GABA<sub>A</sub> Receptor Composition Is Determined by Distinct Assembly Signals within α and β Subunits

Received for publication, October 7, 2002, and in revised form, December 4, 2002
Published, JBC Papers in Press, December 5, 2002, DOI 10.1074/jbc.M210229200

Karen Bollan‡, Dale King‡, Laura A. Robertson‡, Kenneth Brown‡, Pamela M. Taylor§,
Stephen J. Moss§, and Christopher N. Connolly‡

From the §Department of Pharmacology and Neuroscience, Ninewells Medical School, University of Dundee,
Dundee DD1 9SY, Scotland and ¶Medical Research Council Laboratory for Molecular Cell Biology,
University College London, Gordon St., London WC1E 6BT, United Kingdom

Key to understanding how receptor diversity is achieved and controlled is the identification of selective assembly signals capable of distinguishing between other subunit partners. We have identified that the β1–3 subunits exhibit distinct assembly capabilities with the γ2L subunit. Similarly, analysis of an assembly box in α1-(57–68) has revealed an absolute requirement for this region in the assembly of αβ subunits. Furthermore, a selective requirement for a single amino acid (Arg-66), previously shown to be essential for the formation of the low affinity GABA binding site, is observed. This residue is critical for the assembly of α1β2 but not α1β1 or α1β3 receptors. We have confirmed the ability of the previously identified GKER signal in β2 to direct the assembly of βγ receptors. The GKER signal is also involved in driving assembly with the α1 subunit, conferring the ability to assemble with α1<sup>1R66A</sup> on the β2 subunit. Although this signal is sufficient to permit the formation of β2γ2 receptors, it is not necessary for β3γ2 receptor formation, suggesting the existence of alternative assembly signals. These findings support the belief that GABA<sub>A</sub> receptor assembly occurs via defined pathways to limit the receptor diversity.

γ-Aminobutyric acid, type A (GABA<sub>A</sub>)<sup>1</sup> receptors are the major sites of fast synaptic inhibition in the brain. In mammals, they are constructed as pentameric structures from multiple subunits selected predominantly from the following distinct classes: α (1–6), β (1–3), γ (1–3), δ, ε, θ, and σ, creating an incredible (16<sup>n</sup>) potential for structural diversity. However, relatively few functionally distinct receptor compositions are thought to exist in vivo (1). It is possible that multiple receptor types may exist that are functionally equivalent. Their distinct subunit compositions may provide subtle functions such as modulation by endogenous ligands such as neurosteroids (2) or second messenger systems (3, 4), subcellular localization (5), or long term differences in the regulation of receptor surface expression (6, 7). Despite these caveats, GABA<sub>A</sub> receptor heterogeneity occurs via defined pathways to limit receptor diversity (3, 6). Possible mechanisms include brain region-specific (8) and temporal expression (9). However, many neuron types often express multiple receptor subunit mRNAs simultaneously (8), suggesting that subcellular mechanisms for differential receptor assembly may also exist. Potential processes could include the discrete sites of subcellular receptor assembly (10, 11) and/or the presence of assembly signals capable of differential interaction with other subunits.

In support of the existence of differential assembly signals, GABA<sub>A</sub> receptor assembly appears to be strictly controlled, producing receptors with a fixed stoichiometry of α2, β2, and γ1 (12–17). Furthermore, GABA<sub>A</sub> receptor assembly signals have been identified in the α1 (18, 19), β2/3 (19, 20), and γ3 (21) subunits. Although the regions identified in these studies may exhibit subunit class-specific interactions, to date no studies have investigated the ability of GABA<sub>A</sub> receptors to discriminate between subunits of the same class.

Consistent with the location of these assembly signals to intersubunit contact points, the αγ signals (18, 19, 21) are located proximal to the GABA and benzodiazepine binding sites (22, 23) formed at subunit interfaces between the α- and β- and α- and γsubunits, respectively, and also the β2 high affinity GABA site (24). Similarly, the homologous region in α1 is an important component of the GABA binding domain (25).

Given the high degree of homology between the α and γ subunits in this region combined with their differential ability to assemble with β subunits (α1 with β1–3, γ2 with β3, possibly β1, but not β2) (20), we sought to investigate the role of these sequences in the differential assembly of αγ subunits with β subunits. Using site-directed mutagenesis, we have determined that the conversion of a single amino acid in α1 to that of γ2 (R66A) is sufficient to alter the assembly profile of the α1 subunit to that of the γ2, as determined by immunofluorescence and cell surface ELISA. These results demonstrate that the identity of a single amino acid may be critical in determining assembly with particular receptor subunits and may identify a basis for subunit-specific GABA<sub>A</sub> receptor assembly. Furthermore, we present evidence for the existence of alternative assembly signals, which may permit the formation of diverse receptor types, dependent upon subunit availability.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—COS 7 cells (ATCC CRL 1651) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 units/ml penicillin in an atmosphere of 5% CO<sub>2</sub>. Exponentially growing cells were transfected by electroporation (400 V, infinity resistance, 125 microfarads, Bio-Rad Gene Electropulser II). 10 μg of DNA was used per transfection (2 × 10<sup>6</sup> cells) using equimolar ratios of expression constructs. Cells were analyzed 12–48 h after transfection.

**DNA Constructions**—Murine α1, β1–3, and γ2L subunit cDNAs containing either the Myc or FLAG epitope tags (between amino acids 4

This paper is available on line at http://www.jbc.org
and 5 of the mature polypeptide) have been described previously and shown to be functionally silent with respect to receptor pharmacology and physiology (5, 18, 20, 26). The mutant constructs α1S, α1(ρ1), β2GAKKR, and β2GQMTK were generated by site-directed mutagenesis as reported previously (18, 20). The remaining mutant α1/2 constructs were generated by site-directed mutagenesis using the oligonucleotides: α1(Δ1), 5'-CATCCTCCAAGTTGGAGCAGAAAAATATCTGATT-ATC-3', β2A(Δ1), 5'-CTCAAACTTTTGACGAAAAACACATC-3'.

Differential GABA<sub>A</sub> Receptor Assembly

FIG. 1. Sequence alignment of GABA<sub>A</sub> receptor subunits between amino acids 57 and 94 (α1 mouse numbering). Sequence alignments over this region of α (A), β (B), and γ (C) subunits are shown. Consensus sequences are shown for each individual subunit class. An overall consensus sequence is shown for α, β, and γ subunits (D) along with the sequences of e and s subunits. The amino acid underlined (in bold) identifies a single residue completely conserved within each subunit class but absent from all other subunits. Completely conserved tryptophans (in all members of the ligand-gated superfamily) are illustrated in bold.

(Gln-67) and the tryptophan (Trp-69) have been shown to be essential for assembly (18, 28). However, the glutamine is completely conserved among all GABA<sub>A</sub> receptors, and the tryptophan is completely conserved among all members of the ligand-gated ion channel superfamily, indicating a general role in subunit architecture or folding.

To implicate residues that may be involved in subunit class-specific assembly of GABA<sub>A</sub> receptors, we examined the sequences in this region to identify residues completely conserved within a subunit class but absent from the other subunit classes. Surprisingly, despite the high degree of homology evident, only one residue (Arg-66 in α1) fits this criterion. At this position, an arginine (αs), glutamine (βs), or alanine (γs), is always present (histidine in δ, serine in e). Interestingly, this residue in α1 has been shown to be critical for GABA binding (22).

To assess the role of this region in GABA<sub>A</sub> receptor assembly we investigated α1/2 subunit chimeras and mutants for their ability to assemble with the β subunits. The constructs used are α1myc, α1Smyc (lacking residues 57–68), α1v2myc (residues 57–68 replaced with the homologous residues from γ2), α1Kmyc (arginine at position 66 mutated to alanine), γ2Lmyc, γ2L10myc (residues replaced with homologous residues from α1), and γ2L2A10myc. Subunit selection was performed using 9E10 antibodies that recognize the Myc epitope present in all the α1/2L subunits. All β subunits used were epitope-tagged with the FLAG epitope. When expressed alone, all the above α1/2L subunits do not reach the cell surface but are retained
within the ER (5) (results not shown). Thus, we measure the ability of the α1/γ2L subunits to be rescued from the ER and expressed on the surface as heteromeric receptors with β subunits.

Cell Surface Expression of α1/γ2 Subunits with β2—COS7 cells were transfected with α1/γ2LFLAG and immunofluorescence was performed using 9E10 antibodies. As shown previously (5, 26), α1 and β2 subunits are able to assemble and form heteromeric receptors on the cell surface (Fig. 2A). Consistent with the presence of an assembly signal existing between residues 57 and 68 of the α1 subunit, α1S (lacking this region) cannot reach the cell surface despite the presence of the β2 subunit. The α1S is retained within the ER, as evidenced by the classic reticular staining pattern observed (Fig. 2A). To address the possibility that this subunit might misfold, we examined an α1(S1) chimera in which residues 57–68 in the α1 were replaced with the homologous residues from GABA<sub>A</sub> receptor β1 subunit (18). Given the expected structural similarities between β1 and GABA<sub>A</sub> receptor subunits combined with the recent observation that this region in β1 also contributes to the GABA binding site (25), this chimera would be less likely to misfold. In keeping with the inability of the β1 subunit to assemble with the GABA<sub>A</sub> receptor β subunits (29, 30), coexpression of α1(S1) with β2 did not lead to cell surface expression (results not shown).

Receptors composed of β2/γ2 do not access the cell surface but are retained in the ER (5) (Fig. 2). Therefore, we assessed the ability of α1γ2L to assemble with β2. Consistent with a role for α1 residues 57–68 in assembly with β2, cell surface expression of α1γ2L is abolished.

Given the critical role of arginine at position 66 of α1 in GABA binding (22) and the complete conservation of this position within all subunit classes (Fig. 1), we mutated this arginine (in α1) to the corresponding residue (alanine) in γ2 creating α1(R-A). This mutation completely abolished the ability of α1 to assemble with β2.

To determine whether this putative α1 assembly signal might be transplanted into the γ2 subunit, we generated a γ2L(α1) construct containing the residues 57–68 from α1 in place of the homologous γ2 sequence. When expressed with β2, γ2L(α1) is still incapable of assembling with β2 (Fig. 2A). Thus, although the α1 region 57–68 is essential for the assembly of α1 and β2 and critically dependent upon an arginine at position 66, this region is insufficient to direct this assembly event. This is corroborated by the γ2L[γ2] construct, which cannot assemble with β2.

Quantification of these observations was performed by whole cell ELISA to determine receptor cell surface expression (no detergent) versus total (detergent) receptor expression. The values presented here do not reflect a true percentage of surface-expressed receptors for two reasons; first, the α1β2 values have been normalized to 100% and, second, surface values greater than 100% have been detected for α1 homomers (results not shown), suggesting a possible inhibitory influence of prior detergent treatment on this assay. In these experiments (Fig. 2B, Table I) it can be seen clearly that the assembly of β2 with α1 requires residues 57–68 of α1 (α1S = 0.9 ± 1.3%, most notably an arginine at position 66 (α1(R-A) = 5.0 ± 4.3%). In agreement with the results observed by immunofluorescence,
Differential GABA<sub>A</sub> Receptor Assembly

Table I

Summary of cell surface expression for mutant GABA<sub>A</sub> receptors

|        | β1  | β2  | β3  | β2<sup>GluR</sup> | β3<sup>ENTK</sup> |
|--------|-----|-----|-----|-------------------|-------------------|
| α1     | 100 ± 7.6 | 101 ± 8.7 | 100 ± 10.5 | 100.4 ± 18.2 | 100.2 ± 9.7 |
| α1S    | 2.7 ± 3.1 | 0.9 ± 1.3 | 3.5 ± 3.6 | 9.7 ± 6.9 | 7.9 ± 5.6 |
| γ2L    | 10.2 ± 11.8 | 3.0 ± 3.0 | 54.7 ± 19.7 | 74.3 ± 19.9 | 26.8 ± 11.5 |
| α1<sup>R-A</sup> | 108.6 ± 23.4 | 10.2 ± 10.0 | 163 ± 24.6 | 118.6 ± 21.9 | 99.6 ± 5.8 |
| α1<sup>R-A</sup> | 83.9 ± 16.1 | 5.0 ± 4.3 | 812 ± 9.2 | 90.1 ± 24.3 | 61.6 ± 13.2 |
| γ2L<sup>2(A-R)</sup> | 5.6 ± 2.5 | 3.7 ± 3.1 | 50.9 ± 16.2 | 55.8 ± 26.0 | 38.3 ± 20.5 |
| γ2L<sup>2(A-R)</sup> | 4.9 ± 5.0 | 7.5 ± 6.0 | 85 ± 23.3 | 31.3 ± 9.9 | 24.7 ± 13.2 |

Table values are obtained from Fig. 2–6 and are expressed as the percentage cell surface expression relative to α1 (normalized to 100%) ± S.E.

Fig. 3. Surface expression of β1 recombinant GABA<sub>A</sub> receptors requires the presence of the α1 subunit. COS 7 cells coexpressing β1<sup>FLAG</sup> with either α1<sup>mov</sup>, α1S<sup>mov</sup>, γ2L<sup>mov</sup>, α1(γ2)<sup>mov</sup>, α1(R-A)<sup>mov</sup>, γ2L(α1)<sup>mov</sup>, or γ2(A-R)<sup>mov</sup> were examined by immunofluorescence (A) for the presence of the Myc-tagged subunits at the surface and intracellularly. Quantification of surface expression levels (B) were performed by cell ELISA in the absence (Surface) or presence (total) of detergent and normalized to α1 levels. COS 7 cells were coexpressing β1<sup>FLAG</sup> with either α1<sup>mov</sup> (lane 1), α1S<sup>mov</sup> (lane 2), γ2L<sup>mov</sup> (lane 3), α1(γ2)<sup>mov</sup> (lane 4), α1(R-A)<sup>mov</sup> (lane 5), γ2L(α1)<sup>mov</sup> (lane 6), or γ2(A-R)<sup>mov</sup> (lane 7). Each recording represents the mean ± S.E. of at least nine determinants in at least three independent experiments. *, denotes significant difference from α1 control (p < 0.001, t test).

Although this region is critically involved in assembly with β2, transplantation of this signal to γ2 does not confer the ability to assemble with β2 (α1<sup>1γ2</sup> = 3.7 ± 3.1%).

Cell Surface Expression of α1/γ2 Subunits with β1—COS7 cells were transfected with α1/γ2<sup>β1</sup><sup>FLAG</sup>, and immunofluorescence was performed using 9E10 antibodies. As shown previously (26) α1 and β1 subunits are able to assemble and form heteromeric receptors on the cell surface (Fig. 3). Consistent with the presence of an assembly signal existing between residues 57–68 of the α1 subunit, α1S cannot reach the cell surface despite the presence of the β1 subunit (2.7 ± 3.1%). Similar results were observed for the α1<sup>1γ2</sup> (results not shown).

In this study, we could detect only very low levels of γ2L/β1 surface receptors by immunofluorescence (Fig. 3A) that could not be resolved from background (mock-transfected cells) when analyzed quantitatively (10.2 ± 11.8%, Fig. 3B). To determine whether the robust surface expression observed upon α1β1 expression results from the use of the α1 57–68 assembly signal, we assessed the ability of α1<sup>1γ2</sup> to assemble with β1. In contrast to the results observed with the β2 subunit, cell surface expression of α1<sup>1γ2</sup> is not significantly different (108.6 ± 23.4%) from that observed for wild-type α1 (100 ± 7.6%) but higher than that observed for the wild-type γ2L (10.2 ± 11.8%). These findings suggest that, in contrast to the β2 subunit, the β1 subunit does not exhibit the same requirements for this region of the α1 for the assembly of α1β1 heteromeric receptors.
This possibility is corroborated by the efficient assembly of β1 with α1R^A (83.9 ± 16.1%, Fig. 3). However, this region is still essential for α1β1 receptor assembly, as evidenced by the inability of α1S (Fig. 3) and α1α3β1 (results not shown) to assemble with β1. Both of the γ2 mutants (γ2α1^A) and γ2αA^R) were indistinguishable from the wild-type γ2L with respect to cell surface expression with β1, i.e. weak surface immunofluorescence and non-detectable surface levels by ELISA (Fig. 3).

Cell Surface Expression of α1γ2 Subunits with β3—COS7 cells were transfected with α/γ3β3^FLAG, and immunofluorescence was performed using 9E10 antibodies. As shown previously (26) α1 and β3 subunits are able to assemble and form heteromeric receptors on the cell surface (Fig. 3). Consistent with the presence of an assembly signal existing between residues 57 and 68 of the α1 subunit, α1S cannot reach the cell surface (3.5 ± 3.6%) despite the presence of the β3 subunit. Similar results were observed for the α1α3β1 (results not shown).

Receptors composed of β3γ2 have been reported to be capable of forming functional cell surface receptors (18). In this study, we observed robust surface expression by immunofluorescence (Fig. 4A) and ELISA (54.7 ± 19.7%, Fig. 4B). We assessed the ability of α1γ2β3 to assemble with β3. As observed with the β1 subunit, α1γ2β3 is capable of assembling with β3 and even exhibits enhanced levels of surface expression (163 ± 21.9%, p < 0.02, t test) compared with α1 (100 ± 10.5%) or γ2L (54.7 ± 19.7%). These findings suggest that, like the β1 subunit but in contrast to the β2 subunit, β3 does not require this region of the α1 for the assembly of α1β3 heteromeric receptors. This possibility is corroborated by the efficient assembly of β3 with α1α3β3^R^A (81.2 ± 9.2%, Fig. 4).

Both of the γ3 mutants (γ3α1R^A) and γ3αA^R) were indistinguishable from the wild-type γ3L with respect to cell surface expression with β3, i.e. strong surface immunofluorescence and detectable surface levels by ELISA (Fig. 4, γ3Lα1^A = 50.9 ± 16.2% and γ3Lα1^A = 85 ± 23.3%).

Interestingly, the β3 subunit (20, 31) and to a lesser extent the β1 subunit (32, 33) can form functional homomeric ion channels (not GABA-gated), whereas the β2 subunit is incapable of exiting the ER (5). A four-amino acid signal (GKERR) that controls β3 homooligomerization has been identified (20). When transferred to β2 (β2GKER), this subunit is capable of cell surface expression as functional ion channels. The reciprocal construct (β3^DNTR) can no longer assemble into homomeric receptors but is retained in the ER (20). To determine whether the assembly signal identified in the α1 may also recognize the homomeric assembly signal present in the β3 subunit, we analyzed the assembly of α1γ2 subunits with the β2GKER and β3^DNTR subunits.

Cell Surface Expression of α1γ2 Subunits with β2—COS7 cells were transfected with α/γ3β2GKER^FLAG, and immunofluorescence was performed using 9E10 antibodies. As expected, α1 and β2GKER subunits are able to assemble and form heteromeric receptors on the cell surface (Fig. 5). Again, α1S cannot reach the cell surface (9.7 ± 6.9%) despite the presence of the β2GKER subunit. Similar results were observed for the α1αβ3 (results not shown). In contrast to the ER retention of β2γ2L complexes (5) (3.0 ± 3.0% surface expression, Fig. 2), when β2GKER and γ2L are coexpressed, cell surface (74.3 ± 19.9%) receptors are produced (Fig. 5). Furthermore, surface
Cell Surface Expression of α1/γ2 Subunits with β3DNTK—

COS 7 cells were transfected with α1myscDNA, β3DNTK(FLAG), and immunofluorescence was performed using 9E10 antibodies. As expected, α1 and β3DNTK subunits are able to assemble and form heteromeric receptors on the cell surface (Fig. 6). Again, α1S cannot reach the cell surface (7.9 ± 5.6%) despite the presence of the β3DNTK subunit. Similar results were observed for the α1γ2L receptors (54.7 ± 19.7% surface expression, Fig. 6), when β3DNTK and γ2L are coexpressed, cell surface (26.8 ± 11.5%) receptors are produced (Fig. 6). Furthermore, surface expression of α1γ2L with β3DNTK occurred at significant levels (99.6 ± 5.8%). In addition, the ability of the α1(R-A) to assemble with β3DNTK and reach the cell surface (61.6 ± 13.2%) compared with β3 (81.2 ± 9.2%) was unaffected. Analysis of the γ2 mutants reveals that the γ2LN (1) and the γ2L(A-R) are expressed on the cell surface at significant levels (38.3 ± 20.5 and 24.7 ± 13.2%), comparable with those observed for β3 (50.9 ± 16.2 and 85 ± 23.3%, respectively) but not β2 (3.7 ± 3.1% and 7.5 ± 6.0%, respectively). Although the values obtained for the assembly of β3DNTK are consistently lower than that observed for the wild-type β3, the differences are not statistically significant, with the exception of α1γ2L and γ2(L-A-R) (p < 0.005, t test). Paradoxically, results observed for β3DNTK suggests that the β3 homomeric assembly signal is not an essential requirement for the assembly of β3 with either α1 or γ2.

To determine the ability of the α1/γ2 polypeptides to oligomerize with β2, cDNAs were cotransfected into COS 7 cells, [35S]methionine-labeled, and immunoprecipitated via the Myc epitope tag on the α1/γ2 subunits. Only the extracellular domain of β2 was used to eliminate any contribution from other subunit interactions (34) and events at the cell surface such as receptor turnover (6). Bands corresponding to the α1/γ2 and β2 extracellular fragments were excised and quantified using a scintillation counter. The ratio of β2 co-immunoprecipitated with the α1/γ2 subunit was normalized to that of wild-type α1 (39%) such that the ratio (β2:α1) of β2 co-immunoprecipitated by α1 represents 100%. No significant reduction in binding was evident for any of the α1/γ2 subunits (Fig. 7). This is not surprising, because each subunit must possess at least two interfaces (and presumably assembly signals) with other subunits (Fig. 8).

**DISCUSSION**

To date 16 different GABA<sub>A</sub> receptor cDNAs have been isolated from a variety of vertebrates (4). Many of these subunits
though there is extensive evidence for a role of temporal (9) and spatial (8, 35) regulation of subunit expression in determining receptor composition, these mechanisms cannot explain how receptor diversity is limited in neurons co-expressing multiple GABA_A receptor subunits simultaneously (8, 35). To date, there is no supporting evidence for discrete subcellular assembly sites for GABA_A receptors, although emerging evidence for the localized translation of proteins at synapses (11) may be relevant.

The potential for hierarchical assembly signals is supported by the strict control of receptor stoichiometry (12–17) and the observations that α1β3 (versus α13 homomers) and α1β2y2 (versus α1β2) receptors form to the exclusion of the other possible combinations (3, 31, 36). In vivo evidence for hierarchical receptor assembly has been provided by the analysis of α6 knockout mice, which determined that the δ subunit was concomitantly “knocked-out” by a partial α6 polypeptide product that remains able to associate with the δ subunit but cannot produce functional receptors (37).

The discovery of putative GABA_A receptor assembly signals began with the identification of a natural splice variant of α6 that lacked 10 amino acids in the N-terminal extracellular domain (38). This splice variant (termed α6Short) was determined to be incapable of assembling into functional receptors (18, 38). The high degree of homology between all GABA_A receptor subunits within this region suggests a common role in receptor assembly, a possibility vindicated for the α1 (18) (but see Ref. 19) and γ3 (21). Putative assembly signals have also been discovered adjacent to this region in α1 (for binding to γ2)
Differential GABA<sub>A</sub> Receptor Assembly

### A

| Interface 1 | Sequence | Interface 2 | Site | Binding |
|-------------|----------|-------------|------|---------|
| α1A:        | 1) MEYTIDVFPRQSW<sup>5</sup> 2) unknown alternative β1(B3)<sup>6</sup> | β2B | 1 | <GABA> |
| β1B:        | a) MTYRLRNLMASKITTPDIFHH<sup>2</sup> b) unknown<sup>6</sup> | γA | 5 | Bz |
| γ2A:        | MDYTLTMYFQQAW<sup>7</sup> | αB<γB | 2,4 | <GABA> |
| β2B:        | unknown | αA | 1,3 | <GABA> |
| β3A:        | MDYTLTMYFQQAW<sup>7</sup> | αB<γB | 2,4 | <GABA> |
| β3B:        | 1) G-K-→ER<sup>4</sup> 2) Unknown alternative<sup>8</sup> | αA<γA | 1,5 | GABA/<b>2</b> |
| γ2A:        | MEYTDFFAQTWW<sup>7</sup> | αB | 5 | Bz |
| γ2B:        | YDRLKMNST<sup>4</sup> | βA | 4 | - |
| γ3A:        | MEYQDIFFAQT<sup>7</sup> | αB | 5 | Bz |

### B

**Fig. 8. Identification and location of putative assembly signals in GABA<sub>A</sub> receptor subunits.** A, putative assembly signals are illustrated for interface 1 and their opposite interface (interface 2) shown. α1B may form an interface with either a (α) γ or a (β) β subunit. β3B may possess alternative signals capable of interacting with α1A/<b>γ</b>2A. Two different γ2A signals have been identified. <GABA> or >GABA represents the low or high affinity GABA binding sites, respectively. 1, Ref. 18, 2, Ref. 39. 3, Ref. 20, 4, Ref. 19. 5, Ref. 21. *, this study. B, arrangement of subunits in an αβ pentameric receptor indicating subunit interfaces and binding sites for GABA (G) and benzodiazepines (Bz). The mirror image of this structure is equally possible (39).

(39) and γ2 (for binding to α1 and β3) (19). Furthermore, two invariant tryptophans within this region have been shown to be essential for GABA<sub>A</sub> receptor assembly and benzodiazepine, but not GABA, binding (28). Indeed these tryptophans are completely conserved in all members of the ligand-gated ion channel superfamily and may provide some common structural feature necessary for receptor assembly (28).

Given the similarity between the homologous regions between α (MEYTIDVFPRQSW) and γ (MEYTDFFAQTWW) subunits, we examined the possibility that the sequence differences between these subunit classes might be responsible for the differential ability of the γ2 subunit to assemble with β1 and β3 (20, 33), but not β2 (5, 20), whereas the α1 can assemble with all three β subunits (26).

Consistent with previous findings, α1S and α<sup>1α<sub>13</sub></sup> (18) are not able to assemble with β1–3. In addition, γ2L cannot assemble with β2 (20) and only weakly with β1 (Table I) (3). In keeping with the possibility that α1 (57–68) constitutes an assembly signal determining oligomerization with β subunits (18), α<sup>1α<sub>13</sub></sup> is also unable to assemble with β2. In fact, a single site (Arg-66) was found to be critical for the assembly of α1B2 receptors. However, the failure of γ2L<sup>α</sup>13 to assemble with β2 suggests that this putative assembly signal is necessary but not sufficient to direct this oligomerization step.

Interestingly, a striking overlap between the αA site and the GABA binding site (loop D) exists for α1B2 receptors (22). Boileau <i>et al.</i> (22) showed that residues Phe-64, Arg-66, and Ser-68 were found to be part of (or close to) the GABA binding site, with Phe-64 and Arg-66 critical for modulation by GABA. In keeping with other studies (18, 28), these authors found that Gln-67 and Trp-69 were essential for the production of functional receptors. Moreover, this region is not only important in the α1 subunit contribution to GABA binding, but the high affinity GABA binding site has been shown to be produced by the homologous region in β2 (β2A interface to α1) (24). In addition, the benzodiazepine binding site on the γ2 subunit resides within the same homologous region, with A79 (homologous position to Arg-66 in α1) lining the benzodiazepine binding pocket (23). A similar overlap between assembly signal and benzodiazepine binding is observed for the opposing interface on the α1 subunit, with residues 74–123 providing the binding site (40) and residues 81–100 (MTVRLNLMASKWTP-DTFF, see Fig. 1) involved in oligomerization with γ2 (39).

In contrast to the tight correlation between receptor assembly and the formation of the GABA/benzodiazepine binding site in α1B2γ2 receptors, little overlap exists for the opposing side of this interface, the βB site. This side of the interface has been determined to be constructed from 3 distinct regions; loop A between residues 93 and 101, loop B between residues 157 and 160, and loop C between residues 202 and 209 to generate the GABA binding domain on the β2 subunit (41, 42). The residues required for assembly (GDKAVGVER in β3) fall between loops B and C.

In contrast to the findings for the β2 subunit, although α1,


β3, β2GKER, and β3DNTK all require the presence of residues 57–68 from either α1 or α2, but not α1β2, receptor formation. β1, β2, and β3 exhibit distinct assembly profiles with α1 and γ2, utilizing distinct assembly signals in α1/γ2. We have identified an assembly signal in β1 that is sufficient to drive assembly with both α1 and γ2. However, it is not necessary, implicating the presence of multiple assembly signals capable of fulfilling the same function, selecting a subunit with which to assemble.

REFERENCES
1. McKernan, R. M., and Whiting, P. J. (1996) Trends Neurosci. 19, 129–143
2. Wohlfarth, K. M., Bianchi, M. T., and Macdonald, R. L. (2002) J. Neurosci. 22, 1541–1549
3. Angeletti, T. M., and Macdonald R. L. (1993) J. Neurosci. 13, 1418–1428
4. Moss, S. J., and Smart, T. G. (2001) Nat. Rev. Neurosci. 2, 240–250
5. Connolly, C. N., Wooltorton, J. R., Smart, T. G., and Moss, S. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 999–9904
6. Connolly, C. N., Kitzler, J. T., Thomas, P., Uren, J. M., Brandon, N. J., Smart, T. G., and Moss, S. J. (1999) J. Biol. Chem. 274, 36565–36572
7. Wang, Y., Xiong, Z. G., MacNeil, H., Burt, D. R., Allred, C. A., Brain, J. W., Bevan, M. P., MacDonald, J. F., and Wang, Y. T. (1997) Nature 388, 686–690
8. Wisden, W., and Seeburg, P. H. (1998) Curr. Opin. Neurobiol. 8, 263–289
9. Smith, S. S., Gung, Q. S., Li, X., Moran, M. H., Bitran, D., Frye, C. A., and Hsu, F. C. (1998) J. Neurosci. 18, 5275–5284
10. Gardiol, A., Racca, C., and Triller, A. (1999) J. Neurosci. 19, 168–179
11. Jönsson, J., and Eberwine, J. (2001) Nat. Rev. Neurosci. 2, 890–896
12. Im, W. B., Prengeyer, J. F., Binder, J. A., Dillon, G. H., and Alberts, G. L. (1995) J. Biol. Chem. 270, 26063–26066
13. Chang, Y., Wang, R., Barot, S., and Weis, D. S. (1996) J. Neurosci. 16, 5415–5424
14. Trettter, V., Ebyha, N., Fuchs, K., and Sieghart, W. (1997) J. Neurosci. 17, 2728–2737
15. Karrar, S. J., Whiting, P. J., Bonnert, T. P., and McKernan, R. M. (1999) J. Biol. Chem. 274, 10100–10104
16. Sieghart, W., Fuchs, K., Trettter, V., Eberl, V., Jeckling, M., Hogan, H., and Adamikik, D. (1999) Neurochem. Int. 34, 379–385
17. Baumann, S. W., Baur, R., and Siegel, E. (2001) J. Biol. Chem. 276, 36275–36280
18. Taylor, P. M., Connolly, C. N., Kitzler, J. T., Gorrie, G. H., Hosie, A., Smart, T. G., and Moss, S. J. (2000) J. Neurosci. 20, 1297–1306
19. Klauss,er, F. G., and Sieghart, W. (2000) J. Biol. Chem. 275, 8921–8926
20. Taylor, P. M., Thomas, M., Homanics, G. E., Connolly, C. N., Smart, T. G., and Moss, S. J. (1999) J. Biol. Chem. 19, 6360–6371
21. Sarto, I., Klauss,er, F. G., Ebyha, N., Mayer, B., Fuchs, K., and Sieghart, W. (2001) J. Biol. Chem. 276, 9241–9246
22. Boileau, A. J., Evers, A. R., Davis, A. F., and Czajkowski, C. (1999) J. Neurosci. 19, 4847–4854
23. Teissere, J. A., and Czajkowski, C. (2001) J. Neurosci. 21, 4977–4986
24. Smith, S. S., Gung, Q. S., Bateson, A. N., and Dunn, S. M. (2000) J. Biol. Chem. 275, 14198–14204
25. Torres, V. L., and Weiss, D. S. (2002) J. Biol. Chem. 277, 43741–43748
26. Taylor, P. M., Thomas, M., Homanics, G. E., Connolly, C. N., Smart, T. G., and Moss, S. J. (1999) J. Biol. Chem. 271, 89–96
27. Evans, G. I., Lewis, G., Ramsay, G., and Bishop J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
28. Srinivasan, S., Nichols, C. J., Lawless, G. M., Olsen, R. W., and Tobin, A. J. (1999) Neurochem. Int. 34, 195–205
29. Homanics, G. E., Sieghart, W., and Luddens, H. (2002) J. Neurochem. 81, 3–10
30. Bevan, M. P., MacDonald, J. F., and Wang, Y. T. (1997) J. Neurosci. 17, 2916–2925
31. Klauss,er, T., Sarto, I., Ebyha, N., Fuchs, K., Furtmüller, R., Mayer, B., and Sieghart, W. (2001) J. Neurosci. 21, 1192–1193
32. Smith, G. B., and Olsen, R. W. (2000) Neuropharmacology 39, 55–64
33. Boileau, A. J., Newell, J. G., and Czajkowski, C. (2002) J. Biol. Chem. 277, 2931–2937
34. Wagner, D. A., and Chajkowski, C. (2001) J. Neurosci. 21, 67–74
35. Trettter, V., Baur, B., Nusser, Z., Mihalek, R. M., Hogan, H., and Adamikik, D. (1999) Neurochem. Int. 34, 195–205
36. Homanics, G. E., Sieghart, W., and Luddens, H. (2002) Neurosci. 109, 723–743