated with α_1 subunits from $\alpha_1\beta_3\gamma_2$ or α_1*$\beta_3\gamma_2$ transfected cells, the amount of γ_2 subunits (49 kDa) coprecipitated with α_1* subunits was only 32 ± 3% (mean ± SEM, n = 3; from three different transfections) of that coprecipitated with α_1 subunits. Similar results were obtained when the order of detection of subunits was changed and Western blots were first probed with γ_2(1–33) antibodies and after stripping were re-analyzed with α_1(1–9) or β_3(1–13) antibodies. These results indicate that α_1* was able to form receptors with β_3 subunits, but that the ability to form receptors containing γ_2 subunits was reduced by 68%.

Properties of $GABA_A$ receptors composed of α_1*$\beta_3\gamma_2$ or α_1*β_3 subunits

To investigate the properties of the receptors formed, HEK cells cotransfected with α_1*, β_3, and γ_2 subunits were subjected to patch-clamp analysis, and whole-cell recordings were compared with those from cells transfected with α_1, β_3, and γ_2 subunits. GABA exhibited an apparent EC_{50} of 68 ± 10 μM (mean ± SEM; n = 11 cells from different plates; total of four transfections) (Fig. 5*E*) in HEK cells transfected with α_1*, β_3, and γ_2 subunits and elicited a maximal current of 713 ± 170 pA at a GABA concentration of 1000 μM (Fig. 5*A*). In contrast, GABA exhibited an EC_{50} of 7.7 ± 2.3 μM (mean ± SEM; n = 8 cells from different plates; total of four transfections) (Fig. 5*E*) in HEK cells transfected with α_1, β_3, and γ_2 subunits and elicited a maximal current of 2988 ± 469 pA at a concentration of 300 μM (Fig. 5*B*). These data not only indicated that GABA exhibited a 10-fold reduced potency for activating α_1*$\beta_3\gamma_2$ receptors, but also that the maxi-

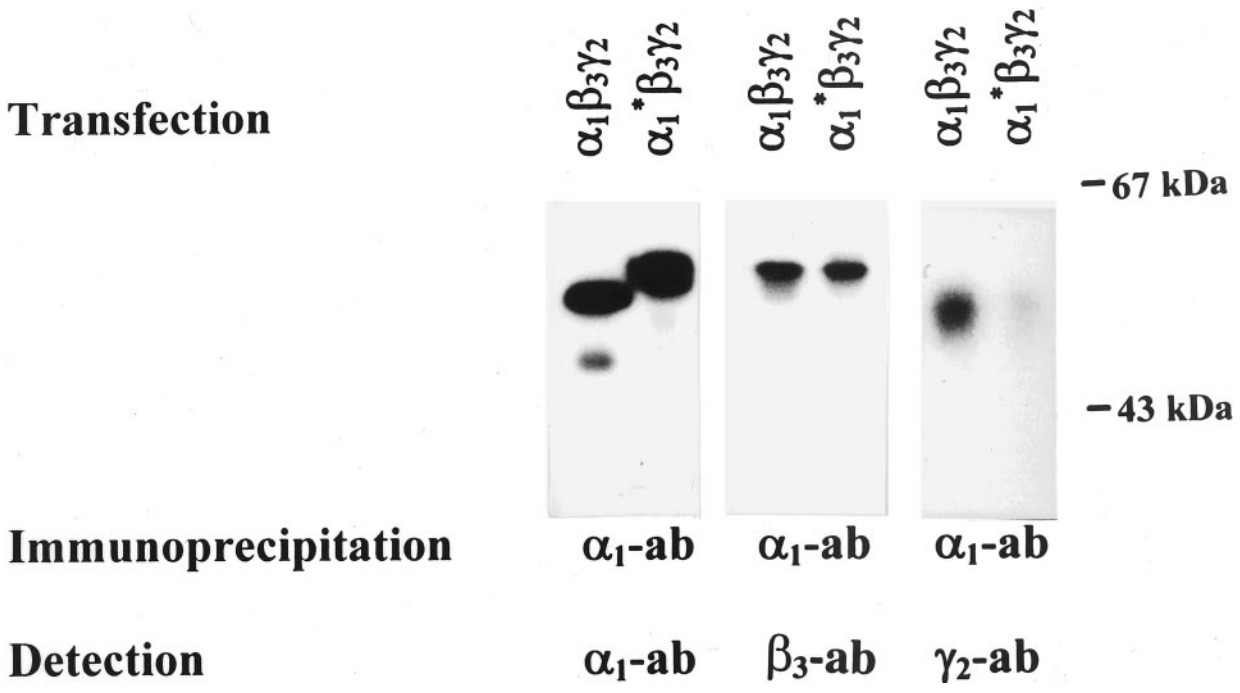

Figure 4. Western blot analysis of $GABA_A$ receptors labeled on the surface of HEK cells. HEK cells were cotransfected with α_1, β_3, and γ_2 subunits or with α_1*, β_3, and γ_2 subunits. $GABA_A$ receptors expressed on the cell surface were immunolabeled by adding α_1(1–9) antibodies to intact cells, and were then extracted, immunoprecipitated, and analyzed by SDS-PAGE and Western blots using digoxygenized α_1(1–9), β_3(1–13), or γ_2(1–33) antibodies.

mal current of cells transfected with $\alpha_1{}^*\beta_3\gamma_2$ subunits was only ~24% of that transfected with $\alpha_1\beta_3\gamma_2$ subunits.

Because surface expression studies indicated a significant formation of $\alpha_1{}^*\beta_3$ receptors in $\alpha_1{}^*\beta_3\gamma_2$ transfected cells, the properties of receptors in $\alpha_1{}^*\beta_3$ transfected cells were also investigated. Although homo-oligomeric receptors composed of β_3 subunits could also have been formed under the conditions used, they would not have contributed to the GABA evoked current because these receptors apparently are not gated by GABA (Connolly et al., 1996b). Using various GABA concentrations, it was demonstrated that GABA exhibited an EC_{50} of 10.5 ± 2.1 μM (mean ± SEM; $n = 8$ cells from different plates; total of four transfections) (Fig. 5*E*) in HEK cells transfected with $\alpha_1{}^*$ and β_3 subunits and elicited a maximal current of 270 ± 63 pA at a concentration of 300 μM (Fig. 5*C*). In contrast, GABA exhibited an EC_{50} of 3.0 ± 1.2 μM (mean ± SEM; $n = 9$ cells from different plates; total of three transfections) (Fig. 5*E*) in cells transfected with α_1 and β_3 subunits and elicited a maximal current of 426 ± 146 pA at a concentration of 100 μM (Fig. 5*D*). These data supported the conclusion that the $\alpha_1{}^*$ construct was able to form functional receptors with β_3 subunits. The potency of GABA for activating $\alpha_1{}^*\beta_3$ receptors, however, was significantly ($p < 0.05$; Student's t test) reduced compared with receptors composed of $\alpha_1\beta_3$ subunits. Similarly, the maximal currents elicited by GABA in $\alpha_1{}^*\beta_3$ transfected cells were significantly smaller than those in $\alpha_1\beta_3$ transfected cells ($p < 0.05$).

Although $\alpha_1{}^*\beta_3$ receptors significantly contribute to receptors formed in $\alpha_1{}^*\beta_3\gamma_2$ transfected HEK cells, as indicated by surface expression studies, because of the low maximum currents observed in $\alpha_1{}^*\beta_3$ receptors (Fig. 5*C*), these receptors overall have a comparatively small contribution to currents elicited in $\alpha_1{}^*\beta_3\gamma_2$ transfected cells (Fig. 5*A*) that is apparent only as a slightly increased range of GABA concentrations that are able to elicit currents in these cells (Fig. 5*E*). Thus, most of the current elicited in the cells investigated was produced by $\alpha_1{}^*\beta_3\gamma_2$ receptors. The low apparent potency of GABA to activate currents in these cells as well as the increased dose range of GABA for stimulation of currents clearly indicated the formation of $\alpha_1{}^*\beta_3\gamma_2$ receptors in addition to $\alpha_1{}^*\beta_3$ receptors.

This conclusion was supported by investigating the effects of 100 μM Zn^{2+} on whole-cell currents stimulated by 100 μM GABA. In agreement with previous results (Draguhn et al., 1990; Gingrich and Burkat, 1998), currents mediated by the wild-type $\alpha_1\beta_3\gamma_2$ receptors were only weakly reduced (86 ± 4% of control; $n = 6$; total of four transfections), whereas currents mediated by $\alpha_1\beta_3$ receptor were reduced to 7 ± 3% ($n = 6$; total of three transfections) (Fig. 5*F*) in the presence of Zn^{2+}. For HEK cells transfected with $\alpha_1{}^*$, β_3, and γ_2 subunits, currents mediated by 100 μM GABA were reduced to 50 ± 4% ($n = 7$; total of four transfections), and for cells transfected with $\alpha_1{}^*$ and β_3 subunits, GABA-mediated currents were reduced to 8 ± 2% ($n = 8$; total of four transfections) in the presence of 100 μM Zn^{2+} (Fig. 5*F*). Because $\alpha_1\beta_3$ and $\alpha_1{}^*\beta_3$ receptors exhibit a comparable Zn^{2+} sensitivity, it is reasonable to assume that the Zn^{2+} sensitivity of $\alpha_1{}^*\beta_3\gamma_2$ and $\alpha_1\beta_3\gamma_2$ receptors was also comparable. The 36% increase in Zn^{2+} sensitivity of $\alpha_1{}^*\beta_3\gamma_2$-transfected cells therefore indicated that ~40% of the $\alpha_1{}^*\beta_3\gamma_2$ current was mediated by the additionally formed $\alpha_1{}^*\beta_3$ receptors. Combined with the observation that the main conductance level of $\alpha\beta$ receptors (15–18 pS) is only half of that of $\alpha\beta\gamma$ receptors (~30 pS; Hevers and Lüddens, 1998), and assuming that the same holds true for $\alpha_1{}^*\beta_3$ and $\alpha_1{}^*\beta_3\gamma_2$ receptors, a ratio of $\alpha_1{}^*\beta_3$:$\alpha_1{}^*\beta_3\gamma_2$ receptors of 80:60 can be calculated, indicating that $\alpha_1{}^*\beta_3\gamma_2$ receptors represented 43% of receptors formed in these cells. Given the many assumptions that had to be made in the course of this calculation, this percentage is in good agreement with the data from the immunoprecipitation experiments shown in Figure 4.

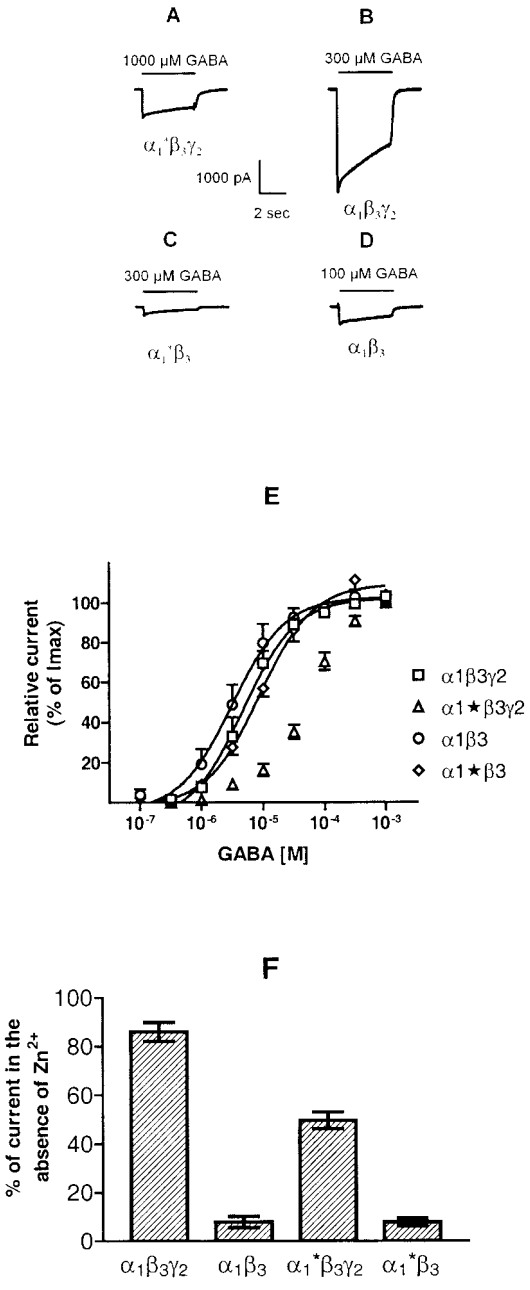

Figure 5. Functional properties of $GABA_A$ receptors containing the α_1* subunit. *A—D,* Whole-cell recordings from HEK cells cotransfected with α_1*, β_3, and γ_2, or α_1, β_3, and γ_2, α_1* and β_3, or α_1 and β_3 subunits after application of GABA concentrations producing maximal currents. Shown are single experiments that were reproduced with similar results 8–11 times in different cells. *E,* Relative currents induced by various GABA concentrations in cells transfected with subunit combinations as indicated. Data shown are mean values ± SEM of relative currents from 8–11 individual dose–response curves obtained from different cells derived from a total of four transfections. *F,* Bar graph showing the effect of 100 μM Zn^{2+} on currents activated by 100 μM GABA. HEK cells were transfected, as indicated. Height of bars indicates fraction of control current remaining in the presence of Zn^{2+}, measured 1.5 sec after application was started; error bars indicate ± SEM (n = 6–8).

Amino acid sequence α_1(80–100) binds to the γ_2(91–104) sequence

Recently it has been demonstrated that incorporation of the amino acid sequence γ_2(91–104) into the fragment α_1(1–100), that per se could not bind to α_1 subunits, resulted in the chimera α_1(1–100)chimγ_2(91–104) that was able to bind to α_1 subunits. From this it was concluded that the amino acid sequence γ_2(91–104) forms the contact site to α_1 subunits (Klausberger et al., 2000). It therefore seemed interesting to investigate whether the γ_2(91–104) sequence directly interacts with the α_1(80–100) sequence.

To clarify this question, it first was investigated whether the α_1(1–100) fragment and the fragment β_3(1–115), which was used to identify the α_1(80–100) contact site (Fig. 2), could bind to each other. For this, fragments β_3(1–115) and α_1(1–100) were cotransfected into HEK cells. The extract of HEK cells expressing β_3(1–115) and α_1(1–100) fragments was then immunoprecipitated with β_3(1–13) antibodies, and the precipitate was subjected to SDS-PAGE and Western blot analysis using digoxygenized α_1(1–9) antibodies. As shown in Figure 6, *A* and *B*, α_1(1–100) fragments were not coprecipitated by β_3(1–13) antibodies, confirming the absence of cross-reactivity of these antibodies with the α_1(1–100) fragments and indicating that β_3(1–115) could not bind to α_1(1–100) fragments. Similarly, the construct β_3(1–115)chimα_1(80–100), which contains the putative binding site for γ_2 subunits, was unable to bind to α_1(1–100) fragments after cotransfection into HEK cells (Fig. 6*A,B*). In the reverse experiment, the construct α_1(1–100)chimγ_2(91–104), containing the binding site for α_1 subunits, could also not bind to the β_3(1–115) fragment. Only when the α_1(80–100) sequence was incorporated into the β_3(1–115) fragment and the γ_2(91–104) sequence was incorporated into the α_1(1–100) fragment, the resulting chimeras could bind to each other (Fig. 6*A,B*). These results indicate that the α_1(80–100) and the γ_2(91–104) sequences can directly bind to each other.

In control experiments (Fig. 6*C*) it was demonstrated that each of the constructs used in this experiment was expressed to a similar extent after single transfection into HEK cells. Constructs α_1(1–100) and α_1(1–100)chimγ_2(91–104) each contained a single glycosylation site and thus, gave rise to two fragments: a weakly labeled of 12 kDa and a strongly labeled of 14 kDa. Construct β_3(1–115) contained two glycosylation sites and thus, formed three fragments, two strongly labeled of 16 and 18 kDa and a weakly labeled fragment of 14 kDa. Construct β_3(1–115)chimα_1(80–100) contained only one glycosylation site and formed a strongly labeled protein band of 14 and weakly labeled band of 16 kDa.

Interestingly, predominantly the unglycosylated α_1(1–100)chimγ_2(91–104) fragment of 12 kDa seemed to assemble with β_3(1–115)chimα_1(80–100) on cotransfection of these fragments into HEK cells, although the glycosylated fragment of 14 kDa was the predominant one expressed after single transfection of HEK cells (Figs. 1*B,* 6*C*). This suggests that assembly of subunit fragments already starts when subunits are not fully glycosylated. This conclusion is supported by previous observations (Klausberger et al., 2000, 2001) as well as by observations with other constructs (Fig. 1*B*).

DISCUSSION

Amino acid sequence α_1(80–100) forms the binding site to γ_2 but not to β_3 subunits

The present study demonstrated that the N-terminal extracellular domain of the α_1 subunit [α_1(1–221)] could bind to full-length γ_2 subunits after coexpression in HEK cells, as indicated by coimmunoprecipitation with subunit-specific antibodies. Binding between α_1(1–221) and γ_2 subunits represented a specific assembly

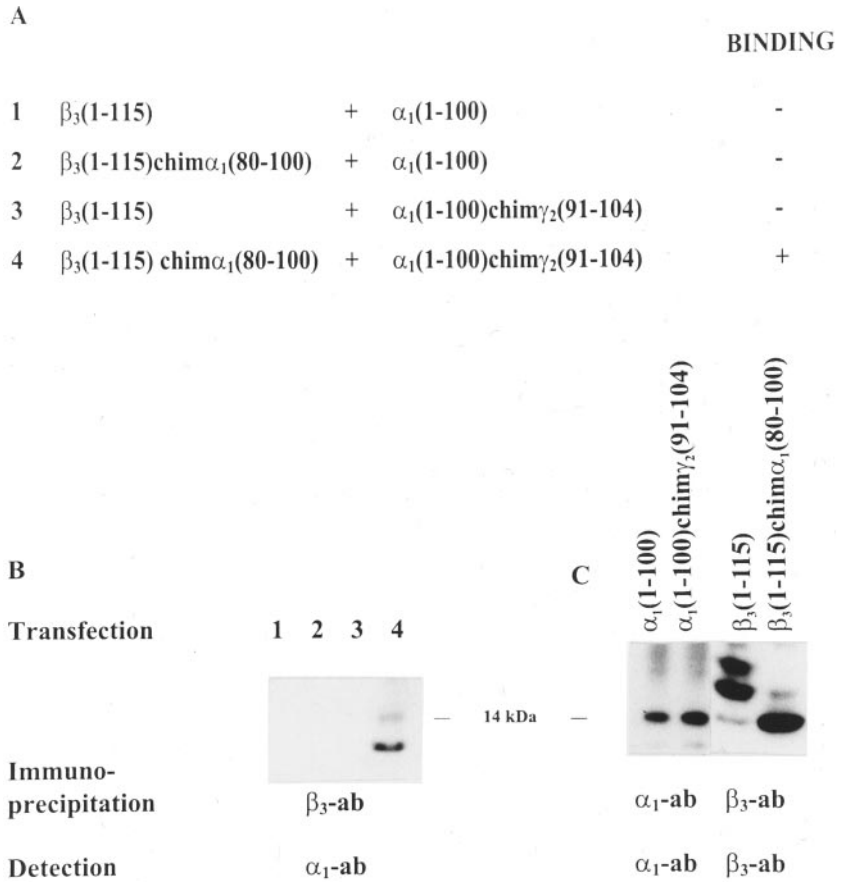

Figure 6. Amino acid sequence α_1(80–100) directly binds to γ_2(91–104). *A, B,* HEK cells were cotransfected with the constructs as indicated. Cell extracts were immunoprecipitated with β_3(1–13) antibodies, and the precipitate was subjected to SDS-PAGE and Western Blot analysis using digoxygenized α_1(1–9) antibodies. *A,* Schematic representation of the experiment. + indicates binding, and − indicates absence of binding between the cotransfected constructs. *B,* Western blots demonstrating binding between constructs under condition "*4*". *C,* Western blots demonstrating the extent of expression of the indicated constructs on single transfection into HEK cells. All experiments were performed three times with similar results.

process, as indicated by the formation of specific [^{3}H]Ro15–1788 binding sites that are assumed to be formed on the interface of α_1 and γ_2 subunits of $GABA_A$ receptors. These results are consistent with previous studies indicating that N-terminal sequences of $GABA_A$ receptor (Hackam et al., 1997; Klausberger et al., 2000) or K^+ channel (Shen et al., 1993) subunits can assemble with full-length subunits.

A subsequent reduction in the size of the truncated subunit indicated that the α_1(1–117), but not the α_1(1–100) construct was still able to bind to γ_2 subunits. The respective binding site was then identified by incorporating various α_1 sequences into the β_3(1–115) fragment. This fragment is homologous to α_1(1–117) but in contrast to the latter construct could not bind to γ_2 subunits after coexpression in HEK cells. The incorporation of the sequence α_1(80–100) into the β_3(1–115) fragment was sufficient to induce binding to γ_2 but not to β_3 subunits, suggesting that the α_1 binding sites for γ_2 and β_3 subunits are different.

The observation that the α_1(1–100) fragment was unable to bind to γ_2 subunits although it contained the α_1(80–100) sequence is consistent with previous results indicating that γ_2(1–113) was the smallest fragment that could bind to α_1 subunits, although the respective binding site was identified to be formed by the γ_2(91–104) sequence (Klausberger et al., 2000). The additional length of the fragments presumably is required for stabilizing the conformation of the actual binding sites located in a more N-terminal position.

In other experiments a chimeric α_1 subunit (α_1*) was constructed in which the α_1(79–100) sequence was replaced by the homologous β_3(77–98) sequence. Chimera α_1* was then coexpressed with β_3 and γ_2 subunits in HEK cells. Confocal immunofluorescence microscopy, whole-cell patch-clamp experiments, as well as immunolabeling and quantification of receptors on the cell surface indicated a 60–70% reduction in receptors containing α_1*, β_3, and γ_2 subunits, although the level of expression of α_1* subunits and its extent of assembly with β_3 subunits was unimpaired. These results confirmed the importance of the α_1(80–100) sequence for assembly with γ_2 but not with β_3 subunits. The remaining formation of α_1*$\beta_3\gamma_2$ receptors can be explained by the existence of additional binding sites between α_1 and γ_2 subunits that partially can compensate for the absence of the α_1(80–100) sequence in α_1* subunits.

Amino acid sequences α_1(80–100) and γ_2(91–104) form part of the α–γ interface and are located close to the benzodiazepine binding site of $GABA_A$ receptors

Recently it was demonstrated that the sequence γ_2(91–104) forms the contact site to α_1 subunits (Klausberger et al., 2000). To investigate whether the sequences α_1(80–100) and γ_2(91–104) directly interact with each other, these sequences were incorporated into $GABA_A$ receptor fragments β_3(1–115) and α_1(1–100), respectively, which could not bind to each other. The observation that α_1(80–100) had to be incorporated into β_3(1–115) and γ_2(91–104) into α_1(1–100) to induce coprecipitation of the fragments indicated that the α_1(80–100) and the γ_2(91–104) sequences can directly bind to each other and thus, form part of the α_1–γ_2 subunit interface. This is the first time that interacting sequences from two different subunits could be identified in this receptor superfamily. It is possible, however that subunits rearrange during assembly (Mitra et al., 2001). Whether the identified sequences also form part of the final intersubunit contact, thus, will have to

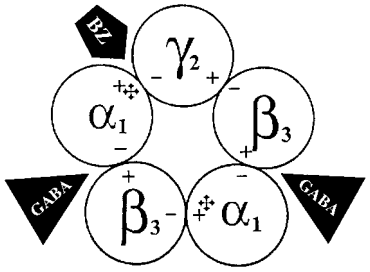

Figure 7. Stoichiometry and subunit arrangement of the recombinant $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptor (Tretter et al., 1997). A mirror image arrangement is equally possible. +, ✣, and − indicate the clockwise and counterclockwise binding sites of a subunit, respectively. BZ or GABA indicate site of interaction of benzodiazepines or GABA with $GABA_A$ receptors, respectively.

be determined by further studies. Interestingly, two amino acid residues (L90 and S92) identified previously as important for homo-oligomeric assembly of glycine receptor α_1 subunits (Griffon et al., 1999) are located in a region homologous to α_1(80–100), but their identity is different in $GABA_A$ receptors. In addition, L90 as well as a further amino acid residue (P79) that also is important for binding between glycine receptor subunits, are located in a region homologous to γ_2(91–104). This indicates that $GABA_A$ receptors, glycine receptors, and possibly also nAChRs (Kreienkamp et al., 1995) use homologous regions with receptor-specific assembly signals for forming intersubunit contacts.

Interestingly, the amino acid H101 of the α_1 subunit that can be photolabeled by [^{3}H]flunitrazepam (Smith and Olsen, 2000) and seems to be involved in the formation of the benzodiazepine binding pocket (Sigel and Buhr, 1997) is located immediately adjacent to the α_1(80–100) sequence forming the intersubunit contacts to γ_2 subunits. In addition, P96 of the α_1 subunit, which also can be photolabeled by [^{3}H]flunitrazepam (Smith and Olsen, 2000), contributes to the α_1–γ_2 subunit interface. This indicates that the benzodiazepine binding site of $GABA_A$ receptors is located close to the intersubunit contact between α_1 and γ_2 subunits.

Other amino acid residues possibly contributing to the benzodiazepine binding site are α_1Y159, α_1G200, α_1S204, α_1T206, α_1Y209, γ_2M130, and γ_2F77 (Sigel and Buhr, 1997). Although located outside the α_1(80–100) and γ_2(91–104) sequences, all these residues are embedded in domains with a hydrophobicity comparable with that of these sequences (data not shown). These residues might thus be involved in the formation of other parts of this interface. In any case, all residues forming the benzodiazepine binding site must be in spatial proximity to α_1H101.

Implications for $GABA_A$ receptor assembly and subunit stoichiometry

The present observation that the α_1(80–100) sequence binds to γ_2 but not to β_3 subunits suggests the existence of at least three distinct subunit binding sites on α_1 subunits: a (+) and a (−) site for binding of β_3 subunits and an additional (✣) site for binding of γ_2 subunits (Fig. 7). Whereas the (+) and the (✣) sites are located at the same side and are possibly situated closely together, the (−) site is located at the other side of the subunit. The recently identified sequence α_1(58–67), which mediates binding to β_3 subunits (Taylor et al., 2000), seems to form part of the (−) site because one of its residues (α_1F64; Smith and Olsen, 1994) contributes to the GABA binding site assumed to be located at the α_1–β_3 interface of $GABA_A$ receptors (Fig. 7).

Interestingly, one α_1 subunit of $GABA_A$ receptors uses the (✣) site for binding to a γ_2 subunit, whereas the other one uses the (+) site for a β_3 subunit (Fig. 7). Previous studies have indicated that cells transfected with α_1, β_3, and γ_2 subunits predominantly form pentameric receptors composed of $2\alpha_1$, $2\beta_3$, and one γ_2 subunit, whereas cells transfected with α_1 and β_3 subunits form tetramers and pentamers (Tretter et al., 1997). This delayed formation of subunit pentamers indicates that a γ_2 subunit can be more easily accommodated into an $\alpha_1\beta_3$ tetramer than an additional β_3 subunit. A preferential use of the α_1(✣) site under these conditions ensures the incorporation of a γ_2 subunit into the receptor. Because the alternate use of the α_1(+) and the α_1(✣) site in the two α_1 subunits of $GABA_A$ receptors seems to be sterically or energetically favored, binding to the α_1(+) site of the second α_1 subunit should be preferred when a γ_2 subunit is already present in an assembly intermediate. In this case the preferred use of the α_1(+) site prevents the incorporation of a second γ_2 subunit into the receptor. This conclusion is supported by most of the experimental data available, suggesting that there is only one γ_2 subunit in $GABA_A$ receptors (for review, see Sieghart et al., 1999) and indicates that the α_1–γ_2 intersubunit contact controls subunit assembly and stoichiometry of $GABA_A$ receptors.

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