Assembly and Cell Surface Expression of Heteromeric and Homomeric $\gamma$-Aminobutyric Acid Type A Receptors*  

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The ability of differing subunit combinations of $\gamma$-aminobutyric acid type A (GABA$_A$) receptors produced from murine $\alpha_1$, $\beta_2$, and $\gamma_2L$ subunits to form functional cell surface receptors was analyzed in both A293 cells and Xenopus oocytes using a combination of molecular, electrophysiological, biochemical, and morphological approaches. The results revealed that GABA$_A$ receptor assembly occurred within the endoplasmic reticulum and involved the interaction with the chaperone molecules immunoglobulin heavy chain binding protein and calnexin. Despite all three subunits possessing the ability to oligomerize with each other, only $\alpha_1/\beta_2$ and $\alpha_1/\gamma_2L$ subunit combinations could produce functional surface expression in a process that was not dependent on N-linked glycosylation. Single subunits and the $\alpha_1/\gamma_2L$ and $\beta_2/\gamma_2L$ combinations were retained within the endoplasmic reticulum. These results suggest that receptor assembly occurs by defined pathways, which may serve to limit the diversity of GABA$_A$ receptors that exist on the surface of neurons.

Accordingly, the expression of cDNA clones has been used to examine the minimal subunit composition required to produce functional GABA$_A$ receptors, determined by electrophysiological methodologies. Expression of unitary subunits has produced conflicting results; some subunits expressed alone appear to be able to produce GABA-gated ion channels (Blair et al., 1988; Pritchett et al., 1988) or channels that are sensitive to inhibition by picrotoxin, a GABA$_A$ receptor channel blocker (Sigel et al., 1989), whereas other studies demonstrate that some single subunits do not produce functional receptors (Sigel et al., 1990; Angelotti and Macdonald, 1993; Krishek et al., 1994). Expression of some binary subunit combinations have also produced conflicting data. For example, GABA-gated channels have been reported upon co-expression of either $\alpha_1/\gamma_2$ or $\beta_2/\gamma_2$ subunits (Verdon et al., 1990; Draguhn et al., 1990). In contrast, the failure of co-expressed $\beta_2/\gamma_2$ and $\alpha_1/\gamma_2L$ subunits to produce functional GABA$_A$ receptors has also been reported (Sigel et al., 1990; Krishek et al., 1994; Angelotti and Macdonald, 1993). There is, however, general agreement that co-expression of $\alpha$ and $\beta$ subunits is sufficient for the production of GABA-gated chloride currents, and the co-expression of $\alpha$ and $\beta$ with either the $\gamma_2$ or $\gamma_3$ subunits produce GABA$_A$ receptors that are sensitive to modulation by benzodiazepines (Pritchett et al., 1988, 1989; Burt and Kamatchi, 1991)

To further investigate these observations and attempt to seek an explanation for the failure of certain subunit combinations to produce functional GABA$_A$ receptors, we have examined the assembly and surface expression of homomeric and heteromeric GABA$_A$ receptors using biochemical, immunological, and electrophysiological methodologies. We have studied the assembly of GABA$_A$ receptors of varying subunit composition produced from $\alpha_1$, $\beta_2$, and $\gamma_2L$ subunits expressed in both Xenopus oocytes and transiently transfected A293 cells. From in situ hybridization and immunochromical analyses these subunits are co-localized in many adult brain regions and comprise up to 30% of all benzodiazepine-sensitive GABA$_A$ receptors in the adult brain (Benke et al., 1994; Fritschy et al., 1992).

In this study we demonstrate that GABA$_A$ receptor assembly occurs in the endoplasmic reticulum (ER), where interactions with the molecular chaperones immunoglobulin heavy chain binding protein (BiP) and calnexin were detected. Access to the cell surface was, however, limited to receptors composed of $\alpha_1/\beta_2$ and $\alpha_1/\gamma_2L$ subunits. Single subunits and the binary combinations of $\alpha_1/\gamma_2L$ and $\beta_2/\gamma_2L$, although capable of oligomerization, were retained within the ER, presumably via interactions with BiP and calnexin. Receptor assembly and transport to the cell surface was not dependent on N-linked glycosylation, although its efficiency was enhanced. These results suggest that GABA$_A$ receptor assembly occurs by defined...
mechanisms, which may serve to regulate the diversity of GABA<sub>A</sub> receptors expressed on the surface of neurons.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Human embryonic kidney 293 cells (ATCC CRL 1573) were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Life Technologies Ltd.) supplemented with 10% fetal bovine serum. Exponentially growing cells were seeded at 2 × 10<sup>5</sup> cells/10-cm dish and transfected by calcium phosphate precipitation as described previously (Mass et al., 1992). 20 µg of DNA was used per 10-cm plate. A293 cells using equimolar ratios of expression constructs. Cells were analyzed 12-18 h (immunofluorescence and immunoprecipitation) or up to 24h (pharmacology and electrophysiology) after transfection. Nuclear injection of Xenopus oocytes with murine GABA<sub>A</sub> receptor subunit constructs was performed as described by Krishke et al. (1994).

**DNA Constructions**—Murine α1, γ2L (Wang et al., 1992; Kofuji et al., 1991), and β2 (Kamatc et al., 1995) subunit cDNAs were cloned as EcoRI fragments into the mammalian expression vector pGEM1 (Mass et al., 1990). Expression of heterologous cDNAs in this vector is under the control of the cytomegalovirus promoter. The subunits were tagged with the 10-amino acid 9E10 epitope (EQKLISEEDL) from c-terminus of the cytomegalovirus promoter. The subunits were tagged with the 10-amino acid 9E10 epitope (EQKLISEEDL) from c-terminus of the cytomegalovirus promoter. The subunits were tagged with the 10-amino acid 9E10 epitope (EQKLISEEDL) from c-terminus of the cytomegalovirus promoter. The subunits were tagged with the 10-amino acid 9E10 epitope (EQKLISEEDL) from c-terminus of the cytomegalovirus promoter. The subunits were tagged with the 10-amino acid 9E10 epitope (EQKLISEEDL) from c-terminus of the cytomegalovirus promoter. The subunits were tagged with the 10-amino acid 9E10 epitope (EQKLISEEDL) from c-terminus of the cytomegalovirus promoter. The subunits were tagged with the 10-amino acid 9E10 epitope (EQKLISEEDL) from c-terminus of the cytomegalovirus promoter.

**GABA<sub>A</sub> Receptor Subunit Combinations:** Capability of Surface Membrane Expression—To study the assembly of murine GABA<sub>A</sub> receptors, heterologous expression of GABA<sub>A</sub> receptor α1, β2, and γ2L (a differentially spliced variant of γ2 containing an 8-amino acid insert in the predicted major intracellular domain, which is lacking from the other splice variant, γ2S) was analyzed by biochemical and morphological analyses. GABA<sub>A</sub> receptor subunits were tagged using the epitopes of 9E10 or FLAG. These epitopes were added to the α1, β2, and γ2L subunits by site-directed mutagenesis between amino acids 4 and 5 of the mature subunits to create α1<sub>4E10</sub>, β2<sub>5E10</sub>, and γ2L<sub>9E10</sub>. The functional effects of these additions were tested by electrophysiological analysis in A293 cells and Xenopus oocytes. Receptors incorporating 9E10-tagged subunits produced GABA-activated responses, which were indistinguishable from receptors comprised of wild-type subunits (Draguhn et al., 1990; Smart et al., 1991; Angelotti and Macdonald, 1993) with regard to zinc insensitivity and benzodiazepine modulation (Fig. 1). Similar results were obtained with subunits containing the FLAG epitope. Therefore, the addition of these small epitopes to the extreme N-terminal domains of GABA<sub>A</sub> receptor subunits appears to be "functionally silent." The subcellular distribution of these tagged GABA<sub>A</sub> receptors expressed in A293 cells was determined by immunofluorescence of the FLAG epitope followed by confocal microscopy. Expression of α1<sub>1FLAG</sub>, β2<sub>2FLAG</sub>, or γ2<sub>2FLAG</sub> alone revealed an ER-like staining pattern similar to that for horseradish peroxidase containing the C-terminal ER retention signal Lys-Asp-Glu-Leu (horseradish peroxidase-KDEL) (Fig. 2A), which is almost exclusively localized to the ER (Connolly et al., 1994). To further investigate the localization of these GABA<sub>A</sub> receptor subunits, immunofluorescence was performed on cells in which the peroxidase reaction had been performed using diaminobenzidine as the substrate. This results in the production of a dark insoluble precipitate, which cross-links all proteins that co-localize with horseradish peroxidase (Courtoy et al., 1984; Ajoka and Kaplan, 1987). The reaction product should therefore prevent the detection of fluorescence (DAB quenching) if the candidate protein shares the same intracellular localization as horseradish peroxidase.

To confirm this, we examined the ability to detect endogenous markers in the presence of DAB reaction product produced from horseradish peroxidase-KDEL. After completion of the DAB reaction, under conditions identical to those shown in Fig. 2B, fluorescence was performed on horseradish peroxidase-KDEL-expressing cells using either antibodies against BIP and calnexin (localized to the ER; Fig. 3A) or fluorescently labeled wheat germ agglutinin and Lens culinaris lectins (markers for the Golgi apparatus; Fig. 3B). In cells expressing horseradish peroxidase-KDEL, no fluorescence was detected using antibodies to the ER markers, BIP, and calnexin, although

Reimmunoprecipitation was performed on immunoprecipitates in reducing sample buffer. These were diluted 20-fold in lysis buffer containing 2% Triton X-100 prior to the second immunoprecipitation with either 9E10 supernatant, anti-calnexin, or anti-BIP as described above. Electrophysiological Analysis—Whole cell recordings from transfected A293 cells and analysis of membrane currents from Xenopus oocytes were performed as described previously (Krishek et al., 1994). Currents from transfected cells were analyzed up to 24 h after transfection, whereas Xenopus oocytes were examined at 48 h after nuclear injection.

**RESULTS**

**GABA<sub>A</sub> Receptor Subunit Combinations:** Capability of Surface Membrane Expression—To study the assembly of murine GABA<sub>A</sub> receptors, heterologous expression of GABA<sub>A</sub> receptor α1, β2, and γ2L (a differentially spliced variant of γ2 containing an 8-amino acid insert in the predicted major intracellular domain, which is lacking from the other splice variant, γ2S) was analyzed by biochemical and morphological analyses. GABA<sub>A</sub> receptor subunits were tagged using the epitopes of 9E10 or FLAG. These epitopes were added to the α1, β2, and γ2L subunits by site-directed mutagenesis between amino acids 4 and 5 of the mature subunits to create α1<sub>4E10</sub>, β2<sub>5E10</sub>, and γ2L<sub>9E10</sub>. The functional effects of these additions were tested by electrophysiological analysis in A293 cells and Xenopus oocytes. Receptors incorporating 9E10-tagged subunits produced GABA-activated responses, which were indistinguishable from receptors comprised of wild-type subunits (Draguhn et al., 1990; Smart et al., 1991; Angelotti and Macdonald, 1993) with regard to zinc insensitivity and benzodiazepine modulation (Fig. 1). Similar results were obtained with subunits containing the FLAG epitope. Therefore, the addition of these small epitopes to the extreme N-terminal domains of GABA<sub>A</sub> receptor subunits appears to be "functionally silent." The subcellular distribution of these tagged GABA<sub>A</sub> receptors expressed in A293 cells was determined by immunofluorescence of the FLAG epitope followed by confocal microscopy. Expression of α1<sub>1FLAG</sub>, β2<sub>2FLAG</sub>, or γ2<sub>2FLAG</sub> alone revealed an ER-like staining pattern similar to that for horseradish peroxidase containing the C-terminal ER retention signal Lys-Asp-Glu-Leu (horseradish peroxidase-KDEL) (Fig. 2A), which is almost exclusively localized to the ER (Connolly et al., 1994). To further investigate the localization of these GABA<sub>A</sub> receptor subunits, immunofluorescence was performed on cells in which the peroxidase reaction had been performed using diaminobenzidine as the substrate. This results in the production of a dark insoluble precipitate, which cross-links all proteins that co-localize with horseradish peroxidase (Courtoy et al., 1984; Ajoka and Kaplan, 1987). The reaction product should therefore prevent the detection of fluorescence (DAB quenching) if the candidate protein shares the same intracellular localization as horseradish peroxidase.

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Functional properties of recombinant GABA<sub>A</sub> receptors incorporating the 9E10 epitope tag expressed in A293 cells. A, GABA equilibrium concentration response curves for α1β2 (●) and α1<sup>9E10</sup>β2<sup>9E10</sup> (●) constructs (n = 3–5 cells). The curves were fitted using a nonlinear least squares Marquart routine with the following logistic state function: \( I / I_{\text{max}} = 1 / (1 + (EC_{50}/[A])^n) \), where \( I \) represents the GABA-activated current at a given concentration of GABA ([A]), and \( I_{\text{max}} \) is the maximum current induced by a saturating concentration of GABA. \( EC_{50} \) describes the concentration of GABA inducing a half-maximal response, and \( n \) is the Hill coefficient. The determined \( EC_{50} \) values and Hill coefficients for the α1β2 are 0.77 ± 0.07 μM and 1.45 ± 0.22, and for the α1<sup>9E10</sup>β2<sup>9E10</sup>, the values are 0.82 ± 0.03 μM and 1.66 ± 0.12. B, whole cell recordings of GABA-activated currents after rapid application of GABA to A293 cells at −50 mV expressing α1<sup>9E10</sup>β2<sup>9E10</sup> constructs. The solid lines indicate the duration of the GABA application. W represents the recovery times (min) for the GABA-induced responses following exposure to 1 μM flurazepam for 10 μM Zn²⁺. C, concentration response curves for the recombinant GABA<sub>A</sub> receptors α1β2-2L (●) and α1<sup>9E10</sup>β2<sup>9E10</sup>-2L (●) expressed in A293 cells. The \( EC_{50} \) values and Hill coefficients were 4.61 ± 0.83 μM and 1.38 ± 0.32 and 4.8 ± 0.63 μM and 0.98 ± 0.1, respectively. D, examples of GABA-induced membrane currents recorded from α1<sup>9E10</sup>β2<sup>9E10</sup>-2L receptors after application of 1 μM flurazepam or 50 μM Zn²⁺. Note the enhanced response to GABA after flurazepam and the reduced inhibition by Zn²⁺ in the 2L subunit containing GABA<sub>A</sub> receptors. The calibration in D applies to the upper and lower series of responses.

Fig. 1. Functional properties of recombinant GABA<sub>A</sub> receptors incorporating the 9E10 epitope tag expressed in A293 cells. A, GABA equilibrium concentration response curves for α1β2 (●) and α1<sup>9E10</sup>β2<sup>9E10</sup> (●) constructs (n = 3–5 cells). The curves were fitted using a nonlinear least squares Marquart routine with the following logistic state function: \( I / I_{\text{max}} = 1 / (1 + (EC_{50}/[A])^n) \), where \( I \) represents the GABA-activated current at a given concentration of GABA ([A]), and \( I_{\text{max}} \) is the maximum current induced by a saturating concentration of GABA. \( EC_{50} \) describes the concentration of GABA inducing a half-maximal response, and \( n \) is the Hill coefficient. The determined \( EC_{50} \) values and Hill coefficients for the α1β2 are 0.77 ± 0.07 μM and 1.45 ± 0.22, and for the α1<sup>9E10</sup>β2<sup>9E10</sup>, the values are 0.82 ± 0.03 μM and 1.66 ± 0.12. B, whole cell recordings of GABA-activated currents after rapid application of GABA to A293 cells at −50 mV expressing α1<sup>9E10</sup>β2<sup>9E10</sup> constructs. The solid lines indicate the duration of the GABA application. W represents the recovery times (min) for the GABA-induced responses following exposure to 1 μM flurazepam for 10 μM Zn²⁺. C, concentration response curves for the recombinant GABA<sub>A</sub> receptors α1β2-2L (●) and α1<sup>9E10</sup>β2<sup>9E10</sup>-2L (●) expressed in A293 cells. The \( EC_{50} \) values and Hill coefficients were 4.61 ± 0.83 μM and 1.38 ± 0.32 and 4.8 ± 0.63 μM and 0.98 ± 0.1, respectively. D, examples of GABA-induced membrane currents recorded from α1<sup>9E10</sup>β2<sup>9E10</sup>-2L receptors after application of 1 μM flurazepam or 50 μM Zn²⁺. Note the enhanced response to GABA after flurazepam and the reduced inhibition by Zn²⁺ in the 2L subunit containing GABA<sub>A</sub> receptors. The calibration in D applies to the upper and lower series of responses.
The α1 subunit contains two consensus sequences (Asn-Xaa-Ser/Thr) for N-linked glycosylation at positions 10 and 110. Treatment of the α1-expressing cells with tunicamycin (a potent inhibitor of N-linked glycosylation) produced a single band coincident with the lowest mass of α1FLAG subunit of 48 kDa (Fig. 4, α, +). Thus, the three α1 forms differ in their extent of N-linked glycosylation, and their sizes are consistent with the presence of zero, one, and two sites of N-linked glycosylation.

The β2<sup>FLAG</sup> subunit exhibits apparent molecular masses of 53 and 56 kDa plus a weak band at 50 kDa (Fig. 4, β, −). Again, this subunit contains two consensus sequences for N-linked glycosylation at positions 8 and 80. Tunicamycin treatment produced a major band at 50 kDa (Fig. 4, β, +), as predicted by cDNA cloning (Kamatchi et al., 1995). Thus, the β2 forms are also consistent with the presence of zero, one, and two sites of N-linked glycosylation. The γ2L<sup>FLAG</sup> subunit migrated as a broad band at approximately 42 kDa (Fig. 4, γ, −) and, following tunicamycin treatment, as a broad band of around 30 kDa (Fig. 4, γ, +); this shift is consistent with the predicted presence of three consensus sites for N-linked glycosylation at positions 13, 90, and 208. The apparent molecular masses of both glycosylated and unglycosylated forms of γ2L are much smaller than its 48-kDa predicted molecular mass derived from cDNA cloning (Pritchett et al., 1989). This may be due to proteolysis, a common observation for this subunit (Moss et al., 1992; Haddingham et al., 1992) and may explain the appearance of a smear rather than discreet bands.

Oligomerization of GABA<sub>A</sub> Receptor Subunits—A possible explanation for the differential ability of GABA<sub>A</sub> subunit combinations to reach the cell surface as functional receptors may be found in their respective abilities to oligomerize with each other, as is often a prerequisite for exit from the ER (Hurtley and Helenius, 1989). The ability of GABA<sub>A</sub> receptor subunits to oligomerize was therefore analyzed by co-immunoprecipitation of binary combinations (α1β2L, β2γ2L, and α1β2).

Consistent with the surface expression of the α1β2L subunit combination, it was found that β2 co-immunoprecipitates both the 48-kDa unglycosylated and the 50-kDa partially glycosylated but not the 52-kDa fully glycosylated forms of the α1 subunit (Fig. 5A, lane 3). The reciprocal co-immunoprecipitation was not so clear and may be complicated by the co-migration of both the α1 and β2 bands at 50 kDa (Fig. 5A, lane 4). Despite being transport incompetent, the α1γ2L subunits could also co-immunoprecipitate each other (Fig. 5B, lanes 3 and 4). As seen with the β2 subunit, the γ2L also binds exclusively to the two lower forms of α1 (Fig. 5B, lane 3). Thus, neither β2 nor γ2L show detectable oligomerization with the 52-kDa fully glycosylated form of the α1 subunit, even though it is the major species present. Similarly, the β2 and γ2L subunits are also capable of oligomerizing despite their transport incompetence. In this case, γ2L binds predominantly to the nonglycosylated 50-kDa form of β2 (Fig. 5C, lane 3). It also appears that both α1 and β2 bind preferentially to lower molecular mass γ2L (Fig. 5, B, lane 4, and C, lane 4, respectively) compared to the major species present when immunoprecipitated directly (Fig. 5, B, lane 2, and C, lane 2). These preferences for subunit binding occur in the presence of the nonbind-
Assembly and Cell Surface Expression of GABA<sub>A</sub> Receptors

Pharmacology of recombinant GABA<sub>A</sub> receptors with and without 9E10 epitope tags

The benzodiazepines (BDZ) diazepam and flurazepam (1 μM), pentobarbitaline (BARB; 50–100 μM), picrotoxin (PTX; 10 μM), bicuculline (BIC; 10 μM), zinc (1–1000 μM), or pregnanolone (PRG; 1 μM) were both applied to Xenopus oocytes or A293 cells expressing GABA<sub>A</sub> receptors of various subunit compositions. N.D., not determined; –, no GABA-activated currents detected; +, membrane currents were induced by GABA<sub>A</sub> receptor agonists; s and t, an enhancement or reduction of the GABA-induced current, respectively, *+, presence of the 9E10 epitope.

| Subunit combination | GABA | BDZ | BARB | PTX | BIC | Zinc | PRG |
|---------------------|------|-----|------|-----|-----|------|-----|
| α1                  | –    | –   | N.D. | N.D. | N.D. | N.D. | N.D. |
| β2                  | –    | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| γ2L                 | ++   | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| α1β2                | ++   | –   | –    | s    | t    | t    | s    |
| α1β2*               | ++   | –   | –    | s    | t    | t    | s    |
| α1γ2L               | –    | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| β2γ2L               | –    | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| α1β2γ2L             | +++  | –   | –    | s    | s    | s    | s    |
| α1β2*γ2L*           | +++  | –   | –    | s    | s    | s    | s    |

TABLE I: Assembly and Cell Surface Expression of GABA<sub>A</sub> Receptors

Fig. 4. Immunoprecipitation and glycosylation of GABA<sub>A</sub> receptor α1, β2, and γ2L subunits from transfected A293 cells. A293 cells expressing α1<sup>9E10</sup> (α), β2<sup>9E10</sup> (β), γ2L<sup>9E10</sup> (γ), or untransfected (C) were [<sup>35</sup>S]methionine-labeled in the absence (−) or presence (+) of 3 μg/ml tunicamycin (present 2 h prior to and during labeling). Receptor subunits were then immunoprecipitated using 9E10 antibody coupled to protein G-Sepharose. Immune complexes were then separated by SDS-polyacrylamide gel electrophoresis using 8% gels. The molecular masses of marker proteins (Bio-Rad) are indicated on the right. A co-immunoprecipitating band migrating at approximately 75 kDa (*) was consistently observed.

Fig. 5. Oligomerization of GABA<sub>A</sub> receptor subunits expressed in A293 cells. A293 cells expressing single or binary combinations of GABA<sub>A</sub> receptor subunits were labeled with [<sup>35</sup>S]methionine, cells were lysed, and GABA<sub>A</sub> receptor subunits were purified using 9E10 antibody coupled to protein G-Sepharose. GABA<sub>A</sub> receptor subunits were then separated by SDS-polyacrylamide gel electrophoresis using 8% gels. Control cells show the presence of a contaminating band at approximately 40 kDa, which is sometimes observed regardless of the antibody used (arrowhead). A, oligomerization of α1 and β2 subunits: lane 1, α1<sup>9E10</sup>β2<sup>9E10</sup>; lane 2, α1β2<sup>9E10</sup>γ2L<sup>9E10</sup>; lane 3, β2<sup>9E10</sup>γ2L<sup>9E10</sup>; lane 4, α1<sup>9E10</sup>β2<sup>9E10</sup>. B, oligomerization of α1 and γ2L subunits: lane 1, α1<sup>9E10</sup>β2<sup>9E10</sup>γ2L<sup>9E10</sup>; lane 2, γ2L<sup>9E10</sup>α1β2<sup>9E10</sup>; lane 3, γ2L<sup>9E10</sup>β2<sup>9E10</sup>α1; lane 4, α1<sup>9E10</sup>β2<sup>9E10</sup>γ2L<sup>9E10</sup>. C, oligomerization of β2 and γ2L subunits: lane 1, β2<sup>9E10</sup>γ2L<sup>9E10</sup>; lane 2, γ2L<sup>9E10</sup>β2<sup>9E10</sup>; lane 3, γ2L<sup>9E10</sup>β2<sup>9E10</sup>α1; lane 4, β2<sup>9E10</sup>γ2L<sup>9E10</sup>. oligomerization could occur in the absence of N-linked glycans, because the nonglycosylated α1 subunit (identified in Fig. 6, lane 1) co-precipitated the nonglycosylated β2 subunit (identified in Fig. 6, lane 2) as well as the nonglycosylated γ2L subunit (30 kDa). Furthermore the oligomerization of the binary subunit complex α1γ2L was also apparently unaffected by tunicamycin treatment as determined by co-precipitation (Fig. 6, lane 4).

Although the inhibition of N-linked glycosylation did not affect subunit oligomerization, N-linked glycosylation may be important for subsequent maturation. Therefore, we examined cell surface receptor expression in the presence of tunicamycin. This treatment caused other effects, most notably a reduction in cell number, reduced transfection efficiency, and changes in morphology. However, cell surface expression was not pre-
sites by site-directed mutagenesis) in the presence of β1 and γ2, which produced functional GABA_A receptors with pharmacological properties similar to those observed for the wild-type receptor but with reduced levels (Buller et al., 1994).

GABA_A Receptor Subunits Interact with ER Chaperone Proteins—The correct folding of many proteins has been shown to involve the interaction of BIP (Pellman, 1989). In addition, another ER chaperone, calnexin, appears to be involved exclusively with glycoproteins (Ou et al., 1993). As seen earlier, immunoprecipitation of either α1, β2, or γ2 GABA_A receptor subunits routinely co-immunoprecipitated a protein of approximately 75 kDa (Fig. 4, asterisk), which may represent BIP. We therefore sought to determine if these two chaperone proteins participate in the assembly of GABA_A receptors by retention of unassembled subunits within the ER, as observed for the α1, β2, γ2L, α1γ2L, and β2γ2L combinations. Cells expressing the α1γ2L subunit alone (which is transport-incompetent and therefore retained in the ER) were first immunoprecipitated with 9E10 antibody. This precipitate was then reprecipitated with either anti-BIP, anti-calnexin, or 9E10 antibodies, and bands migrating with expected molecular masses for both BIP and calnexin were evident (Fig. 8A). In addition, weak bands co-migrating with the nonprecipitated proteins (e.g. BIP and calnexin when performed via 9E10) were also present (weakly visible in Fig. 8A).

This interaction with BIP and calnexin is not unique to the α1γ2L subunit, because the α1β2L, β2β2L, and γ2L9E10 subunits can also be co-immunoprecipitated with anti-calnexin antibodies (Fig. 8B) with no apparent preference for different forms. In all three cases, a protein migrating at the same molecular mass as BIP is also co-immunoprecipitated, suggesting some overlap in the binding abilities of BIP and calnexin. This overlap appears to occur with some endogenous proteins in A293 cells as evidenced by the untransfected control lane (Fig. 8B), in which anti-calnexin antibody co-immunoprecipitated a band coincident with BIP. When these apparent complexes were immunoprecipitated using 9E10, calnexin is only weakly observed, consistent with its low turnover rate and subsequently low [35S]methionine incorporation (Hammond et al., 1994). Upon expression of all three, subunits BIP is still immunopre-
cipitated (Moss et al., 1995). However, whether this is due to interactions occurring between unitary, binary, or tertiary subunit complexes under these conditions is difficult to ascertain.

DISCUSSION

To date, 15 different GABA<sub>A</sub> receptor cDNAs have been isolated from a variety of vertebrates (Burt and Kamatchi, 1991). Many of these subunits exhibit differing patterns of both spatial and developmental expression in the CNS, with many neurons often expressing multiple numbers of receptor subunits (Wisden and Seeburg, 1992). A major challenge in trying to analyze the diversity of GABA<sub>A</sub> receptor structure in the brain is determining what processes control receptor assembly. Regulation of receptor assembly could occur at numerous stages, including subunit oligomerization or export to the cell surface. Unfortunately, to date there is little experimental data on these important questions. To address this, we have examined the assembly of GABA<sub>A</sub> receptors of differing subunit compositions constructed from those of α1, β2, and γ2 subunits expressed in both A293 cells and Xenopus oocytes using immunological, biochemical, and electrophysiological methodologies. These subunits are thought to co-localize in many brain regions and comprise up to 30% of all benzodiazepine-sensitive GABA<sub>A</sub> receptors in the adult brain (Fritschy et al., 1992; Benke et al., 1994). For immunological and biochemical analyses the 9E10 (Evans et al., 1985) or the FLAG epitopes were added between amino acids 4 and 5 of each of these subunits. As demonstrated by electrophysiological analyses, these additions appeared to be functionally silent.

Using immunolocalization, epitope tagged α1, β2, or γ2 subunits expressed alone are incapable of leaving the ER, as are the binary subunit combinations of α1γ2L and β2γ2L, demonstrated by co-localization with horseradish peroxidase-KDEL (Connolly et al., 1994). The only combinations of receptors produced from these subunits that are capable of exiting the ER and accessing the cell surface are α1β2 and α1β2γ2L. In agreement with this only the latter two subunit combinations exhibited resolvable GABA-gated currents when expressed in either A293 cells or Xenopus oocytes.

Previous studies have produced conflicting data on the expression of single subunits and the combinations of α1γ2L and β2γ2L, as determined by electrophysiological analysis. Blair et al. (1988) reported the production of GABA-gated channels on the expression of single α1 or β1 subunits in Xenopus oocytes, and Sigel et al. (1989) reported finding chloride currents that could be blocked by picrotoxin on the expression of the rat β1 subunit. Moreover, Verdoorn et al. (1990) and Draguhn et al. (1990) found robust receptor expression from α1γ2L subunits and smaller currents from β2γ2L subunits in A293 cells. In contrast to these results Angelotti and Macdonald (1993) and Krishke et al. (1994) found no GABA-gated currents when expressing α1γ2L or β1γ2L subunits in L929 or A293 cells. These discrepancies could be due to differences in the expression systems used or differences in the type of β subunit used in some of these experiments (β1 versus β2). Expression of murine β1 subunits alone in both A293 cells and Xenopus oocytes can produce low levels of surface expression, in common with the observations of Blair et al. (1988) and Sigel et al. (1989). Therefore some of this variability in expression may be subunit-specific. A second reason for such discrepancies may result from over-expression. It is possible that the longer expression periods used in many previous studies (48–72 h in A293 cells and 2–6 days in oocytes (Blair et al., 1988; Verdoorn et al., 1990) compared with 12–24 h and 2–3 days for 293 cells and Xenopus oocytes, respectively, used in this study) may have resulted in the escape of normally transport-incompetent receptor complexes through saturation of the ER retention system, resulting in low levels of surface expression.

In spite of the apparent inability of the α1γ2L and β2γ2L subunit combinations to leave the ER, they were still capable of rapid oligomerization. In fact, all oligomerization events were not dependent on N-linked glycosylation and could occur with unglycosylated subunits. Furthermore, N-linked glycosylation was not required for the transport of receptor heteroligomers to the cell surface. Interestingly, the β2 subunit appeared to oligomerize more efficiently to lower molecular mass forms of the α1 (and possibly the γ2 subunits), and the γ2L subunit appeared to oligomerize more efficiently to lower molecular mass forms of both the α1 and the β2 subunits. The significance of these interactions is uncertain; they may represent preferred patterns of subunit oligomerization if these incompletely processed forms are represented in cell surface receptors in the presence of glycosylated forms. Whether these interactions are important in controlling the final assemblies of GABA<sub>A</sub> receptors produced warrants further investigation.

GABA<sub>A</sub> receptor subunits interacted with at least two ER chaperone proteins, BiP and calnexin, whose function is to retain misfolded and unassembled proteins. Interactions with BiP are thought to result from the exposure of hydrophobic domains in incorrectly folded proteins (Pelham, 1989), whereas calnexin appears to show specificity for glycoproteins containing partially “glucose-trimmed” carbohydrate side chains (Hammond et al., 1994). Calnexin is thought to hold glycoproteins in the ER until folding/assembly is complete, thus preventing their aggregation and/or premature exit from the ER (Hochstenbach et al., 1992; Ou et al., 1993; Hammond et al., 1994). Presumably single GABA<sub>A</sub> receptor subunits and the binary combinations of α1γ2L and β2γ2L are retained in the ER via the interaction with chaperone proteins such as calnexin and BiP.

The demonstration of the intracellular localization of GABA<sub>A</sub> receptor heteroligomers has important consequences for our understanding of GABA<sub>A</sub> receptor structure. To date, the method of choice for examining the complexity of GABA<sub>A</sub> receptor structure in the brain has been the use of subunit-specific antisera to immunoprecipitate receptor complexes from solubilized brain tissue (e.g. Duggan and Stephenson, 1990; Mertens et al., 1993; Mckernan et al., 1991; Mossier, 1994). These procedures are complex and often yield contradictory results (cf. Pollard et al. (1993), Quirk et al. (1994), and Khan et al. (1994)). It is possible that some of the interactions seen following immunoprecipitation in these experiments and thereby proposed to represent cell surface receptor subunit combinations may in fact represent ER-retained forms of GABA<sub>A</sub> receptors. Such complexes as demonstrated in our study may have differing subunit combinations from GABA<sub>A</sub> receptors expressed on the surface of neurons.

Finally, the inability of GABA<sub>A</sub> receptor heteroligomers consisting of α1γ2L and β2γ2L subunits to reach the cell surface suggests that assembly of GABA<sub>A</sub> receptors might share similar mechanisms to those employed by muscle acetylcholine receptors. Assembly of these receptors is believed to utilize intracellular dimer or trimer intermediates (Green and Miller, 1995). Further studies will elucidate whether the ER-retained subunit heteroligomers described in our work are important intermediates in the production of fully assembled pentameric GABA<sub>A</sub> receptors. This may be of significance because the consensus of opinion (Burt and Kamatchi, 1991; Pritchett et al., 1988, 1989), derived from a combination of molecular, pharmacological, and electrophysiological methodologies, suggests that
in vivo benzodiazepine-responsive GABA<sub>A</sub> receptors are composed of αβγ in unknown stoichiometries. The relevance of these α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>L and β<sub>2</sub>γ<sub>2</sub>L intracellular subunit complexes to the final functional GABA<sub>A</sub> receptors produced is currently under investigation.

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Assembly and Cell Surface Expression of Heteromeric and Homomeric \-Aminobutyric Acid Type A Receptors

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