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Stoichiometry and Assembly of a Recombinant $GABA_A$ Receptor Subtype

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$GABA_A$ receptors are ligand-gated chloride ion channels that are presumed to be pentamers composed of α, β, and γ subunits. The subunit stoichiometry, however, is controversial, and the subunit arrangement presently is not known. In this study the ratio of subunits in recombinant $\alpha1\beta3\gamma2$ receptors was determined in Western blots from the relative signal intensities of antibodies directed against the N terminus or the cytoplasmic loop of different subunits after the relative reactivity of these antibodies had been determined with $GABA_A$ receptor subunit chimeras composed of the N-terminal domain of one and the remaining part of the other subunit. Via this method a subunit stoichiometry of two α subunits, two β subunits, and one γ subunit was derived. Similar experiments investigating the composition of $\alpha1\beta3$ receptors expressed on the surface of human embryonic kidney (HEK) 293 cells cotransfected with $\alpha1$ and $\beta3$ subunits resulted in a stoichiometry of two α and three β subunits. Density gradient centrifugation studies indicated that combinations of $\alpha1\beta3\gamma2$ or $\alpha1\beta3$ subunits expressed in HEK 293 cells are able to form pentamers, whereas combinations of $\alpha1\gamma2$ or $\beta3\gamma2$ subunits predominantly form heterodimers. These results provide valuable information on the mechanism of $GABA_A$ receptor assembly and support the conclusion that $GABA_A$ receptors are pentamers in which a total of four alternating α and β subunits are connected by a γ subunit.

Key words: $GABA_A$ receptor; stoichiometry; assembly; subunit arrangement; human embryonic kidney 293 cells; chimeric subunits; density gradient centrifugation; Western blot

GABA, the major inhibitory transmitter in the CNS, mediates fast synaptic inhibition by opening the chloride ion channel intrinsic to the $GABA_A$ receptor. This receptor is a hetero-oligomeric protein and the site of action of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, steroids, anesthetics, and convulsants (Sieghart, 1995). So far, six α, three β, three γ, one δ, and two ρ subunits of these receptors, as well as several alternatively spliced isoforms of some of these subunits, have been identified in mammalian brain (Macdonald and Olsen, 1994; Sieghart, 1995). Expression studies have indicated that an α, a β, and a γ subunit have to combine to produce $GABA_A$ receptors with a pharmacology resembling that of receptors found in the brain and that, depending on the subunits used for transfection of cells, receptors with distinct pharmacological and electrophysiological properties do arise (Sieghart, 1995). Overall it is assumed, however, that a total of five subunits have to combine to form functional $GABA_A$ receptors (Nayeem et al., 1994).

A variety of subunit-specific antibodies has been raised to investigate the subunit composition of $GABA_A$ receptors. Immunocytochemical studies demonstrating the colocalization of subunits in $GABA_A$ receptor clusters on neuronal membranes (Fritschy et al., 1992; Caruncho and Costa, 1994; Fritschy and Möhler, 1995; Somogyi et al., 1996), as well as studies investigating the subunit composition of isolated receptors (Benke et al., 1991), have indicated that receptors consisting of $\alpha1$, $\beta2/3$, and $\gamma2$ subunits presumably are the major $GABA_A$ receptors in the brain. Other studies have demonstrated that two different α subunits are present in at least some $GABA_A$ receptors (Duggan et al., 1991; Lüddens et al., 1991; Zezula and Sieghart, 1991; Mertens et al., 1993). Discrepant results were obtained on a possible colocalization of different γ subunits in the same $GABA_A$ receptor. Whereas in one study (Mossier et al., 1994) it was demonstrated that $GABA_A$ receptors seem to contain only a single type of γ subunit, other studies have indicated a significant colocalization of the alternatively spliced short and long form of the $\gamma2$ subunit (Khan et al., 1994) or of the $\gamma2$ and $\gamma3$ subunits (Quirk et al., 1994) in the same receptor.

To resolve this discrepancy and to determine directly the subunit stoichiometry of $GABA_A$ receptors, we developed a method in the present study that allows the determination of subunit ratios in multimeric proteins. Via this method the subunit stoichiometry of recombinant $\alpha1\beta3\gamma2$ $GABA_A$ receptors expressed in human embryonic kidney (HEK) 293 cells was determined. In addition, density gradient centrifugation experiments investigating assembly intermediates of recombinant $GABA_A$ receptors in HEK 293 cells transfected with $\alpha1\beta3$, $\alpha1\gamma2$, $\beta3\gamma2$, or $\alpha1\beta3\gamma2$ subunits provided important information on the subunit arrangement within recombinant $\alpha1\beta3\gamma2$ receptors as well as on the mechanism of $GABA_A$ receptor assembly.

MATERIALS AND METHODS

Generation and purification of antibodies. The anti-$\alpha1$(1–9) antibody was generated and affinity-purified as described previously (Zezula and Sieghart, 1991). Peptide $\beta3$(1–13) was custom-synthesized with an additional C-terminal cysteine and was coupled to keyhole limpet hemocyanin. Rabbits were immunized with this adduct, and anti-$\beta3$(1–13) antibodies were purified from the serum of the rabbits by affinity chromatography on a column consisting of the peptide $\beta3$(1–13) coupled to thiopropyl–Sepharose. The anti-$\alpha1$(328–382) and the anti-$\gamma2$(319–366) antibodies were generated by immunizing rabbits with a maltose binding protein (MBP)-$\alpha1$(328–382)-7His or MBP-$\gamma2$(319–366)-7His fu-

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sion protein, respectively (Mossier et al., 1994). Then the antibodies were purified by using the corresponding glutathione *S*-transferase fusion protein coupled to Affi-Gel 10 (Bio-Rad, Hercules, CA).

Cloning of chimeric receptor subunits. For the generation of recombinant receptors, α1, β3, and γ2 subunits of $GABA_A$ receptors from rat brain were cloned and subcloned into the pCDM8 expression vector (Invitrogen, San Diego, CA) as described previously (Fuchs et al., 1995). To generate chimeric receptor subunits, we cloned the cDNAs for the α1, β3, and γ2 subunits into the vector pALTER-1 (Promega, Madison, WI), using *Hin*dIII and *Not*I restriction sites of the polylinker. Then oligonucleotide-directed mutagenesis was performed to introduce a unique *Bgl*II site at the 5′-end of the first transmembrane region of each subunit. Mutagenesis was performed with the Altered Sites II *in vitro* Mutagenesis System (Promega) according to the instructions of the manufacturer. The following oligonucleotides were used: α1-mut, 5′-TTG AAT AAC AAA GTA GCC GAT AGA TCT CTT CAA GTG AAA GTG AGT-3′; β3-mut, 5′-CTG AAG TAT GAA GTA CCC AAT AGA TCT CTT CAA CCG AAA ACT-3′; γ2-mut, 5′-CTG GAT GGT AAA GTA CCC CAT AGA TCT GCT CAG ATC AAA GTA-3′. Mutations were confirmed by dideoxy–DNA sequencing. Chimeras were generated by cutting with restriction enzymes (*Hin*dIII and *Bgl*II for the N-terminal part or *Bgl*II and *Not*I for the C-terminal part of the genes) and isolating and religating of the desired fragments with the *Hin*dIII and *Not*I cut expression vector pCDNA I Amp (Invitrogen). Chimeras were confirmed by selective restriction enzyme cleavage and by sequencing across the chimera boundaries. Large-scale DNA purification for transfections was performed with the Qiagen plasmid purification procedure (Qiagen GmbH, Hilden, Germany).

Expression of recombinant $\alpha1\beta3\gamma2$ receptors and chimeric receptor proteins. HEK 293 cells (CRL 1573) from American Type culture collection (Rockville, MD) were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin G, and 100 μg/ml streptomycin and nonessential amino acids (Life Technologies) in 75 cm^2 Petri dishes by the use of standard cell culture techniques. HEK 293 cells (3×10^6) were transfected with a total of 21 μg of subunit cDNA (cDNA ratio $\alpha1{:}\beta3{:}\gamma2$ = 1:1:1 or 1:1:4) or with 20 μg of individual chimera cDNA via the calcium phosphate precipitation method (Chen and Okayama, 1988). The cells were harvested 44 hr after transfection.

Purification of recombinant $\alpha1\beta3\gamma2$ receptor. The transfected HEK cells from a total of 150 Petri dishes were harvested, and the pelleted cells were extracted in 50 ml of deoxycholate buffer [0.5% deoxycholate, 0.05% phosphatidylcholine and (in mM) 10 Tris-HCl, pH 8.0, 150 NaCl, 0.3 PMSF, and 1 benzamidine with 100 mg/l bacitracin] for 30 min at 4°C. The extract was centrifuged for 40 min at 150,000 × *g* at 4°C and was applied to a column consisting of the benzodiazepine Ro 7-1986 coupled to Affi-Gel 15 (Bio-Rad) (Fuchs and Sieghart, 1989). The column was washed with 3 vol of deoxycholate buffer, 6 vol of immunoprecipitation (IP) high buffer (50 mM Tris-HCl, 0.5% Triton X-100, 600 mM NaCl, and 1 mM EDTA, pH 8.3), and 6 vol of IP low buffer (50 mM Tris-HCl, 0.2% Triton X-100, 150 mM NaCl, and 1 mM EDTA, pH 8.0) and was eluted with 6 M guanidine-HCl, 0.2 M acetic acid, and 0.5% Triton X-100. Before electrophoresis the receptor was precipitated with methanol/chloroform (Wessel and Flügge, 1984), and the pellet was dissolved in sample buffer [108 mM Tris-sulfate, pH 8.2, 10 mM EDTA, 25% (w/v) glycerol, 2% SDS, and 3% dithiothreitol] for SDS-PAGE.

Immunopurification of chimeric receptor subunits. Formaline-fixed *Staphylococcus aureus* cells (1 ml; Immunoprecipitin, Life Technologies, Gaithersburg, MD) were pelleted and resuspended in 900 μl of buffer containing 3% SDS/10% β-mercaptoethanol in PBS. The suspension was heated at 95°C for 30 min, centrifuged at 8000 × *g*, and washed three times with IP low buffer. Finally, the pellet was suspended in 900 μl of IP low buffer containing 100 mg/l bacitracin, 1 mM benzamidine, and 0.3 mM PMSF, and the suspension was used for immunoprecipitation.

Transfected HEK cells were grown at 37°C for 44 hr, and receptor chimeras were extracted with deoxycholate buffer (see above) for 30 min at 4°C at a concentration of 1.3 mg of protein per milliliter of buffer. The extract was centrifuged at 150,000 × *g* for 30 min at 4°C, and the clear supernatant was incubated overnight at 4°C under gentle shaking with 20 μg of antibody per milliliter. After addition of Immunoprecipitin and 0.5% nonfat dry milk powder and shaking for an additional 3 hr at 4°C, the precipitate was washed three times with IP low buffer. The precipitated protein was dissolved in sample buffer for SDS-PAGE.

SDS-PAGE, Western blot, and chemiluminescence detection. SDS-PAGE was performed according to Neville and Glossmann (1974) with 10% acrylamide/0.27% bisacrylamide. Various dilutions of receptor and chimeric subunit protein were applied to SDS-PAGE. Samples that should be compared directly (chimeras and/or receptors detected with two different antibodies) were run on the same gel. After electrophoresis, gels containing the receptor samples or the chimeras were tank-blotted onto prewetted polyvinylidene fluoride membranes in the same sandwich under identical conditions. After blocking with 1.5% nonfat dry milk powder in PBS and 0.1% Tween 20 for 1 hr at room temperature, we incubated the membranes overnight with digoxigenated primary antibody at the following concentrations: anti-α1(1–9), 2 μg/ml; anti-β3(1–13), 2 μg/ml; anti-γ2(319–366), 1 μg/ml; anti-α1(328–382), 1 μg/ml. After extensive washing [1.5% (w/v) dry milk powder and 0.1% Tween 20 in PBS], the membranes were incubated with anti-digoxigenin $F(ab)_2$ fragments coupled to alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) for 45 min at room temperature. Membranes were washed extensively and equilibrated in assay buffer (0.1 M diethanolamine and 1 mM $MgCl_2$, pH 10.0) for 10 min. Then membranes were incubated with 1 ml of 0.25 mM CSPD or CDP-star reagent (Tropix, Bedford, MA) diluted in assay buffer. After 5 min the fluid was removed and the membranes were sealed in a foil and exposed to x-ray films (X-Omat S, Kodak, Rochester, NY) for various time periods. Signals were quantified by a gel documentation system (Docu Gel 2000i; software: RFLP-Scan; MWG Biotech, Ebersberg, Germany).

Determination of surface expression of receptors. HEK 293 cells were transfected with various combinations of $GABA_A$ receptor subunits. After 44 hr the culture medium was removed and the cells were washed once with PBS containing (in mM) 2.7 KCl, 1.5 KH_2PO_4, 0.14 NaCl, and 4.3 Na_2HPO_4, pH 7.3. Cells then were detached from the Petri 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 × *g*. The pellet from two dishes was incubated with 30 μg of antibody in 3 ml of the same medium for 30 min at 37°C. Cells again were pelleted and washed twice with 10 ml of DMEM and 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, and antibody-labeled receptors were isolated by immunoprecipitation. The resulting protein pellet was dissolved in sample buffer for SDS-PAGE.

Density gradient centrifugation. Transfected HEK 293 cells from eight Petri dishes were harvested and extracted in 1.6 ml of Lubrol extraction buffer [1% Lubrol PX, and (in mM) 150 NaCl, 5 EDTA, 50 Tris-HCl, pH 7.4, 0.3 PMSF, 1 benzamidine with 0.18% phosphatidylcholine, and 100 mg/l bacitracin] overnight at 4°C. The clear extract (250 μl) was layered onto the top of a density gradient (5–20% sucrose in Lubrol extraction buffer). For the determination of sedimentation coefficients, 2 μg of digoxigenated catalase (sedimentation coefficient 11 *s*), 1.2 μg of digoxigenated alkaline phosphatase (sedimentation coefficient 6.1 *s*) and 1 μg of digoxigenated carbonic anhydrase (sedimentation coefficient 3.3 *s*) were included in the overlays. The gradients were centrifuged at 120,000 × *g* at 4°C for 23 hr and then were fractionated by piercing at the bottom. Protein in individual fractions was precipitated (Wessel and Flügge, 1984) and dissolved in sample buffer for SDS-PAGE. Then the samples were analyzed by Western blotting and densitometry.

RESULTS

Identification of individual receptor subunits in recombinant $GABA_A$ receptors

HEK 293 cells were transfected with a mixture of cDNAs encoding α1, β3, and γ2 subunits of $GABA_A$ receptors. To eliminate incompletely assembled receptors, we extracted recombinant receptors that formed by using a deoxycholate-containing buffer and subjected them to affinity chromatography on a column containing the benzodiazepine Ro 7-1986 (Fuchs and Sieghart, 1989). Affinity-purified $GABA_A$ receptors then were subjected to SDS-PAGE and Western blot analysis by the use of polyclonal antibodies specific for the α1, β3, or γ2 subunits. In agreement with previous results (Zezula and Sieghart, 1991), anti-α1(1–9), antibodies identified a protein with apparent molecular mass of 51 kDa (Fig. 1). A protein with identical apparent molecular mass was identified by anti-α1(328–382) antibodies. Anti-β3(1–13) an-

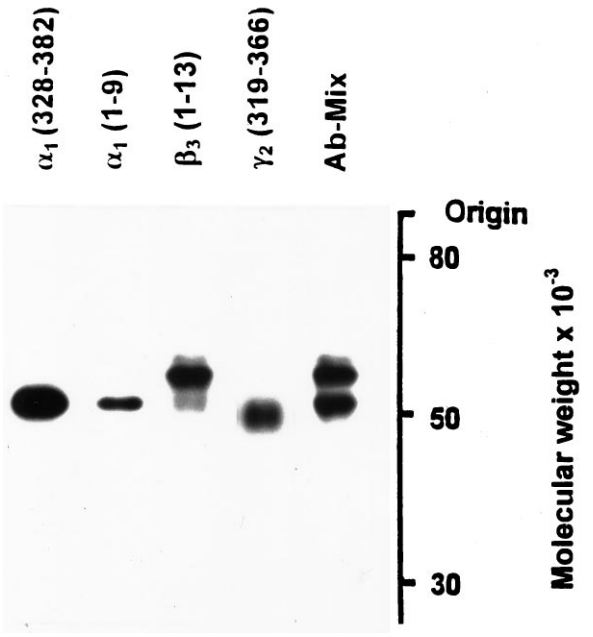

Figure 1. Identification of $GABA_A$ receptor subunits in affinity-purified recombinant $\alpha1\beta3\gamma2$ receptors. HEK 293 cells were transfected with $\alpha1\beta3\gamma2$ subunits. Receptors were extracted, purified by affinity chromatography, and subjected to SDS-PAGE and Western blot analysis by using the digoxigenated subunit-specific antibodies and chemiluminescence detection, as indicated. Chemiluminescence exposure time was varied to result in comparable signal intensities for the individual antibodies. Antibody mix (*Ab-Mix*): a mixture of anti-peptide *α1(1–9)*, anti-peptide *β3(1–13)*, and anti-peptide *γ2(319–366)* antibodies was used for the detection of $GABA_A$ receptor subunits. The experiment was performed three times with similar results.

tibodies strongly identified a protein with apparent molecular mass of 54 kDa and weakly recognized additional proteins with a slightly higher or lower molecular mass, as was shown previously with anti-β3(345–408) antibodies (Slany et al., 1995) or the monoclonal antibody bd 17 (Fuchs et al., 1989). Anti-γ2(319–366) antibodies identified a protein with apparent molecular mass of 49 kDa (Mossier et al., 1994). As shown in Figure 1 (lane labeled *Ab-Mix*), the similarity of the apparent molecular masses of the individual proteins and the microheterogeneity of the labeled protein bands precluded a direct quantification of recombinant receptor subunits after pulse labeling with radiolabeled methionine, a method that has been applied successfully for the determination of the subunit stoichiometry of the nicotinic ACh receptor (Anand et al., 1991).

Determination of subunit ratios in $GABA_A$ receptors

Determination of subunit ratios using β3–α1 chimeras

Immunological signals as shown, for instance, in Figure 1, because of differences in the reactivity of the antibodies used (avidity differences, different number of epitopes identified), do not reflect directly the amount of the respective protein present in the purified receptor preparation. To determine the relative reactivity of the anti-β3(1–13) and the anti-α1(328–382) antibodies used, we constructed a chimeric $GABA_A$ receptor subunit consisting of the extracellular domain of the β3 subunit and the four transmembrane domains and the cytoplasmic loop of the α1 subunit (Fig. 2). Because the epitopes identified by the anti-β3 and anti-α1 antibodies now were present in the same protein, the reactivity of the individual antibodies against their epitopes could be compared directly.

The chimeric β3–α1 protein was expressed in HEK 293 cells and purified by immunoprecipitation with anti-β3(1–13) antibodies. Increasing amounts of the chimera then were subjected to SDS-PAGE and Western blot analysis with digoxigenated anti-β3(1–13) and anti-α1(328–382) antibodies. As shown in Figure 2, the digoxigenated antibodies identified the same protein with the expected molecular mass, and the signal intensity of the antibodies increased linearly with increasing amounts of chimeric protein applied to the gel. The ratio of the slopes (signal intensity per protein unit) of the standard curves indicated that the anti-α1(328–382) antibodies reacted 4.04 times stronger with the chimeric protein than the anti-β3(1–13) antibodies (Fig. 2). This was not much of a surprise, because the number of epitopes recognized by the anti-α1(328–382) antibodies (directed against 45 amino acids) probably was larger than that recognized by the anti-β3(1–13) antibodies (directed against 13 amino acids).

In the same experiment increasing amounts of affinity-purified $\alpha1\beta3\gamma2$ receptor were subjected to SDS-PAGE and Western blot analysis with the same digoxigenated antibodies. Each of the antibodies now identified the respective $GABA_A$ receptor subunits, and the signals obtained again increased linearly with increasing amounts of receptor protein applied to the gel. The slope ratio obtained indicated that the reaction of the anti-α1(328–382) antibodies with the α1 subunit was 4.82 times stronger than the reaction of the anti-β3(1–13) antibodies with the β3 subunit. Taking into account the 4.04 times higher reactivity of the anti-α1(328–382) antibodies, we calculated the ratio of the α1 and β3 subunits in the recombinant $\alpha1\beta3\gamma2$ receptors to be 1.19:1 (Fig. 2). This experiment was performed independently a total of five times, resulting in an average subunit ratio of $\alpha1{:}\beta3 = 0.95 \pm 0.25$ (mean ± SD).

Determination of subunit ratios using β3–γ2 chimeras

To compare the reactivity of the anti-β3(1–13) and anti-γ2(319–366) antibodies, we constructed a chimeric $GABA_A$ receptor subunit consisting of the N terminus and extracellular domain of the β3 and the four transmembrane domains and the cytoplasmic loop of the γ2 subunit. The chimeric protein was identified by the anti-β3(1–13) as well as by the anti-γ2(319–366) antibodies, and increasing amounts of the purified chimeric protein resulted in a linear increase of both signals (Fig. 3). The slope ratio indicated that the signal obtained with the anti-γ2(319–366) antibodies in this experiment was 8.48 times stronger than that obtained with the anti-β3(1–13) antibodies. In the same experiment and under the same conditions, increasing amounts of affinity-purified $\alpha1\beta3\gamma2$ receptor were subjected to SDS-PAGE and Western blot analysis. The signals obtained with the anti-γ2(319–366) and anti-β3(1–13) antibodies again increased linearly with the protein concentration applied to the gel (Fig. 3). The reaction of the anti-γ2(319–366) antibodies with the γ2 subunit was 4.55 times stronger than the reaction of the anti-β3(1–13) antibodies with the β3 subunit. After correction of this result for the difference in the response of the two antibodies toward the chimeric β3–γ2 protein, a $\beta3{:}\gamma2$ subunit ratio of 1:0.53 was obtained. This experiment was performed independently a total of six times, resulting in an average subunit ratio of $\beta3{:}\gamma2 = 2.19 \pm 0.63$ (mean ± SD).

Determination of subunit ratios using α1–γ2 chimeras

Finally, a chimeric protein was constructed consisting of the N terminus and extracellular domain of the α1 and the four trans-

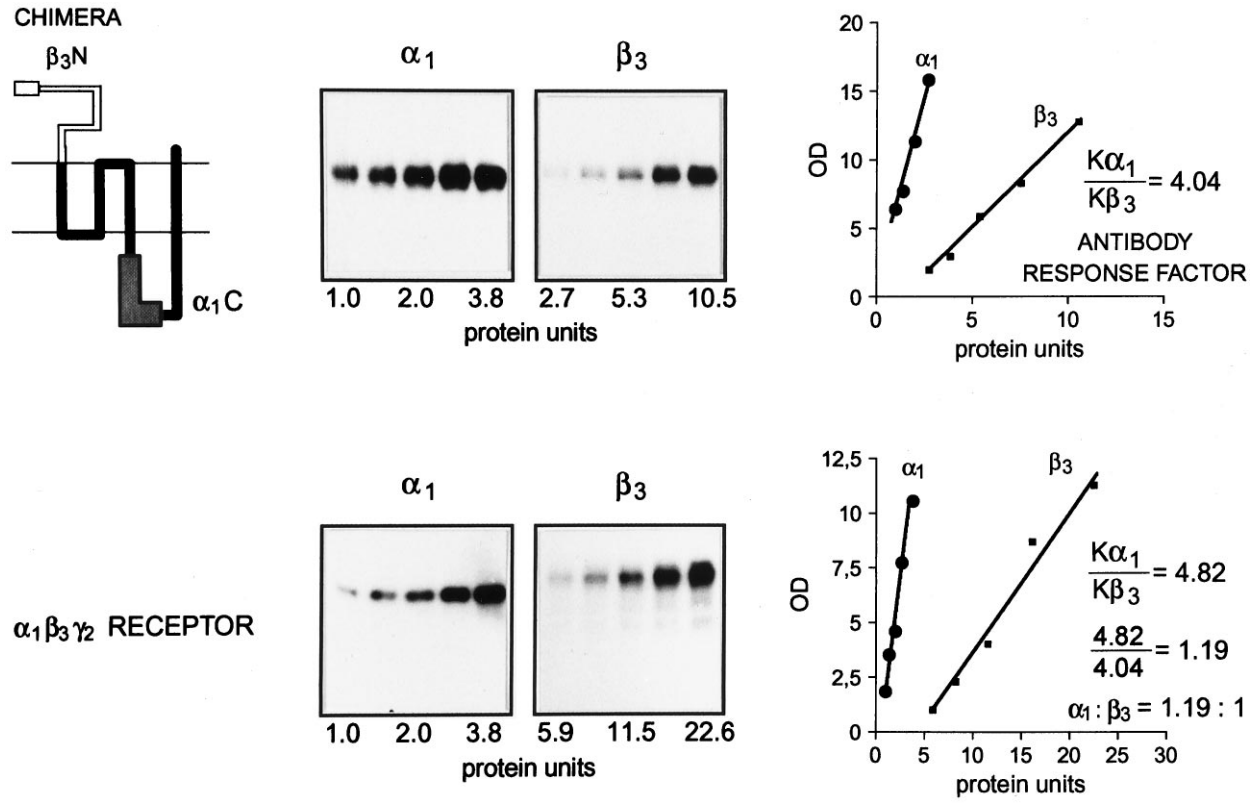

Figure 2. Determination of the α1:β3 subunit ratio using a β3–α1 chimera. A chimeric protein consisting of the N terminus of the β3 subunit and the four transmembrane domains plus the cytoplasmic loop of the α1 subunit was expressed in HEK 293 cells. The chimera was extracted and precipitated using anti-β3(1–13) antibodies; increasing amounts of this protein were subjected to SDS-PAGE and Western blot analysis with digoxigenated anti-β3(1–13) or anti-α1(328–382) antibodies, anti-digoxigenin F(ab)$_2$ fragments coupled to alkaline phosphatase, and the chemiluminescence substrate CDP-star. In the same experiment affinity-purified recombinant $\alpha1\beta3\gamma2$ receptors were subjected to SDS-PAGE and Western blot analysis by using the same primary and secondary antibodies and substrate. Chemiluminescence signals were detected by exposure to x-ray film and were quantified with a gel documentation system. Signals obtained were subjected to linear regression analysis. Shown is a typical experiment that was repeated a total of five times with comparable results.

membrane domains and the cytoplasmic loop of the γ2 subunit. The protein was expressed and purified; after SDS-PAGE and Western blot analysis this protein was identified by the anti-α1(1–9) as well as by the anti-γ2(319–366) antibodies. As shown in Figure 4, the signal obtained from the anti-γ2(319–366) antibodies was 6.16 times that obtained with the anti-α1(1–9) antibodies. When affinity-purified $\alpha1\beta3\gamma2$ receptor was investigated in the same experiment, the signals obtained by the reaction of the anti-γ2(319–366) antibodies with the γ2 subunit was 3.12 times larger than that obtained from the reaction of the anti-α1(1–9) antibodies with the α1 subunit. When these results were corrected for the individual antibody response factor, an α1:γ2 subunit ratio in recombinant $\alpha1\beta3\gamma2$ receptors of 1:0.51 was obtained (Fig. 4). This experiment was performed independently a total of six times, resulting in an average subunit ratio of $\alpha1{:}\gamma2 = 1.99 \pm 0.51$ (mean ± SD).

Stoichiometry of recombinant $\alpha1\beta3\gamma2$ receptors

Combined results obtained from five to six separate experiments for each chimera and subunit pair indicated a subunit stoichiometry for $\alpha1{:}\beta3{:}\gamma2$ of 2:2:1. The same subunit stoichiometry was obtained whether subunit cDNA ratios of $\alpha1{:}\beta3{:}\gamma2 = 1{:}1{:}1$ or 1:1:4 were used for transfection of HEK 293 cells, supporting previous results (Chang et al., 1996) indicating that stoichiometry did not depend on the relative availability of GABA$_A$ receptor subunits.

A variety of control experiments was performed to account for factors possibly influencing the results obtained. Thus, in the first experiments of this study a less-sensitive immunological detection system (chemiluminescence substrate CSPD instead of the ten times more sensitive CDP-star) was used. Consequently, larger amounts of protein had to be applied to the gels to detect the proteins. Nevertheless, these experiments resulted in the same stoichiometry, indicating that the results obtained were not influenced by the amount of receptor or chimeric protein applied to the gel. In addition, results obtained were independent from the receptor or chimera preparation used and were identical whether chimeras were isolated by immunoprecipitation with antibodies directed against the N terminus or against the cytoplasmic loop. Because saturating concentrations of antibodies were used for the detection of proteins in Western blots, results obtained were not influenced by the antibody concentration. Finally, results obtained were not influenced by the degree of digoxigenation of the antibodies. Depending on the degree of digoxigenation, the reactivity of the antibodies and, thus, the antibody response factors was different. In the course of this study, antibody response factors of anti-β3:anti-α1 antibodies between 1:2 and 1:5 were obtained. Antibody response factors of anti-β3:anti-γ2 antibodies were between 1:3 and 1:8, and those for anti-α1:anti-γ2 antibodies were between 1:2 and 1:8. Nevertheless, the subunit ratios obtained were the same as long as these ratios were determined with the

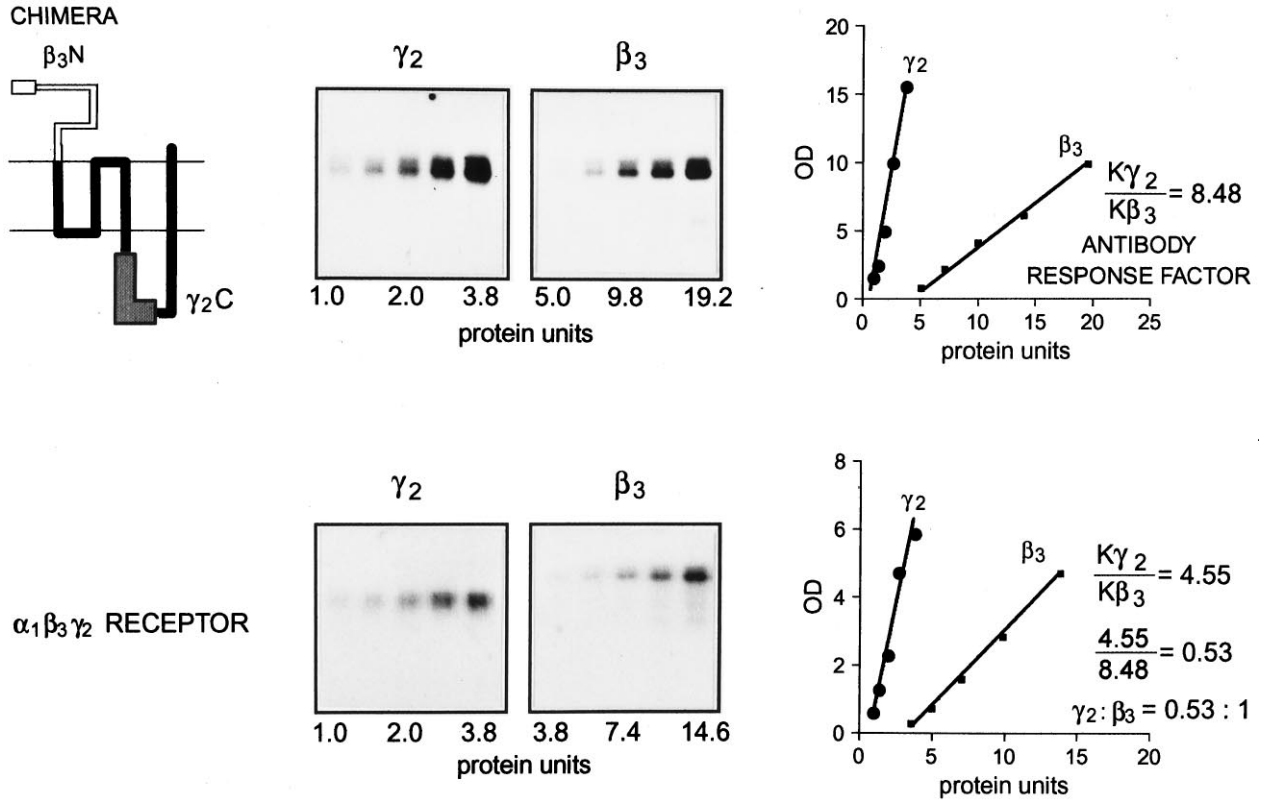

Figure 3. Determination of the β3:γ2 subunit ratio by using a β3–γ2 chimera. A chimeric protein consisting of the N terminus of the β3 subunit and the four transmembrane domains plus the cytoplasmic loop of the γ2 subunit was expressed in HEK 293 cells. The chimera was extracted and precipitated using anti-β3(1–13) antibodies; increasing amounts of this protein were subjected to SDS-PAGE and Western blot analysis with digoxigenated anti-β3(1–13) or anti-γ2(319–366) antibodies, anti-digoxigenin $F(ab)_2$ fragments coupled to alkaline phosphatase, and the chemiluminescence substrate CDP-star. In the same experiment affinity-purified recombinant α1β3γ2 receptors were subjected to SDS-PAGE and Western blot analysis by using the same primary and secondary antibodies and substrate. Chemiluminescence signals were detected by exposure to x-ray film and were quantified with a gel documentation system. The double band observed with the chimeric protein might have resulted from differential glycosylation. Identical antibody response factors were obtained when the signals from both proteins or from the individual proteins were compared. Signals obtained were subjected to linear regression analysis. Shown is a typical experiment that was repeated a total of six times with comparable results.

same antibodies, in the same experiment, and under the same conditions as the antibody response factors.

$GABA_A$ receptor assembly

α1β3γ2 coexpression

There are six possible subunit arrangements in pentameric receptors consisting of two α, two β, and one γ subunit (Fig. 5). Arrangements 5*A* and 5*B*, as well as 5*C* and 5*D*, are mirror images and are characterized by γ subunits being in contact with α as well as with β subunits. In arrangement 5*E* the γ subunit is in contact with two α and in arrangement 5*F* with two β subunits. To identify the subunit arrangement actually formed, we investigated whether it was possible to isolate intermediates of the assembly process of recombinant α1β3γ2 receptors. For this, HEK 293 cells were transfected with α1, β3, and γ2 subunits, and the receptors that formed were extracted with an extraction buffer containing Lubrol. Then receptors were subjected to density gradient centrifugation on sucrose gradients. Under these conditions, depending on their molecular mass, monomeric and multimeric proteins migrate into the gradient with different sedimentation coefficients. Gradients were fractionated, and the proteins in individual fractions were precipitated and subjected to SDS-PAGE and Western blot analysis with subunit-specific antibodies. *s* values of receptors and receptor intermediates were determined by analyzing the sedimentation of standard proteins with the known *s* value added to each gradient. As shown in Figure 6*A*, in HEK cells transfected with α1β3γ2 subunit cDNAs, the α1, β3, as well as the γ2 subunit protein sedimented at a single peak at 8.7 *s*. This sedimentation coefficient is identical with that of $GABA_A$ receptors isolated from adult brain (experiments not shown) and is comparable with that observed with the pentameric nicotinic ACh receptor (9 *s*; Green and Claudio, 1993). The cosedimentation of α1, β3, and γ2 subunits of $GABA_A$ receptors in a single protein peak at 8.7 *s* thus indicates the formation of pentameric $GABA_A$ receptors. The protein shoulder above 8.7 *s* of the α1, β3, and γ2 subunits also has been observed with subunits of the nicotinic ACh receptors (Gu et al., 1991; Green and Claudio, 1993) and presumably is caused by an aggregation of pentameric receptors. The absence of α1, β3, and γ2 protein peaks with lower *s* values (Fig. 6*A*) indicated that most of the $GABA_A$ receptors formed in α1β3γ2 transfected cells are pentamers consisting of α1β3γ2 subunits and that assembly intermediates could not be identified under these conditions.

α1β3 coexpression

In a further attempt to identify possible assembly intermediates, we transfected HEK 293 cells with α1 and β3 subunits of $GABA_A$ receptors only. Density gradient centrifugation indicated that under these conditions α1 as well as β3 subunit proteins again

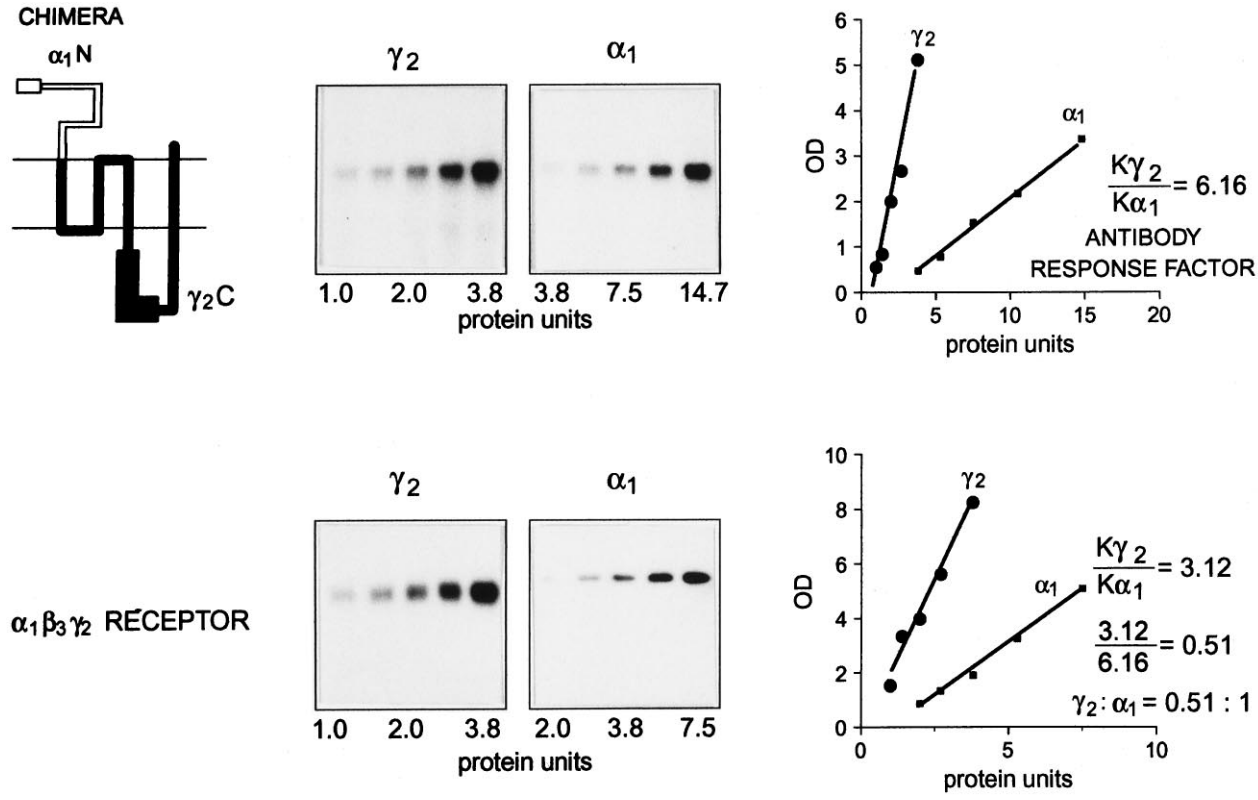

Figure 4. Determination of $\alpha 1$:$\gamma 2$ subunit ratio using an $\alpha 1$–$\gamma 2$ chimera. A chimeric protein consisting of the N terminus of the $\alpha 1$ subunit and the four transmembrane domains plus the cytoplasmic loop of the $\gamma 2$ subunit was expressed in HEK 293 cells. The chimera was extracted and precipitated using anti-$\alpha 1$(1–9) antibodies; increasing amounts of this protein were subjected to SDS-PAGE and Western blot analysis with digoxigenated anti-$\alpha 1$(1–9) or anti-$\gamma 2$(319–366) antibodies, anti-digoxigenin $F(ab)_2$ fragments coupled to alkaline phosphatase, and the chemiluminescence substrate CDP-star. In the same experiment affinity-purified recombinant $\alpha 1\beta 3\gamma 2$ receptors were subjected to SDS-PAGE and Western blot analysis by using the same primary and secondary antibodies and substrate. Chemiluminescence signals were detected by exposure to x-ray film and were quantified with a gel documentation system. Signals obtained were subjected to linear regression analysis. Shown is a typical experiment that was repeated a total of six times with comparable results.

sedimented at a peak of 8.7 *s*, indicating the formation of pentameric $\alpha 1\beta 3$ receptors (Fig. 6*B*).

In addition to the $\alpha 1\beta 3$ pentamers, however, especially $\beta 3$ subunits were able to form subunit complexes with lower sedimentation coefficients. This is indicated by the presence of additional protein peaks at 7.4, 6.7, 5.5, and 3.3 *s* in the gradient shown in Figure 6*B*. In studies investigating the nicotinic ACh receptor, it has been demonstrated that monomeric subunits of this receptor show sedimentation between 3 and 4 *s* (Green and Millar, 1995). Sedimentation of subunit dimers was observed at 6 *s*, trimers sedimented at 7 *s*, and tetramers at 8 *s* (Green and Claudio, 1993). Given the similarity of the sedimentation coefficients observed in experiments investigating the structurally similar $GABA_A$ receptors and nicotinic receptors, it is reasonable to assume that the peaks with 3.3, 5.5, 6.7, and 7.4 *s* represented mono-, di-, tri-, and tetramers of $GABA_A$ receptor $\beta 3$ subunits, respectively. The difference in the sedimentation coefficients between subunit oligomers of nACh receptors and of $GABA_A$ receptors might have been attributable to differences in the apparent molecular mass of nACh receptor and $GABA_A$ receptor subunits. The comparatively broad peak of the $\alpha 1$ subunit sedimentation and its overlap with the 7.4 *s* peak of the $\beta 3$ subunit might indicate that, in addition to $\beta 3$ homotetramers, $\alpha 1\beta 3$ tetramers had been formed to a significant extent under these conditions (Fig. 6*B*). The peaks at 6.7 and 5.5 *s*, however, seem to have consisted predominantly of $\beta 3$ homo-oligomers.

Stoichiometry of recombinant $\alpha 1\beta 3$ receptors expressed at the cell surface

The actual assembly of pentameric $\alpha 1\beta 3$ receptors, their incorporation into the cell membrane, and their stoichiometry were investigated by incubating intact HEK cells transfected with $\alpha 1$ and $\beta 3$ subunits with anti-$\alpha 1$(1–9) antibodies. Under these conditions these N-terminal antibodies interacted only with receptors expressed on the cell surface. Then cells were washed to remove excess of antibodies, and the receptors were extracted with a buffer containing 1% Triton X-100. Under these conditions the interaction between antibodies and receptors was reasonably stable. Receptors previously labeled by the antibody on the cell surface thus could be precipitated by the addition of Immunoprecipitin. Then the pellet was subjected to SDS-PAGE and Western blot analysis, and the ratio of the $\alpha 1$ and $\beta 3$ subunits in these receptors was investigated by using the $\beta 3$–$\alpha 1$ chimera as described above (experiments not shown). Results from two separate experiments indicated that the ratio of $\alpha 1$:$\beta 3$ subunits was 2:2.8 and 2:3.4, indicating that these receptors consisted of three $\beta 3$ and two $\alpha 1$ subunits.

$\alpha 1\gamma 2$ or $\beta 3\gamma 2$ coexpression

When HEK cells were transfected with $\alpha 1$ and $\gamma 2$ subunits, protein complexes containing $\alpha 1$ subunits sedimented at 6.7 and 5.5 *s*, whereas those containing $\gamma 2$ subunits sedimented as a single protein peak at 5.5 *s* (Fig. 6*C*). This seems to indicate that $\alpha 1$ and

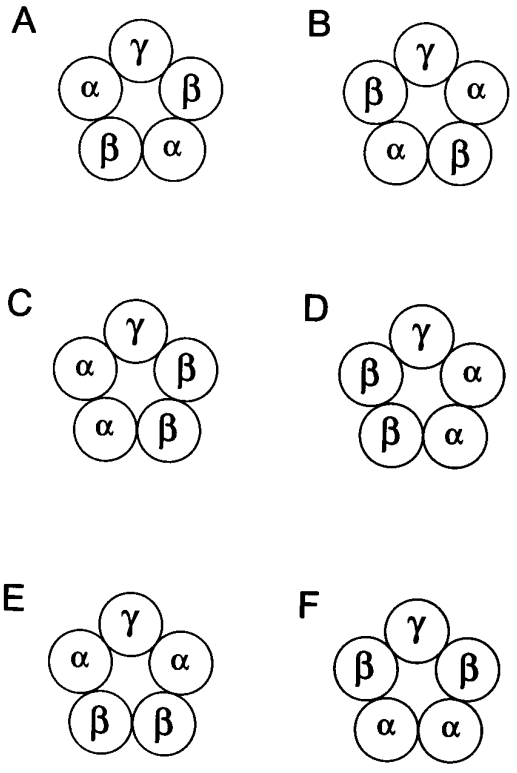

Figure 5. Possible subunit arrangements of receptors composed of two α subunits, two β subunits, and one γ subunit.

$\gamma 2$ subunits readily can form dimers, whereas predominantly the $\alpha 1$ subunits seem to be able to form trimers. Similarly, after transfection of HEK cells with $\beta 3$ and $\gamma 2$ subunits, the largest part of the $\beta 3$ and $\gamma 2$ subunits sedimented at 5.5 *s*, whereas a small proportion of predominantly the $\beta 3$ subunit sedimented with a higher sedimentation coefficient (Fig. 6*D*). This again seems to indicate that $\beta 3$ and $\gamma 2$ subunits readily form dimers but rather inefficiently form higher molecular mass complexes. Other experiments demonstrated that, in contrast to $\alpha 1\beta 3\gamma 2$ and $\alpha 1\beta 3$ receptors, $\alpha 1\gamma 2$ or $\beta 3\gamma 2$ subunit complexes could not be identified on the surface of HEK 293 cells (experiments not shown).

Subunit arrangement of recombinant $\alpha 1\beta 3\gamma 2$ receptors

The present results are in agreement with experiments from the nicotinic ACh receptor, indicating that the assembly of multimeric receptors is an ordered and step-wise process in which each newly added subunit causes a conformational change in the assembly complex that is necessary for the addition of further subunits. Failure to associate with the right subunit then might prevent proper folding and thereby lead to degradation (Green and Millar, 1995).

The formation of pentamers in cells cotransfected with $\alpha 1$ and $\beta 3$ subunits is consistent with all subunit arrangements shown in Figure 5. Each of these arrangements allows for an uninterrupted assembly of two α and two β subunits. The addition of a further β subunit finalizes the pentameric receptor that then is transported to the cell surface.

The observation that $\alpha 1$ and $\gamma 2$ or $\beta 3$ and $\gamma 2$ subunits predominantly form dimers, however, is consistent only with the subunit arrangements shown in Figure 5, *A* or *B*. Only these subunit arrangements predict an interruption of the assembly process at the level of an $\alpha 1\gamma 2$ or $\beta 3\gamma 2$ dimer, because at this stage the

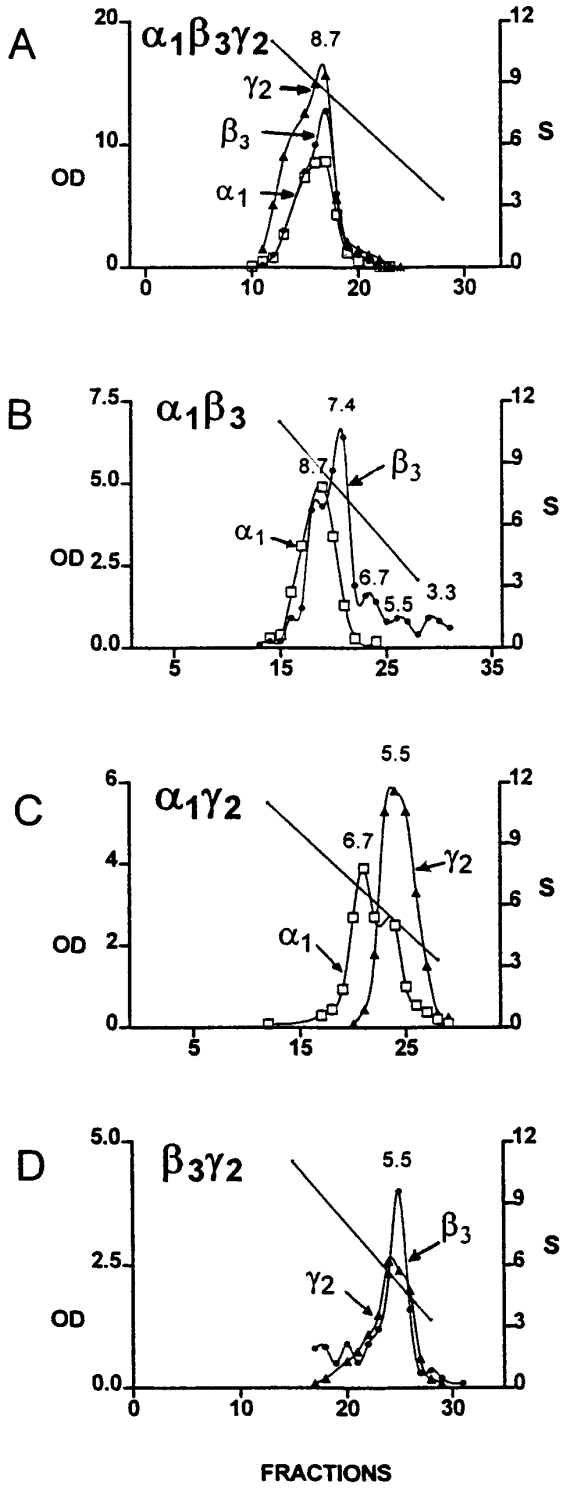

Figure 6. Sucrose density gradient centrifugation of $GABA_A$ receptors extracted from HEK cells transfected with various combinations of $\alpha 1$, $\beta 3$, and $\gamma 2$ subunits. HEK 293 cells were transfected with (*A*) $\alpha 1\beta 3\gamma 2$, (*B*) $\alpha 1\beta 3$, (*C*) $\alpha 1\gamma 2$, or (*D*) $\beta 3\gamma 2$ subunits. Receptors were extracted and size-fractionated on 5–20% linear sucrose density gradients. Gradients were fractionated, and protein in individual fractions was precipitated and subjected to SDS-PAGE and Western blot analysis with anti-$\alpha 1$(1–9), anti-$\beta 3$(1–13), or anti-$\gamma 2$(319–366) antibodies. *s* values were measured by including digoxigenated standard proteins with known *s* values in each gradient. *OD*, Optical density (arbitrary units). Symbols used: *open squares*, $\alpha 1$; *filled circles*, $\beta 3$; *filled triangles*, $\gamma 2$. The experiments were performed three to four times with comparable results.

presence of a $\beta 3$ or $\alpha 1$ subunit, respectively, is necessary to induce the proper conformational change for an efficient further assembly. Subunit arrangements 5*C* or 5*D*, in the absence of a $\beta 3$ or $\alpha 1$ subunit, predict an interruption of the assembly process at the level of trimers consisting of one $\gamma 2$ and two $\alpha 1$ or of one $\gamma 2$ and two $\beta 3$ subunits, respectively. Similarly, subunit arrangement 5*E* in the absence of a $\beta 3$ subunit predicts an interruption of the assembly process at the level of trimers composed of one $\gamma 2$ and two $\alpha 1$ subunits but, in addition, suggests that $\gamma 2$ and $\beta 3$ subunits do not combine with each other. Thus, in the absence of $\alpha 1$ subunits, arrangement 5*E* predicts the formation of $\beta 3$ homodimers and of monomeric $\gamma 2$ subunits. Finally, arrangement 5*F* in the absence of an $\alpha 1$ subunit predicts the formation of trimers composed of one $\gamma 2$ and two $\beta 3$ subunits and, in addition, suggests that $\gamma 2$ and $\alpha 1$ subunits do not combine with each other. In the absence of a $\beta 3$ subunit, arrangement 5*F* predicts the formation $\alpha 1$ homodimers and of $\gamma 2$ subunit monomers. Because previous studies have demonstrated the formation of receptors consisting of $\alpha 1\gamma 2$ subunits (Sigel et al., 1990; Knoflach et al., 1992) and of $\beta 2\gamma 2$ subunits (Draguhn et al., 1990; Sigel et al., 1990; Verdoorn et al., 1990), in agreement with the present conclusion the actual formation of arrangement 5*E* and 5*F* seems not to be very probable.

DISCUSSION

Stoichiometry of recombinant $\alpha 1\beta 3\gamma 2$ receptors

In the present study chimeric proteins composed of the complete N-terminal domain of one $GABA_A$ receptor subunit and of the four transmembrane domains plus the cytoplasmic loop of another subunit were used to compare the reactivity of subunit-specific N-terminal or cytoplasmic loop antibodies in Western blots. Using this information, we could determine the ratio of the respective subunits in purified $GABA_A$ receptors from the relative signal intensities of the antibodies in Western blots. Results obtained indicated that recombinant $\alpha 1\beta 3\gamma 2$ $GABA_A$ receptors are composed of two α, two β, and one γ subunit.

Our conclusions are tied strongly to the assumption that the antibodies exhibited the same reactivity toward the chimeric proteins and the respective subunits. Theoretical considerations do support this assumption. Thus, the epitopes identified by the antibodies used are located at the very N-terminal end or at the cytoplasmic loop of the subunits and are surrounded by amino acid domains that are identical in the original as well as in the chimeric subunits. An identical reactivity of the antibodies with original and chimeric subunits, therefore, is highly likely.

This conclusion is strengthened by the observation that, overall, results on $\alpha 1\beta 3\gamma 2$ receptors obtained from the investigation of three different chimeras and subunit ratios with four different antibodies were highly consistent. Thus, subunit ratios $\alpha 1{:}\beta 3 = 1{:}1$ and $\beta 3{:}\gamma 2 = 2{:}1$ are consistent with the ratio $\alpha 1{:}\gamma 2 = 2{:}1$ and support the observation that native $GABA_A$ receptors do form subunit pentamers (Nayeem et al., 1994). A differential reactivity of the antibodies for receptor subunits and chimeras would have resulted in discrepant subunit ratios.

Finally, assembly studies indicating that $\alpha 1$ and $\beta 3$ subunits can form pentamers (and possibly significant amounts of tetrameric intermediate products) whereas $\alpha 1$ and $\gamma 2$ or $\beta 3$ and $\gamma 2$ subunits can form dimers only with each other (Fig. 6) support a subunit stoichiometry of $\alpha 1{:}\beta 3{:}\gamma 2 = 2{:}2{:}1$. Any other subunit stoichiometry would have resulted in $\alpha 1\beta 3$ complexes smaller than tetramers and in $\alpha 1\gamma 2$ or $\beta 3\gamma 2$ complexes larger than dimers.

Comparison with other studies on recombinant $GABA_A$ receptors

The present results on the subunit stoichiometry of recombinant $GABA_A$ receptors are supported partially by an electrophysiological study (Backus et al., 1993) investigating the degree of outward rectification of the GABA-evoked current in recombinant $\alpha 3\beta 2\gamma 2$ receptors induced by charged substitutions in homologous positions of the putative pore-forming domain of the individual subunits. Although this study favored a subunit stoichiometry of two α, one β, and two γ subunits, a subunit composition of two α, two β, and one γ subunit was only slightly less probable. Given the possibility that the degree of the effect on outward rectification of changing the charge might not be identical in different subunits, as is assumed in this study (Backus et al., 1993), the agreement of this study with the present investigation is reasonable.

Two other electrophysiological studies, however, fully support the present conclusions on the subunit stoichiometry of $\alpha 1\beta 3\gamma 2$ receptors. Thus, Im et al. (1995), investigating ion channels formed from $GABA_A$ receptor subunits and tandem constructs of $\alpha 6$-$\beta 2$ subunits, and Chang et al. (1996), using site-directed mutagenesis to increase the GABA sensitivity of recombinant receptors in proportion to the number of incorporated mutant subunits, concluded that recombinant $\alpha 6\beta 2\gamma 2$ and $\alpha 1\beta 2\gamma 2$ $GABA_A$ receptors, respectively, are pentamers composed of two α subunits, two β subunits, and one γ subunit. The observation that several different groups investigating distinct recombinant $GABA_A$ receptor subtypes with different methods end up with the same subunit stoichiometry strongly supports the conclusion that $GABA_A$ receptors in most cases are composed of two α subunits, two β subunits, and one γ subunit.

Comparison with native $GABA_A$ receptors

This conclusion is in agreement with results obtained with native $GABA_A$ receptors. Several investigations have indicated that two different α subunits can be present in native $GABA_A$ receptors (Duggan et al., 1991; Lüddens et al., 1991; Zezula and Sieghart, 1991; Mertens et al., 1993; Pollard et al., 1995), whereas only a single type of γ subunit was detected in these receptors isolated by highly specific anti-γ antibodies, although the other γ subunits could be identified easily in the original extracts (Mossier et al., 1994).

A stoichiometry of two α subunits, two β subunits, and one γ subunit, however, is in apparent contrast to experiments from Benke et al. (1994), suggesting that native $GABA_A$ receptors may contain only a single type of β subunit. This conclusion was reached from the observation that native $GABA_A$ receptors immunoprecipitated by anti-$\beta 1$, anti-$\beta 2$, or anti-$\beta 3$ antibodies sum up to ~100% of all receptors present in the brain extract. This interpretation, however, did not take into account a possible incomplete immunoprecipitation of receptor subtypes by the antibodies used or a possible predominant presence of two β subunits of the same isoform in $GABA_A$ receptors.

Quirk et al. (1994) or Khan et al. (1994) observed that the sum of the percentages of native $GABA_A$ receptors immunoprecipitated by anti-$\gamma 2$ and anti-$\gamma 3$ or by anti-$\gamma 2$S and anti-$\gamma 2$L antibodies, respectively, was larger than that obtained by a combination of the antibodies. In both cases the data were used to conclude that a minor fraction of the $GABA_A$ receptors can contain more than one type of γ subunit. In addition, Quirk et al. (1994), using Western blot analysis, were able to demonstrate the presence of $\gamma 2$ subunits in $GABA_A$ receptors precipitated by anti-γ_3 antibodies, again suggesting that $\gamma 2$ and $\gamma 3$ subunits can coexist in the

same $GABA_A$ receptor. The discrepancy of these studies with that mentioned above (Mossier et al., 1994) is not clear at present. It should be kept in mind, however, that well characterized and initially very specific antibodies might become unspecific in the course of immunization and that the addition of too much antibody (for instance when receptors are precipitated simultaneously with several antibodies directed against different subunits) often decreases the overall efficiency of immunoprecipitation. In addition, it is possible that the stoichiometry of $GABA_A$ receptors depends on the particular α, β, and γ subunit isoform.

Stoichiometry of recombinant $\alpha1\beta3$ receptors

Via an investigation of $\alpha1\beta3$ receptors incorporated into the cell membranes, it was demonstrated that these receptors are composed of two α and three β subunits. Interestingly, from the observation that the $\alpha6$–$\beta2$ tandem constructs are able to form GABA-activated channels with $\alpha6$ or $\gamma2$, but not with $\beta2$ subunits, Im et al. (1995) concluded that $\alpha6\beta2$ receptors are composed of three $\alpha6$ and two $\beta2$ subunits. The discrepancy between this conclusion and the present results on the composition of $\alpha1\beta3$ receptors again could indicate that the stoichiometry of $\alpha\beta$ receptors depends on the specific subunits present in these receptors. Alternatively, it might have been attributable to changes in the structure of subunits induced by the 10 glutamine residues linking the C-terminal of the $\alpha6$ to the N-terminal of the $\beta2$ subunit in the $\alpha6$–$\beta2$ tandem construct (Im et al., 1995).

$GABA_A$ receptor assembly

The present results support previous conclusions (Connolly et al., 1996a) that only $\alpha1\beta3\gamma2$ or $\alpha1\beta3$ subunits, but not $\alpha1\gamma2$ or $\beta3\gamma2$ subunits, give rise to completely assembled pentameric $GABA_A$ receptors that then are transported to the cell surface. Whereas in cells transfected with $\alpha1\beta3\gamma2$ subunits no significant accumulation of assembly intermediates was observed, in cells transfected with $\alpha1\beta3$ subunits lower oligomers of $\beta3$ subunits and possibly $\alpha1\beta3$ tetramers do accumulate within the cell. In cells transfected with $\alpha1\gamma2$ or $\beta3\gamma2$ subunit combinations, however, predominantly heterodimers consisting of $\alpha1\gamma2$ or $\beta3\gamma2$ subunits are formed.

The present data are consistent with the hypothesis that the assembly of $GABA_A$ receptors starts with the formation of $\alpha1\beta3$, $\alpha1\gamma2$, and/or $\beta3\gamma2$ dimers. In the presence of the respective third subunit, $\alpha1\beta3\gamma2$ pentamers are formed efficiently. Whereas further assembly of $\alpha1\gamma2$ or $\beta3\gamma2$ dimers is reduced dramatically in the absence of $\beta3$ or $\alpha1$ subunits, respectively, the assembly of $\alpha1\beta3$ subunits presumably can continue with reduced efficiency in the absence of the $\gamma2$ subunit, resulting in $\alpha1\beta3$ tetramers and pentamers. Because of the reduced assembly efficiency of $\alpha1\beta3$ subunits, homo-oligomers of $\beta3$ subunits, which also can be formed with low efficiency (Slany et al., 1995; Connolly et al., 1996b), might become relatively enriched in cells transfected with $\alpha1\beta3$ subunits. Further experiments will have to confirm this hypothesis.

Arrangement of the subunits

The formation of tetramers and pentamers from $\alpha1\beta3$ subunit combinations and of dimers from $\alpha1\gamma2$ or $\beta3\gamma2$ combinations suggests that $GABA_A$ receptors are pentamers composed of a total of four alternating α and β subunits connected by a γ subunit (Fig. 5*A* or 5*B*). Results obtained, however, cannot distinguish between these two mirror image arrangements. Cross-linking or mutagenesis experiments will have to be performed to confirm this subunit arrangement and to decide whether it corresponds with that shown in Figure 5*A* or 5*B*. In any case, knowledge on the subunit stoichiometry and arrangement is essential for further studies on the structure and function of $GABA_A$ receptors.

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