Identification of Amino Acid Residues within GABA<sub>A</sub> Receptor β Subunits that Mediate Both Homomeric and Heteromeric Receptor Expression

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GABA<sub>A</sub> receptors are believed to be heteropentamers that can be constructed from six subunit classes: α(1–6), β(1–4), γ(1–3), δ, ε, and π. Given that individual neurons often express multiple receptor subunits, it is important to understand how these receptors assemble. To determine which domains of receptor subunits control assembly, we have exploited the differing capabilities of the β2 and β3 subunits to form functional cell surface homomeric receptors. Using a chimeric approach, we have identified four amino acids in the N-terminal domain of the β3 subunit that mediate functional cell surface expression of this subunit compared with β2, which is retained within the endoplasmic reticulum. Substitution of these four amino acids—glycine 171, lysine 173, glutamate 179, and arginine 180—into the β2 subunit was sufficient to enable the β2 subunit to homo-oligomerize. The effect of this putative “assembly signal” on the production of heteromeric receptors composed of αβ and βγ subunits was also analyzed. This signal was not critical for the formation of receptors composed of either αβ2 or αβ3 subunits, suggesting that mutation of these residues did not disrupt subunit folding. However, this signal was important in the formation of βγ2 receptors. These residues did not seem to affect the initial association of β2 and γ2 subunits but appeared to be important for the subsequent production of functional receptors. Our studies identify, for the first time, key residues within the N-terminal domains of receptor β subunits that mediate the selective assembly of GABA<sub>A</sub> receptors.

Key words: GABA receptor; homomeric; heteromeric; assembly; benzodiazepine; cell surface

GABA<sub>A</sub> receptors are the major sites of fast synaptic inhibition in the brain. Molecular cloning has revealed a multiplicity of GABA<sub>A</sub> receptor subunits that can be divided by sequence homology into six subunit classes: α(1–6), β(1–3), γ(1–4), δ, ε, and π. Alternative splicing further increases the repertoire of GABA<sub>A</sub> receptors (Macdonald and Olsen, 1994; Rabow et al., 1995; Davies et al., 1997; Hedblom and Kirkness, 1997). Localization experiments have revealed a large spatial and temporal variation in subunit expression, with many individual neurons expressing multiple subunits (Laurie et al., 1992; Macdonald and Olsen, 1994; Rabow et al., 1995). Clearly, to understand the diversity of GABA<sub>A</sub> receptors expressed in neuronal membranes it is important to gain some insights into how these receptor subunits are assembled into functional hetero-oligomers.

To address this question, the assembly of recombinant receptors has been analyzed, focusing on receptors composed of α1, β2, and γ2 subunits, because this combination is believed to account for up to 50% of all benzodiazepine-sensitive receptors in the adult brain (Laurie et al., 1992; Benke et al., 1994; Macdonald and Olsen, 1994; Rabow et al., 1995). Collectively, it is apparent that GABA<sub>A</sub> receptors are assembled in the endoplasmic reticulum (ER), where access to the cell surface is limited to receptors composed of either α1β2 or α1β2γ2 subunits (Connolly et al., 1996a,b). The α1γ2 and β2γ2 combinations and homomeric subunits are retained within the ER (Connolly et al., 1996a,b; Gorrie et al., 1997). ER-retained unassembled subunits are rapidly degraded (Gorrie et al., 1997). Recent studies focusing on the β3 subunit have shown that in contrast to homomeric α1, β2, or γ2 subunits, this protein has the capacity to access the cell surface on homomeric expression as determined by immunofluorescence (Connolly et al., 1996b). In addition, homomeric β3 subunits produce spontaneously gated ion channels on expression in either Xenopus oocytes or mammalian cells (Connolly et al., 1996b; Wooltorton et al., 1997).

Using subunit chimeras, we have exploited the differences in cell surface expression between the β2 and β3 subunits to identify key residues that are important in controlling receptor assembly. This approach has identified four amino acids in the N-terminal domain of the β3 subunit that mediate subunit homomemerization and cell surface expression. These residues also selectively affect assembly with the γ2 subunit but not the α1 subunit. Together, these observations demonstrate that defined signals in the N-terminal domains of GABA<sub>A</sub> receptor subunits mediate selective subunit oligomerization and play a critical role in controlling receptor assembly.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney 293 (A293) cells and African green monkey kidney (COS) cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 U/ml streptomycin (Sigma, St. Louis, MO), and 100 U/ml penicillin (Sigma). Cells were electroporated (400 V, infinite
The primary antibodies were applied for 1 hr at the following concentrations: the α1, α2L, and α2S (Whiting et al., 1990; Kofuji et al., 1991) subunits with the 9E10 epitope (between amino acids 4 and 5) and the β2 subunit cDNA with the FLAG epitope (between amino acids 4 and 5) in the cytomegalovirus-based pGW1 expression vector have been described previously (Connolly et al., 1996a). The β3 subunit cDNA in pGW1 was tagged with the FLAG epitope using the oligonucleotide CATGGTCCCAGGGGTCTGTCATCTGTCGGTCTGGATGCTCTGGCTCGT by site-directed mutagenesis as described previously (Kunkel, 1985).

To generate the β2β3 chimera, a PstI/HindIII fragment encoding the C terminal of β2 was ligated into the (FLAG)β2 pGW1 HindIII/PstI vector. An Xhol site was introduced into both the β2 and β3β2 pGW1 constructs at a position corresponding to residue 154 of the mature proteins by site-directed mutagenesis using the oligonucleotides GCCGACCTTGCTATCTC GAGCTCTTCAGTCCAGTCC (β3) (Kunkel, 1985). A SacII/Xhol fragment encoding residues 1–153 of the β3 subunit was ligated into the (FLAG)β2 pGW1 Xhol/SacII vector, and a Xhol/PstI fragment encoding residues 153–224 of the β3 subunit was ligated into the (FLAG)β2 pGW1 Xhol/PstI vector to produce more refined chimeras. Further mutants were generated by site-directed mutagenesis using the oligonucleotides GCCGACCTTGCTATCTC GAGCTCTTCAGTCCAGTCC (β3) and GCCGACCTTGCTATCTC GAGCTCTTCAGTCCAGTCC (β3). Western blotting was performed as described previously (Connolly et al., 1996a) using an enhanced chemiluminescent substrate (Pierce Supersignal Substrate).

Immunoprecipitation. Cells were labeled with [35S]methionine (ICN/Flow) at 200 μCi/ml. Immunoprecipitation using FLAG or 9E10 antibodies was performed as described previously.

Electrophysiological analysis. Whole-cell recordings from transfected A293 cells were performed as described previously (Wooltorton et al., 1997) up to 70 hr after transfection. Drugs were applied via a modified U-tube. The expression of functional cell-surface homomeric β subunit receptors was assessed by their sensitivity to Zn2+ (10 μM), picrotoxin (10 μM), and pentobarbital (1 mM). For αβ and βy heteromeric receptors, GABA sensitivity was assessed. Control untransfected cells did not elicit membrane currents or change membrane conductances when exposed to these ligands.

RESULTS

GABA<sub>A</sub> receptor β2 and β3 subunits differ in their ability to access the cell surface

To examine the mechanisms underlying the assembly of GABA<sub>A</sub> receptors, receptor β subunits modified with reporter epitopes were expressed in A293 cells. Addition of reporter epitopes between residues 4 and 5 of selected GABA<sub>A</sub> receptor subunits has been shown to be functionally silent (Connolly et al., 1996a,b). Receptor expression was analyzed by immunofluorescence with or without membrane permeabilization. Homomeric expression of (FLAG)β2 in A293 cells did not produce surface staining (Fig. 1). The staining pattern in permeabilized cells showed that this subunit is retained within the ER on homomeric expression (Connolly et al., 1996a,b; Gorrie et al., 1997). In contrast, homomeric expression of (FLAG)β3 produced robust surface expression in unpermeabilized cells (Fig. 1), as demonstrated previously in Madin–Darby canine kidney (MDCK) cells (Connolly et al., 1996b). Similar differences in surface expression of β2 and β3 were observed in both COS and baby hamster kidney cells, suggesting that this phenomena is not likely to be host cell specific (data not shown).

Specific residues within the N-terminal domain of GABA<sub>A</sub> receptor β subunits control cell surface expression

To determine the molecular basis of the differential ability of homomeric β subunits to access the cell surface, chimeras between (FLAG)β2 and (FLAG)β3 were produced. These constructs were produced at amino acid glutamine 224 within transmembrane domain 1 (TM1), which is identical in all β subunits (Yemer et al., 1989; Macdonald and Olsen, 1994; Rabow et al., 1995). Two chimeras were constructed in which the N-terminal and C-terminal portions of the (FLAG)β3 and (FLAG)β2 subunits were exchanged. These chimeras, (FLAG)β2/β3 and (FLAG)β3/β2, were expressed in A293 cells, and subunit localization was analyzed by immunofluorescence. The (FLAG)β3/β2 chimera, containing the N terminus of β3, was capable of robust cell surface expression as defined by staining in unpermeabilized cells, comparable to that seen with (FLAG)β3 (Fig. 1D). In contrast, the (FLAG)β2/β3 chimera containing the N terminus of β2 was not able to access the cell surface (Fig. 1C). However, this protein could be seen in permeabilized cells where it appeared to be retained in the ER, like (FLAG)β2 (Connolly et al., 1996a). From this approach, it is clear that the N-terminal domain of (FLAG)β3 is important for determining cell surface expression.

To identify the regions of (FLAG)β3 responsible for mediating homomeric cell surface expression more precisely, further chi-
meras were produced. An alignment of the β2 and β3 subunit N-terminal domains is shown in Figure 2. There are 20 amino acid residues within the N terminus that differ between the β2 and β3 subunits. These differences are clustered in two distinct portions of the N-terminal domain (Fig. 2). Exchange of amino acids between isoleucine 154 and glutamine 224 from the (FLAG)β3 to the (FLAG)β2 subunit resulted in cell surface expression (Fig. 3B). In contrast, substitution of residues 1–153 from (FLAG)β3 into (FLAG)β2 resulted in intracellular retention (Fig. 3A). These studies clearly identify a role for amino acids between residues 154 and 224 within the β3 subunit in mediating cell surface homomeric expression. Using systematic site-directed mutagenesis, four amino acids were identified—G171, K173, E179, and R180 (single letter amino acid code)—within the (FLAG)β3 subunit that were critical in conferring cell surface expression on (FLAG)β2 (Fig. 3D). The individual mutation of D(171)G, N(173)K, T(179)K, or K(180)R in β2 did not promote cell surface homomeric expression (data not shown). As a control, the corresponding residues from (FLAG)β2, D(171)N(173)T(179)K(180), were used to replace GKER in (FLAG)β3. Mutant (FLAG)β3(DNTK) was unable to access the cell surface and appeared to be ER-retained like (FLAG)β2 (Fig. 3C).

Figure 1. Surface expression of homomeric (FLAG)β subunits in A293 cells. Expression was determined by immunofluorescence using anti-FLAG M2 mouse monoclonal antibody and fluorescein-conjugated secondary antibodies with (+) or without (−) permeabilization 15–18 hr after transfection. Images were collected by confocal microscopy. The structure of each construct is indicated above the image, with the β2 sequence in white and β3 in gray. The four transmembrane domains in the C-terminal half of the subunits are represented by boxes. A, (FLAG)β2 (+); B, (FLAG)β3 (−); C, (FLAG)β2β3 (+); D, (FLAG)β3β2 (−). Scale bar, 10 μm.

Figure 2. Sequence alignment of the N-terminal domains of the β2 and β3 subunits. Amino acids that differ between the β2 and β3 subunits are indicated (*). The joins between the two subunits in the (FLAG)β2β3 chimeras are shown by arrows. The four residues that affect cell surface expression are in bold. The presumed Cys–Cys loop is indicated. The boxed region indicates the first presumed transmembrane domain.
In addition to immunofluorescence studies, flow cytometrical sorting (FACS) was used to determine the levels of cell surface expression of homomeric β subunits. Live A293 cells were labeled by immunofluorescence using FLAG antibody followed by an Alexa 488-conjugated secondary antibody and analyzed by FACS. Figure 4A shows typical results for mock-transfected A293 cells or cells expressing (FLAG)β2 or (FLAG)β3. Expression of (FLAG)β3 on the cell surface results in a clear shift of the histogram peak to higher fluorescence intensity. This shift in fluorescence was expressed as a percentage of cells expressing the FLAG epitope on the cell surface. Typically 30% of (FLAG)β3-transfected cells expressed the FLAG epitope. This value reflects transfection efficiency; therefore, when different subunits were compared, cell surface expression was calculated as a percentage of the cell surface expression seen for (FLAG)β3 in each experiment, which was normalized to 100%. Despite the fact that (FLAG)β2 cannot be detected on the cell surface by immunofluorescence microscopy, very low levels (~2%) of (FLAG)β2 could sometimes be detected by FACS analysis. This is likely to represent cells that have become permeabilized during the staining procedure. The values obtained for (FLAG)β2 were not significantly different from mock-transfected cells (p > 0.05) (Fig. 4B). The levels of cell surface expression for the (FLAG)β2(GKER) mutant were found to be variable but not significantly different from the (FLAG)β3 subunit (p > 0.05) (Fig. 4B). Similarly, the number of (FLAG)β3(DNTK)-transfected cells in which the FLAG epitope was detected on the cell surface was not significantly different from that for (FLAG)β2-transfected cells (p > 0.05) (Fig. 4B) and is significantly less than for (FLAG)β3-transfected cells (p > 0.05).

To determine whether the differing wild-type and mutant subunits were expressed at similar levels, cells expressing FLAG-tagged versions of these constructs were metabolically labeled with [35S]methionine. Receptor β subunits were then immunoprecipitated and separated by SDS-PAGE (Fig. 4C). (FLAG)β3 migrated with a molecular mass of between 57 and 59 kDa, and (FLAG)β2 migrated as bands of 54 and 50 kDa, as determined previously (Connolly et al., 1996a; McDonald et al., 1998). This approach determined that β2, β2(GKER), β3, and β3(DNTK) were all expressed to similar levels (Fig. 4C).

**Functional properties of β subunit chimeras**

The ability of different β subunits to form functional homooligomeric receptors was also measured. Whole-cell currents generated in response to the application of various ligands from transfected A293 cells were recorded at a holding potential of −40 mV. A293 cells expressing β2 show no response to the
application of GABA, pentobarbital, Zn$^{2+}$, or picrotoxin (Fig. 5A). In contrast, cells expressing β3 subunits are insensitive to the application of up to 1 mM GABA but display large inward currents with associated rebound currents in response to pentobarbital (Fig. 5B) (Wooltorton et al., 1997). The characteristic spontaneous gating of homo-oligomeric β3 receptors can be demonstrated by the generation of outward currents in response to the GABA$_A$ receptor inhibitors Zn$^{2+}$ and picrotoxin (Fig. 5B). This is because the pipette electrolyte and external Krebs’ composition caused $E_{C1}$ to approximate 0 mV. Thus, the spontaneous gating of β3 homomers was manifest by a persistent inward current at the −40 mV holding potential. In agreement with the immunofluorescence studies, recordings made from cells expressing either the β3β2 or β2β3β3 chimeras resulted in functional cell surface receptors because 1 mM pentobarbital activated inward currents. These chimeras also exhibited spontaneous gating as the addition of 10 μM Zn$^{2+}$ or 10 μM picrotoxin elicited outward membrane currents (Fig. 5C, D). In contrast, β2β3 or β3β2β3 chimeras exhibited no sensitivity to pentobarbital (1 mM), Zn$^{2+}$ (10 μM), or picrotoxin (10 μM) (n = 3; data not shown).

Whole-cell recordings were also made from cells expressing β subunit point mutants. Cells transfected with β3$^{GKER}$ are insensitive to the application of GABA, pentobarbital, Zn$^{2+}$, and picrotoxin (Fig. 5E), confirming that β3$^{DNTK}$ does not assemble into functional homomeric receptors. However, A293 cells expressing β2$^{GKER}$ exhibited a weak response to 1 mM pentobarbital (Fig. 5F), indicating that the four substitutions enable the β2 subunit to form functional homo-oligomeric receptors. In contrast, cells expressing β2$^{GKER}$ do not clearly display outward currents in response to Zn$^{2+}$ or picrotoxin, suggesting that these subunits do not appear to form spontaneously open Cl$^-$ channels.

Therefore, the data derived from the immunofluorescence, FACS, and electrophysiological studies clearly identify four N-terminal amino acid residues, GKER, within (FLAG)β3 that are necessary for homomeric cell surface expression and are also sufficient to confer homomeric cell surface expression on the (FLAG)β2β3 subunit after mutation. Given that similar surface levels of β2$^{GKER}$ and β3 are seen (Fig. 4), the differences in the physiological properties of these homomeric receptors are of interest. These observations suggest that although the residues G171, K173, E179, and R180 are sufficient to mediate cell surface expression, other distinct residues are responsible for the unique pharmacological and physiological properties of β3 homomers.

**Sucrose density gradient fractionation of receptor β subunits**

The ER retention of (FLAG)β2 and the cell surface expression of (FLAG)β3 may reflect differences between the abilities of these surface fluorescence for mock-transfected cells (top panel) and cells transfected with either (FLAG)β3 (middle panel) or (FLAG)β2 (bottom panel) cDNAs. B. Relative levels of (FLAG)β3 subunit cell surface expression. The number of cells expressing the flag epitope on the cell surface was expressed as a percentage of the number of (FLAG)β3-transfected cells expressing the flag epitope on the cell surface (mock, n = 5; (FLAG)β3, n = 9; (FLAG)β2, n = 4; (FLAG)β2$^{GKER}$, n = 6; (FLAG)β3$^{DNTK}$, n = 5). C. Expression levels of (FLAG)β2$^{GKER}$ (lane 2), (FLAG)β3$^{GKER}$ (lane 3). (FLAG)β3$^{DNTK}$ (lane 5) or control untransfected COS cells (lane 1) were assessed in COS cells labeled for 2 hr with 100 μCi/ml $[^{35}]$S)methionine. Expressing cells were then lysed, and receptor subunits were immunoprecipitated with FLAG antibody, resolved by SDS-PAGE, and visualized by autoradiography. The migration of molecular mass standards is indicated on the left.
two proteins to homo-oligomerize, because oligomerization is a prerequisite for ER exit (Hammond and Helenius, 1995). To analyze the oligomerization of receptor β subunits, detergent-solubilized cell extracts were fractionated on 5–20% linear sucrose density gradients. For these studies, expression in COS cells was used because they gave higher expression levels than A293 cells, facilitating biochemical analysis. Gradient fractions were subjected to Western blotting or immunoprecipitation with 9E10 antibody. The behavior of the β3(DNTK), β2(GKER), β2, and β3 subunits was identical in both COS and A293 cells (Fig. 1–3) with regard to cell surface expression (data not shown).

The (FLAG)β3 subunit exhibited a sedimentation coefficient of 9S as determined by reference to standards (Fig. 6A,B). The sedimentation coefficient of (FLAG)β3 is distinct from (FLAG)β2, which exhibits a 5S coefficient (Fig. 6A–C) (Gorrie et al., 1997). In contrast, β2 exhibits a coefficient of 9S when coexpressed with the α1 or the α1 and γ2 subunits to form functional cell surface receptors (Gorrie et al., 1997; Tretter et al., 1997). The distinct sedimentation coefficients of (FLAG)β2 and (FLAG)β3, combined with differential ER retention, suggested that these subunits differ in their abilities to homo-oligomerize. This issue was explored further by determining the sedimentation coefficients of (FLAG)β2(GKER) and (FLAG)β3(DNTK), which differ in their ability to access the cell surface (Figs. 3, 4). (FLAG)β3(DNTK), which is ER-retained, exhibited a sedimentation coefficient of approximately 5S (Fig. 6A,B) like (FLAG)β2 (Fig. 6A,C) (Gorrie et al., 1997). In contrast, (FLAG)β2(GKER), which like (FLAG)β3 can access the cell surface, exhibited a 9S coefficient (Fig. 6A,C). Given that the (FLAG)β2, (FLAG)β2(GKER), (FLAG)β3, and (FLAG)β3(DNTK) proteins are all expressed to similar overall levels (Fig. 4C), these observations strongly suggest that the amino acids GKER in (FLAG)β3 mediate cell surface expression by facilitating subunit homo-oligomerization.

The amino acids responsible for mediating β3 subunit homo-oligomerization mediate cell surface expression with the γ2 subunit but not the α1 subunit

To determine whether the amino acids that control β3 subunit homo-oligomerization influence hetero-oligomerization, various (FLAG)β2 and (FLAG)β3 constructs were coexpressed with (9E10)γ2L or (9E10)α1 subunits. Both (9E10)α1 and (9E10)γ2L are ER-retained on homomeric expression (Connolly et al., 1996a,b), so expression was monitored by detecting the 9E10 reporter epitope at the cell surface. Coexpression of (9E10)α1 with either the (FLAG)β2(GKER) or (FLAG)β3(DNTK) constructs resulted in robust expression of both reporter epitopes on the cell surface (Fig. 7). Likewise, both β2(GKER) and β3(DNTK) were able to assemble with the α1 and γ2 subunits to produce functional α1β2γ2 receptors (data not shown). Because both the β2 and β3 subunits can produce functional receptors on coexpression with

Figure 5. Functional analysis of β subunit homomers. Whole-cell currents were recorded from transfected A293 cells after the application of 1 mM GABA, 1 mM pentobarbital (PB), 10 μM Zn²⁺, or 10 μM picrotoxin (PTX) from A27.1p93 cells expressing A, β2; B, β3; C, β3β2; D, β2β3β2; E, β3(DNTK); and F, β2(GKER) constructs. Calibration bar: A–D, 200 pA; E, F, 100 pA. All of the cells were voltage-clamped at −40 mV, and each trace is representative of observations made from four to five determinations.
a1 and γ2 subunits, this result is not unexpected (Macdonald and Olsen, 1994; Rabow et al., 1995). However, coassembly with the a1 subunit indicates that the four mutations do not disturb the folding of β subunit polypeptides.

Coexpression of (FLAG)β2 with (9E10)γ2L resulted in ER retention of both subunits, in agreement with earlier observations (Connolly et al., 1996a,b) (Fig. 8A). However, coexpression of (FLAG)β3 and (9E10)γ2L resulted in robust cell surface expression of (9E10)γ2L (Fig. 8B). These results suggest clear differences in the ability of (FLAG)β2 and (FLAG)β3 to assemble with (9E10)γ2L.

To determine whether the amino acids that mediate homomeric expression of β3 influence heteromeric expression, selected β subunit mutants were coexpressed with (9E10)γ2L. In contrast to the lack of surface expression of wild-type (FLAG)β2 with (9E10)γ2L, coexpression of (FLAG)β2(GKER) with (9E10)γ2L produced robust cell surface expression of both subunits (Fig. 8C). When (9E10)γ2L was expressed with (FLAG)β3(DNTK), cell surface expression of both subunits was also observed, although at reduced levels compared with cells coexpressing (FLAG)β3 and (9E10)γ2L (Fig. 8D). Identical assembly behavior was seen with
both the γ2L and γ2S splice variants. These observations indicate that the amino acid residues that mediate β3 subunit homooligomerization and cell surface expression also mediate heterooligomeric interactions between β3 and γ2 subunits.

**Cell surface βγ2 receptors also form functional ion channels**

To investigate the abilities of the β subunits to form heterooligomeric receptors when coexpressed with the γ2S subunit, whole-cell currents generated in response to the application of various ligands in transfected A293 cells were recorded. Cells coexpressing β2 and γ2S subunits were insensitive to GABA, pentobarbital, Zn2+, and picrotoxin (Fig. 9A), as described previously (Connolly et al., 1996a). In contrast, cells coexpressing β3 and γ2S exhibited both GABA- and pentobarbital-gated membrane currents (Fig. 9B). The pharmacology of these channels was distinct from that of β3 homomers, which are insensitive to GABA (Connolly et al., 1996a; Wooltorton et al., 1997). Application of the GABA_A receptor inhibitors Zn2+ (10 μM) and picrotoxin (10 μM) to cells expressing β3 and γ2S resulted in the generation of outward currents (Fig. 9B), indicating that β3γ2S channels show a degree of spontaneous activity or that the cells express a mixed population of β3 homomers and β3γ2S receptors.

Cells coexpressing γ2S with β2(γKER) exhibit currents similar to those generated by cells expressing β3γ2S receptors. Application of GABA or pentobarbital generated inward currents; in contrast, both Zn2+ and picrotoxin blocked spontaneously open channels resulting in the generation of outward currents (Fig. 9C). Interestingly, cells coexpressing the β3(DNTK) mutant with γ2S also displayed inward currents in response to GABA or pentobarbital, but these currents were much smaller than those observed for β3γ2S or β2(γKER)γ2S receptors (Fig. 9D). This was a consistent feature that was independent of the transfection efficiency. Small outward currents were induced in response to the application of 10 μM Zn2+ or picrotoxin, indicating that some of the β3(DNTK)γ2S receptors gate spontaneously (Fig. 9D). It is unlikely that mixed populations of β3(DNTK) homomers and β3γ2S receptors are expressed, because the former fail to form functional cell surface receptors. These electrophysiological observations correlate well with the immunofluorescence data, which suggested that β3(DNTK) formed cell surface receptors when coexpressed with γ2, but with a reduced efficiency compared with wild-type β3 subunits. Identical behavior was seen in these experiments with either splice variant of the γ2 subunit (data not shown).

**Quantitation of βγ2 cell surface expression**

To confirm that the reduction in whole-cell currents produced on expression of β3(DNTK) and γ2 subunits compared with β3 and γ2 subunits was caused by a reduction in cell surface expression, coexpressing cells were subjected to flow cytometry to quantify heteromeric βγ2 receptor cell surface expression. This was achieved by monitoring the level of surface (9E10)γ2L using 9E10 antibody. In cells coexpressing β2 and (9E10)γ2L, the level of cell surface expression was low and similar to that observed for untransfected cells (Fig. 10). For cells coexpressing β3 and (9E10)γ2L, (9E10)γ2L could be detected on the surface of ~15% of cells, and this was used to normalize the cell surface expression of constructs in each experiment (Fig. 10). In (9E10)γ2Lβ3(DNTK)_transfected cells, (9E10)γ2L surface fluorescence was significantly decreased (p < 0.05) (Fig. 10) compared with (9E10)γ2Lβ3-expressing cells (Fig. 10) but was significantly greater than for both (9E10)γ2Lβ2 and mock-transfected cells (p < 0.05) (Fig. 10).

**Interaction of the γ2S subunit with β subunits**

To determine whether changes in subunit oligomerization are responsible for the reduced cell surface expression of β3(DNTK)γ2S constructs compared with β3γ2S or β2(γKER)γ2S, subunit oligomerization was analyzed by immunoprecipitation. For these
experiments, a (9E10)γ2S construct was expressed with each of the β subunit constructs used in this study (Fig. 11). The migration of homomeric β2, β3, and γ2S subunits is also shown for clarity in Figure 11. The β2 and β3 subunits migrated as bands of 50–54 and 57–59 kDa, respectively, whereas (9E10)γ2S migrated as a diffuse band of between 45 and 49 kDa. Association between the γ2 and β subunits was examined by immunoprecipitation using 9E10 antibody. Coimmunoprecipitation of β3 (57 and 59 kDa) and β3(ΔNTK) (57 and 59 kDa) with (9E10)γ2S was clearly observed because these proteins have distinct migrations on SDS-
Because the close migration of b2 and g2S, coimmunoprecipitation was more difficult to detect; however, the higher molecular mass species of b2 and b2(GKER) (54 kDa) (Fig. 11) coprecipitated with (9E10)g2S. These results suggested that the reduced efficiency of surface expression of b3(DNTK)g2S and the failure of b2g2S to access the cell surface is not attributable to an inability to oligomerize, in agreement with earlier observations (Connolly et al., 1996a). Instead, the efficiency of assembly is likely to be affected at a later stage, possibly with the b2 and b3(GKER)g2 combinations forming dimeric or trimeric complexes, which are processed inefficiently into functional receptors. Alternatively, these residues may affect the transport or targeting of assembled b/g oligomers to the cell surface.

DISCUSSION

To identify amino acid residues that control GABA_A receptor assembly, we have used a chimeric approach to analyze the selective cell surface expression of the b3 subunit compared with the b2 subunit. This resulted in the identification of four amino acids—G171, K173, E179, and R180—within the N-terminal domain of b3 that are capable of conferring homomeric cell surface expression on b2. The sedimentation of b subunit constructs was compared by sucrose density gradient centrifugation. b2 migrated as a 5S complex; in contrast, b3 migrated as a 9S complex. Interconversion of the sedimentation coefficients of b2 and b3 could be achieved by replacing the amino acids GKER in b3 with DNTK from b2 and vice versa. Together, these observations suggest that these amino acids identified in our study mediate b3 subunit oligomerization. However, whether residues

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**Figure 9.** Functional analysis of bγ2S hetero-oligomers. Whole-cell currents were recorded in response to 1 mM GABA, 1 mM pentobarbital (PB), 10 μM Zn2+, or 10 μM picrotoxin (PTX) from A293 cells expressing A, b2γ2S; B, b3γ2S; C, b2(GKER)γ2S; and D, b3(DNTK)γ2S. Cells were voltage-clamped at −40 mV holding potential, and each trace is representative of four to five determinations.

**Figure 10.** Relative levels of (9E10)γ2L subunit cell surface expression when expressed with different b subunits. Cell surface γ2L subunits were labeled by immunofluorescence on nonpermeabilized cells using 9E10 antibody and an Alexa-488 conjugated anti-mouse secondary antibody. The number of cells expressing the 9E10 epitope on the cell surface was measured by FACS analysis and expressed as a percentage of the number of (9E10)γ2Lb3 transfected cells expressing the 9E10 epitope on the cell surface (n = 3 for each subunit combination).
observations suggest that the residues GKER are sufficient to produce functional receptors. In contrast, pentobarbital-activated unit. COS cells or cells expressing (Connolly et al., 1996) has been reported. Other studies have reported that coexpression of α and γ2S (Angelotti et al., 1993) or β2 and γ2L (Connolly et al., 1996a) does not result in the formation of functional receptors. The observation that β2 and γ2 do not coassemble into cell surface receptors is consistent with earlier results (Connolly et al., 1996a,b). However, β3 was able to assemble with γ2 to form functional GABA-gated cell surface receptors. These results indicate clear differences in the abilities of the β2 and β3 subunits to assemble with the γ2 subunit. Substitution of G171, K173, E179, and R180, key residues in β3 subunit homo-oligomerization, into the β2 was sufficient to allow assembly with the γ2 subunit. However, substitution of these residues for the corresponding amino acids from β2, DNTK, reduced the efficiency of, but did not completely prevent, assembly with the γ2 subunit. Immunoprecipitation of γ2 from COS cell lysates results in the communoprecipitation of coexpressed β2, β3, β3(GKER), or β3(DNTK)1, suggesting that the initial steps of βγ oligomerization are unaffected by these amino acids. However, the production of a 9S complex that presumably represents functional receptors (Gorrie et al., 1997; Tretter et al., 1997) appeared to be drastically reduced for the β3(DNTK) construct compared with either wild-type β3 or β2(GKER) constructs. Together, these observations suggest that the GKER residues within the β3 subunit are not essential for the initial steps in βγ subunit oligomerization but play a critical role in facilitating the production of functional tetrameric or pentameric subunit assemblies.

The role of the residues identified in our study in the production of receptors composed of αβγ subunits that are believed to account for most GABA<sub>A</sub> receptor subtypes in the brain remains to be determined (Macdonald and Olsen, 1994; Rabow et al., 1995). Although the production of receptors composed of α1β2γ3 and α1β2γ3 was unaffected by the amino acids mediating β3 subunit homo-oligomerization, they may play a role in the assembly of receptors containing other α subunit variants. Alternatively, they may also be of importance in the production of heteromeric receptors where the γ subunit is substituted for either the δ or ε subunits (MacDonald and Olsen, 1994; Rabow et al., 1995; Davies et al., 1997).

The four residues that control the interactions of the β3 subunit during assembly are not located in a region of the polypeptide sequence that is homologous to the assembly domains that have been identified for nicotinic ACh receptor subunits or glycine receptor subunits (Gu et al., 1991; Chavez et al., 1992; Kuhse et al., 1993; Kreienkamp et al., 1995). However, an asparagine residue in the N terminus of the ρ1 subunit that mediates cell surface homomeric expression is located in a region homologous to the β3 assembly signal defined here (Hackam et al., 1997). Presumably this assembly signal within the β3 subunit will interact in concert with other as yet undefined assembly signals to ensure the fidelity of GABA<sub>A</sub> receptor assembly.

In conclusion, the results of this study provide the first direct evidence of defined signals in the N-terminal domain of GABA<sub>A</sub> receptor subunits that are important in mediating subunit selective receptor assembly.

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