A GABA$_A$ Receptor $\alpha_1$ Subunit Tagged with Green Fluorescent Protein Requires a $\beta$ Subunit for Functional Surface Expression*

(Received for publication, August 14, 1998)

Jolien X. Connor, Andrew J. Boileau, and Cynthia Czajkowski‡

From the Department of Physiology, University of Wisconsin, Madison, Wisconsin 53706

$\gamma$-Aminobutyric acid, type A (GABA$_A$) receptors, the major inhibitory neurotransmitter receptors in the central nervous system, are heteropentameric proteins assembled from distinct subunit classes with multiple subtypes, $\alpha$(1–6), $\beta$(1–4), $\gamma$(1–3), $\delta$(1), and $\epsilon$(1). To examine the process of receptor assembly and targeting, we tagged the carboxyl terminus of the GABA$_A$ receptor $\alpha_1$ subunit with red-shifted enhanced green fluorescent protein (EGFP). Xenopus oocytes were injected with cRNA of this fusion protein, $\alpha_1$-EGFP, alone or in combination with cRNA of GABA$_A$ receptor $\beta_2$, $\gamma_2$, or $\beta_2+\gamma_2$ subunits. Within 72 h after injection, EGFP fluorescence was visible in all fusion protein-injected cells. The fluorescence was associated with the plasma membrane only when the $\beta_2$ subunit was co-injected with $\alpha_1$-EGFP. Texas Red-conjugated immunolabeling of EGFP on non-permeabilized cells demonstrated that EGFP was localized extracellularly. Hence, the COOH terminus of the $\alpha_1$ subunit is extracellular. Two-electrode voltage clamp of $\alpha_1$-EGFP, $\beta_2$, and $\alpha_1$-EGFP, $\beta_2+\gamma_2$-injected oocytes demonstrates that these cells express functional receptors, with EC$_{50}$ values for GABA and diazepam similar to wild-type receptors. Thus, a COOH-terminal tag of the $\alpha_1$ subunit appears to be functionally silent, providing a useful marker for studies of GABA$_A$ receptor expression, assembly, transport, targeting, and clustering. Moreover, the $\beta_2$ subunit is required for receptor assembly and surface expression.

GABA$_A$ receptors are the major inhibitory neurotransmitter receptors in the mammalian brain and are members of a ligand-gated ion channel superfamily (1), which includes receptors for acetylcholine, glycine, and serotonin. The native GABA$_A$ receptor is likely to be a heteropentameric protein (2) assembled from several different classes and isoforms of GABA$_A$ receptor subunits, including $\alpha_6$, $\alpha_4$, $\beta_3$, $\gamma_1$, $\delta_1$ and $\epsilon_1$ subunit subtypes (3–6). Hydropathy plots of the sequences of all the subunits predict an extracellular NH$_2$-terminal domain of about 200 amino acids, four putative membrane-spanning domains, referred to as M1, M2, M3, and M4, a cytoplasmic domain of variable length between M3 and M4, and an extracellular carboxyl terminus. Theoretically, a large number of different pentameric subunit combinations derived from the various subunit subtypes can be present in the brain. Localization of the GABA$_A$ receptor gene products, including mRNA by in situ hybridization and polypeptides by immunocytochemistry, has revealed that the various subunits and subunit subtypes show different regional as well as developmental distributions (7–11) with many neuronal cell types expressing multiple receptor subunits (12, 13). GABA$_A$ receptors consisting of different subunit isoforms are most likely responsible for the diversity of inhibitory synaptic responses observed in some regions of the brain, and it is possible that different types of GABA$_A$ receptors are present even within single neurons (14). Thus, elucidating the cellular and molecular mechanisms involved in the regulation of the assembly, membrane targeting and subcellular distribution of GABA$_A$ receptors is important for ultimately understanding how neurons control their fast inhibitory responses.

In general, antibody labeling of receptors has been used to study receptor trafficking and localization. This approach, however, can be labor intensive and is generally not useful for monitoring receptors in intracellular compartments. More recently, green fluorescent protein (GFP) from the jellyfish Ae. quorea victoria has been used in a variety of studies to examine gene expression and protein subcellular localization (15–17). GFP is particularly useful because it fluoresces in living cells without requiring additional reagents or cofactors, and its fluorescence is resistant to photobleaching. GFP has been used successfully as a protein tag on both the amino and carboxyl termini of a wide range of cytosolic and membrane-bound proteins (for review, see Cubitt et al. (18)) and provides a way to directly measure levels of protein expression, to visually identify cells expressing the protein, and to localize the protein as it is being processed.

To specifically examine GABA$_A$ receptor expression, assembly and membrane targeting, we attached red-shifted GFP (EGFP, CLONTECH) to the COOH terminus of the GABA$_A$ receptor $\alpha_1$ subunit. This fusion protein, $\alpha_1$-EGFP, was expressed in Xenopus oocytes alone or with $\beta_2$, $\gamma_2$, or $\beta_2+\gamma_2$ GABA$_A$ receptor subunit combinations. Oocytes expressing the fusion protein are clearly fluorescent and show distinct patterns of fluorescence, depending on the subunits cojected. Voltage clamp studies provide evidence that the fusion protein forms a functional channel when coexpressed with $\beta_2$ or $\beta_2\gamma_2$ subunits, and that the GFP tag is functionally silent. In addition, GFP immunolabeling on non-permeabilized cells provides direct experimental evidence that the COOH terminus of the $\alpha_1$ subunit is extracellular, consistent with the four-transmembrane subunit topology model based on hydrophobicity analysis.

MATERIALS AND METHODS

Construction of $\alpha_1$-EGFP Fusion cDNA—By recombinant polymerase chain reaction, the stop codon of $\alpha_1$ was changed to proline, which created a new AgeI site, using the oligonucleotide 5'-CTAAAAGAC-

* This work was supported in part by a March of Dimes Basil O’Connor Starter Scholar Research Award and NINDS, National Institutes of Health Grant NS34727 (to C. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Recipient of the Burroughs Welcome Fund New Investigator Award in the Basic Pharmacological Sciences. To whom correspondence should be addressed: Dept. of Physiology, Rm. 197 MSC, University of Wisconsin, 1300 University Ave., Madison, WI 53706. Tel.: 608-265-5863; Fax: 608-265-3500; E-mail: czajkowski@physiology.wisc.edu.

1 The abbreviations used are: GABA$_A$, $\gamma$-aminobutyric acid, type A; GABA, $\gamma$-aminobutyric acid; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; M, transmembrane domain.
**Cell Surface Expression of GFP-tagged GABA<sub>A</sub> Receptors**

28907

**Fig. 1. Visualization of GABA<sub>A</sub> receptor α<sub>1</sub> subunit distribution in Xenopus oocytes using an α<sub>1</sub>-EGFP fusion protein.** Injection of oocytes with the cRNA of EGFP or the fusion protein α<sub>1</sub>-EGFP, alone or in combination with the cRNA of the β<sub>2</sub> or γ<sub>2</sub> subunits, produces cells with strong green fluorescence. In oocytes expressing EGFP, α<sub>1</sub>-EGFP, or α<sub>1</sub>-EGFP<sub>β2</sub>, the fluorescent signal is distributed throughout the cell (upper row). In oocytes expressing α<sub>1</sub>-EGFP<sub>β2</sub> or α<sub>1</sub>-EGFP<sub>β2γ2</sub>, the green fluorescent signal appears to be localized to the surface plasma membrane (bottom row).

CGTTGATGGGTTGTAAGG-C. A polymerase chain reaction product using this primer and a 5′-upstream α<sub>1</sub>-specific primer resulted in a 581-base pair fragment of α<sub>1</sub>, with a modified COOH terminus. The α<sub>1</sub> fragment was digested with AgeI and BamHI and subcloned into the pEGFP-N1 vector (CLONTECH) which fused the EGFP gene, in frame, to the COOH terminus of the α<sub>1</sub> fragment. This recombinant plasmid was digested with BamHI and XbaI, and the resulting α<sub>1</sub>-EGFP fragment was subcloned into a pGHI vector, which contained the entire wild-type α<sub>1</sub> cDNA (19) for expression in Xenopus oocytes. Thus, the 3′ end of the wild-type α<sub>1</sub> subunit, from the BamHI site to the stop codon, was replaced with the α<sub>1</sub>-EGFP fusion fragment. The α<sub>1</sub>-EGFP fusion cDNA was verified by restriction digest and double-stranded DNA sequencing using standard techniques (20).

**Expression in Oocytes—**Oocytes from *Xenopus laevis* were prepared as described previously (19). GABA<sub>A</sub> receptor rat α<sub>1</sub>, β<sub>2</sub>, and γ<sub>2</sub> subunits and α<sub>1</sub>-EGFP fusion protein were expressed by injection of cRNA into *Xenopus* oocytes (molar ratios of 1:1, αβ, αγ, or 1:1:10, αβγ) using approximately 0.7–2.0 fmol of α<sub>1</sub> or α<sub>1</sub>-EGFP. Capped cRNA coding for the wild-type subunits and α<sub>1</sub>-EGFP fusion protein were synthesized by *in vitro* transcription from *Nhe*I-linearized cDNA template using the mMessage mMACHINE T7 kit (Ambion). The oocytes were maintained in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 137 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.1). Antibodies were diluted in the corresponding blocking buffer. The primary antibody was an anti-GFP antibody (CLONTECH), diluted at 1:2000; the secondary antibody was a biotinylated goat anti-rabbit (Jackson), diluted 1:1000.

**Voltage-Clamp Analysis—**Oocytes under two electrode voltage-clamp (V<sub>hold</sub> = −80 mV) were perfused continuously with ND96 recording solution at a rate of 5 ml/min. Drugs and reagents were dissolved in ND96. The stock diazepam solution was made in MeSO. GABA responses were scaled for run-down or run-up by comparison to a low, nondesensitizing concentration of drug applied just prior to the drug concentration tested. Diazepam potentiation was recorded at approximately EC<sub>7</sub> to EC<sub>20</sub> for GABA. Potentiation is defined as (I<sub>GABA</sub>/I<sub>GABA-1</sub>), where I<sub>GABA</sub> is the current response in the presence of diazepam (DZ) and I<sub>GABA-1</sub> is the control GABA current. Standard two-electrode voltage-clamp recording was performed using a GeneClamp 500 (Axon Instruments) interfaced to a computer with an IT-16 A/D device (Instrutech). Electrodes were filled with 3 M KCl and had a resistance of 0.5–1.5 megohms.

**Fluorescence Analysis and Confocal Laser-scanning Microscopy—**Oocytes were immunoreacted either live or following fixation in 10% neutral-buffered formalin. Removal of the vitelline membrane was not found to be necessary. The cells were blocked in 1% bovine serum albumin in ND96 or 1% bovine serum albumin in phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 137 mM NaCl, 14 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.1). Antibodies were diluted in the corresponding blocking buffer. The primary antibody was an anti-GFP antibody (CLONTECH), diluted at 1:2000; the secondary antibody was a biotinylated goat anti-rabbit (Jackson), diluted 1:1000. The final incubation was in Texas Red-conjugated streptavidin (Jackson), diluted at 1:400. After the last washes, all oocytes were maintained in ND96.

**Oocytes were mounted under a glass coverslip on a glass dual-well slide.**
The slide was fastened upside down on the stage of a Bio-Rad MRC 1024 confocal laser scanning microscope and visualized with a 20× lens. EGFP excitation/emission was achieved with a filter set (488 nm/510 nm) designed for fluorescein detection. Texas Red excitation/emission was achieved with a filter set specific for Texas Red (595 nm/615 nm). All images were recorded at the same adjustments of laser power and photomultiplier sensitivity and were later processed by using Adobe Photoshop software (ADOBE Systems, Mountain View, CA) with identical values for contrast and brightness.

RESULTS

Distinct Cellular Distribution of the α1-EGFP Subunit—To determine whether the α1-EGFP fusion cRNA produced a fluorescent protein, oocytes were injected with six different groups of cRNA, α1-EGFP alone, α1-EGFP + β2, α1-EGFP + γ2, α1-EGFP + β2 + γ2, β2 alone, and EGFP alone (Fig. 1). Two to four days after injection, the oocytes were visualized by epifluorescence microscopy for the development of green fluorescence. Oocytes injected with β2 alone show very low levels of autofluorescence and defined base-line fluorescence. Oocytes injected with EGFP alone were used as positive controls and produced fluorescent protein that appears to be distributed throughout the cytoplasm of the cells.2 Oocytes injected with the α1-EGFP fusion cRNA all produce fluorescent protein. In cells expressing α1-EGFP alone and α1-EGFP γ2, the fluorescent fusion protein appears to be distributed throughout the cell; whereas in cells expressing α1-EGFPβ2 and α1-EGFPβ2γ2 the protein seems to localize to the surface plasmalemma. These results suggest that the presence of a GABA<sub>A</sub> receptor β subunit is needed for efficient translocation of α1-EGFP to the cell surface.

Surface Expression of α1-EGFPβ2 and α1-EGFPβ2γ2 GABA<sub>A</sub> Receptors—To determine whether the α1-EGFP fusion protein fluorescence was extracellular or intracellular, intact oocytes expressing α1-EGFP, α1-EGFPγ2, α1-EGFPβ2γ2, and EGFP alone were labeled with anti-EGFP antibodies and Texas Red (see “Materials and Methods”). Using this protocol, extracellularly EGFP is exclusively tagged, since the cells are not permeabilized. Oocytes expressing α1-EGFP, α1-EGFPγ2, and EGFP alone are not labeled with Texas Red (Fig. 2) even though they produce green fluorescent protein (Fig. 1). Thus, the α1-EGFP fusion protein, when expressed alone or in combination with the γ2 subunit, is localized intracellularly. In contrast, oocytes expressing α1-EGFPβ2 and α1-EGFPβ2γ2 receptors are specifically labeled with Texas Red (Fig. 2), indicating that the α1-EGFP protein is localized on the surface membrane of the oocyte and that the EGFP antibody epitope is facing extracellularly. When images obtained from monitoring Texas Red and EGFP fluorescence separately are overlaid, the majority of both signals colocalize (Fig. 3), demonstrating that the α1-EGFP

2 The apparent heterogeneity of α1-EGFP distribution in oocytes expressing α1-EGFP, α1-EGFPγ2, and EGFP alone (Fig. 1, top row) is attributable to differential adsorption of fluorescent signal by the pigmented animal pole versus the nonpigmented vegetal pole.
fusion protein, when expressed in combination with the \( b_2 \) or \( b_2 \gamma_2 \) subunits, is preferentially localized to the surface plasma-lemma with EGFP facing extracellularly. Since EGFP is fused to the carboxyl terminus of the \( \alpha_1 \) subunit, these results directly demonstrate that the COOH terminus of the \( \alpha_1 \) subunit is extracellular.

**GFP-tagged GABA\(_A\) Receptors Are Functionally Unaltered**—To test whether the \( \alpha_1 \)-EGFP fusion protein could assemble into a functional GABA\(_A\) receptor, oocytes expressing the fusion protein were tested with a two-electrode voltage-clamp for the ability of GABA to activate a Cl\(^-\) current. The traces in Fig. 4 (top) show Cl\(^-\) currents activated by increasing concentrations of GABA from oocytes expressing \( \alpha_1 \)-EGFP\(b_2\) and \( \alpha_1 \)\(\beta_2\) GABA\(_A\) receptors. Fig. 4 (bottom) plots the GABA-activated current for \( \alpha_1 \beta_2 \), \( \alpha_1 \)-EGFP\(\beta_2\), \( \alpha_1 \beta_2 \gamma_2 \), and \( \alpha_1 \)-EGFP\(\beta_2 \gamma_2\) receptors as a function of GABA concentration. The GABA EC\(_{50}\) values for \( \alpha_1 \)-EGFP-containing receptors are similar to wild-type receptors (Table I). Oocytes expressing \( \alpha_1 \)-EGFP\(\beta_2 \gamma_2\) receptors were also tested for the ability of diazepam to potentiate the GABA response for \( \alpha_1 \)-EGFP\(\beta_2 \gamma_2\) receptors as a function of GABA concentration. The GABA EC\(_{50}\) values for \( \alpha_1 \)-EGFP-containing receptors are similar to wild-type receptors (Table I). Oocytes expressing \( \alpha_1 \)-EGFP\(\beta_2 \gamma_2\) receptors were also tested for the ability of diazepam to potentiate the GABA-mediated Cl\(^-\) current. As seen in Fig. 5, the EC\(_{50}\) for diazepam potentiation of the GABA response for \( \alpha_1 \)-EGFP\(\beta_2 \gamma_2\) receptors is similar to wild-type receptors (Table I). The maximum potentiation obtained at 1 \( \mu \)M diazepam was somewhat lower for \( \alpha_1 \)-EGFP\(\beta_2 \gamma_2\) receptors, but still within reported ranges (19, 21). Hill coefficients were not significantly different between \( \alpha_1 \)-containing versus \( \alpha_1 \)-EGFP-containing receptors (data not shown). Taken together, these results indicate that COOH-terminal labeling of the \( \alpha_1 \) subunit of the GABA\(_A\) receptor with EGFP yields fully functional ion channels in *Xenopus* oocytes when expressed with \( \beta_2 \) or \( \beta_2 \gamma_2 \) subunits.

In oocytes expressing \( \alpha_1 \)-EGFP\(\beta_2 \gamma_2\) receptors, the emission output of \( \alpha_1 \)-EGFP was measured before and during a bath application of GABA to determine whether agonist-dependent rearrangements in the GABA\(_A\) receptor would induce changes in the fluorescence of \( \alpha_1 \)-EGFP. No change in fluorescence was measured during a 100 \( \mu \)M GABA application (n = 3).

**DISCUSSION**

Because the response properties of neurons are ultimately controlled by the types of ion channels and neurotransmitter receptors present at a particular synapse, a fundamental question in neurobiology is to understand the regulation and the processes involved in the assembly, expression, and subcellular targeting of these proteins. Since GABA\(_A\) receptors are heteropentameric proteins assembled from five distinct subunit classes with multiple subtypes, this question is particularly complex (22). To address this, we created a fusion protein of the GABA\(_A\) receptor \( \alpha_1 \) subunit with a naturally fluorescent protein, EGFP. We then characterized \( \alpha_1 \)-EGFP expression in *Xenopus* oocytes, alone or with other receptor subunit combinations, using microscopic, immunological, and electrophysiological techniques. Attachment of GFP to a protein allows rapid, noninvasive detection of the tagged protein in living cells and provides a powerful tool for studying protein trafficking. Ideally, the labeled protein should be relatively unperturbed by its fluorescent tag and exhibit little or no change in its behavior.

Attachment of EGFP to the COOH terminus of the GABA\(_A\) receptor \( \alpha_1 \) subunit produces a bright green fluorescent protein when expressed in *Xenopus* oocytes. When expressed alone or in combination with the \( \gamma_2 \) subunit, \( \alpha_1 \)-EGFP shows a gener-
potentiation measured; ND, not determined.

functional receptors in some cases (26, 27), but not in others expressing of a subunit. The data. Parameters from the curve fits are shown in Table I. α1β2 and α1β2γ2 receptors (open symbols, solid lines); α1-EGFPβ2 and α1-EGFPβ2γ2 receptors (filled symbols, dashed lines).

FIG. 4. α1-EGFP containing GABA_A receptors are functional and display wild-type sensitivity to GABA. Two electrode voltage-clamp of oocytes expressing α1-EGFPβ2, α1β2, α1-EGFPβ2γ2, and α1β2γ2 receptors. Oocytes were treated with a range of GABA concentrations, and dose-response curves were fit to the data. Parameters from the curve fits are shown in Table I. α1β2 and α1β2γ2 receptors (open symbols, solid lines); α1-EGFPβ2 and α1-EGFPβ2γ2 receptors (filled symbols, dashed lines).

FIG. 5. Diazepam potentiates α1-EGFP containing GABA_A receptors. Oocytes expressing α1-EGFPβ2γ2 and α1β2γ2 receptors were treated with a range of diazepam concentrations in the presence of 1 μM GABA. Potentiation response ratios were calculated by dividing peak GABA current in the presence of diazepam by peak response to GABA alone and normalized. Parameters from the curve fits are displayed in Table I. The EC50 for diazepam potentiation of the GABA receptor for α1-EGFPβ2γ2 receptors is similar to wild-type receptors. α1β2γ2 receptors (open symbols, solid lines); α1-EGFPβ2γ2 receptors (filled symbols, dashed lines).

TABLE I
Summary of voltage-clamp results

Dose-response data for wild-type and α1-EGFP-containing subunit combinations for GABA and diazepam potentiation of GABA-mediated Cl− current in Xenopus oocytes are tabulated. Two-electrode voltage-clamp and data analysis was performed as described (see “Materials and Methods”). Shown are means ± S.D. for EC50 values calculated from dose-response data (Figs. 4 and 5) using Prism software. I_max is the range of maximal GABA-gated currents measured, maximum potentiation is the fold increase over GABA-gated current at 1 μM diazepam, and n represents the number of oocytes tested. NS, no significant potentiation measured; ND, not determined.

| GABA     | Diazepam potentiation | n | EC50 | Max. Pot. | n |
|----------|-----------------------|---|------|-----------|---|
|          |                       |   |       |           |   |
| α1β2     | 8.2 ± 0.4             | 5 | NS   | NS        | 5 |
| α1-EGFPβ2| 11.5 ± 1.1            | 3 | NS   | NS        | 6 |
| α1β2γ2S  | 12.7 ± 0.7            | 5 | 51 ± 6| 2.91 ± 0.09| 6 |
| α1-EGFPβ2γ2S|15.7 ± 0.5          | 3 | 70 ± 23|2.16 ± 0.12| 5 |

alized fluorescence, which is distributed throughout the cell (Fig. 1). Immunolabeling with an anti-GFP antibody and Texas Red established that when expressed alone or with γ2 subunits targeted to the basolateral membrane and β2 subunits targeted both subunits to the apical membrane. Thus, even though both α1 and β2 subunits are capable of being subcellularly targeted, the β2 subunit when present regulates the targeting of the α1 subunit. In contrast, Connolly et al. (29) showed that α1β1 coexpression in Madin-Darby canine kidney cells resulted in a nonpolarized surface distribution, and neither α1

Table I. The EC50 for diazepam potentiation of the GABA response for GABA_A receptors. Oocytes expressing α1-EGFPβ2γ2 and α1β2γ2 receptors were treated with a range of diazepam concentrations in the presence of 1 μM GABA. Potentiation response ratios were calculated by dividing peak GABA current in the presence of diazepam by peak response to GABA alone and normalized. Parameters from the curve fits are displayed in Table I. The EC50 for diazepam potentiation of the GABA receptor for α1-EGFPβ2γ2 receptors is similar to wild-type receptors. α1β2γ2 receptors (open symbols, solid lines); α1-EGFPβ2γ2 receptors (filled symbols, dashed lines).

GABA_A receptor targeting studies, carried out in polarized Madin-Darby canine kidney cells, have mixed results. One study (28) demonstrated that α1 subunits expressed alone were targeted to the basolateral membrane and β1 subunits expressed alone to the apical membrane. Coexpression of α1 and β1 targeted both subunits to the apical membrane. Thus, even though both α1 and β1 subunits are capable of being subcellularly targeted, the β1 subunit when present regulates the targeting of the α1 subunit. In contrast, Connolly et al. (29) showed that α1β1 coexpression in Madin-Darby canine kidney cells resulted in a nonpolarized surface distribution, and neither α1
nor β subunits expressed alone were inserted into any surface membrane. In the same study, αβ coexpression resulted in a basolateral surface distribution, and αβ in an apical distribution which switched to a basolateral distribution over time. Thus, while it appears that the type of β subunit may guide membrane targeting, it is less clear if it is a required element. In our study, we show that α subunits expressed alone are not targeted to nor inserted into the oocyte surface membrane and that the β subunit is required for targeting and assembling the α subunit into a functional cell surface receptor.

The transmembrane topology of a GABA receptor subunit is predicted by hydrophobicity analysis to have an extracellular NH2-terminal domain, four putative membrane-spanning domains, and an extracellular COOH terminus (30). Experimental support of this model is not well established for the GABA receptor family, and as demonstrated for the ionotropic glutamate receptor family (15, 31), hydrophobicity analysis is not always correct. For GABA receptor subunits, the extracellular placement of the amino-terminal end of the subunit has been verified by antibody labeling of intact cells (25) and mutagenesis of glycosylation sites (32). In vitro and in vivo phosphorylation studies position part of the peptide loop between M3 and M4 intracellularly (33, 34). In this study, since EGFP is fused to the carboxyl terminus of the α subunit, GFP immunocytochemical labeling of nonpermeabilized oocytes expressing a NH2-terminal domain, four putative membrane-spanning domains, predicted by hydrophobicity analysis to have an extracellular region, and a COOH-terminal tagging of the β subunit is extracellular. This is the first report of successfully tagging GFP to a member of the ligand-gated ion channel superfamily, which includes neuronal and muscle nicotinic acetylcholine receptors, GABA receptors, glycine receptors, and serotonin receptors and establishes the general usefulness of this approach for studying their assembly, transport, targeting, and clustering. It should now be possible to identify specific regions of the receptor essential for these processes by using GFP-tagged subunit chimeras.

Acknowledgments—We thank Dr. Meyer Jackson for help with experiments measuring EGFP fluorescent emission and Dr. David Wagner for critical reading of the manuscript. Confocal microscopy was performed at The Kroc Neural Imaging Laboratory, University of Wisconsin-Madison.

REFERENCES
1. Orteils, M. O., and Lunt, G. G. (1995) Trends Neurosci. 18, 121–127
2. Nayem, N., Green, T. P., Martin, I. L., and Barnard, E. A. (1994) J. Neurochem. 62, 815–818
3. Sieghart, W. (1995) Pharmacol. Rev. 47, 181–234
4. Rabow, L. E., Russek, S. J., and Farb, D. H. (1995) Synapse 21, 189–274
5. Davies, P. A., Hanna, M. C., Hales, T. G., and Kirkness, E. F. (1997) Nature 385, 820–823
6. Hedblom, E., and Kirkness, E. F. (1997) J. Biol. Chem. 272, 15346–15350
7. Wisden, W., Laurie, D. J., Monyer, H., and Seeburg, P. H. (1992) J. Neurosci. 12, 1040–1062
8. Zhang, J. H., Sato, M., and Tsuchiya, M. (1991) J. Comp. Neurol. 308, 586–613
9. Pollard, S., Duggan, M. J., and Stephenson, F. A. (1993) J. Biol. Chem. 268, 3753–3757
10. Endo, S., and Olsen, R. W. (1993) J. Neurochem. 60, 1388–1398
11. Speker, G., Schwarzer, C., Tsunashima, K., Fuchs, K., and Sieghart, W. (1997) Neuroscience 80, 987–1000
12. Wisden, W., and Seeburg, P. H. (1992) Curr. Opin. Neurobiol. 2, 263–269
13. Wisden, W., Korpi, E. B., and Bahn, S. (1996) Neuropharmacology 33, 1139–1160
14. Nusser, Z., Sieghart, W., and Somogyi, P. (1998) J. Neurosci. 18, 1693–1703
15. Marshall, J., Molloy, R., Moss, G. W., Howe, J. R., and Hughes, T. E. (1995) Neuron 14, 211–215
16. Barak, L. S., Ferguson, S. S., Zhang, J., Martensson, C., Meyer, T., and Caron, M. G. (1997) Mol. Pharmacol. 51, 177–184
17. Grabner, M., Dirksen, R. T., and Beam, K. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1903–1908
18. Cohut, A. B., Heim, R., Adams, S. E., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995) Trends Biochem. Sci. 20, 448–455
19. Boileau, A. J., Kucken, A. M., Evers, A. R., and Czajkowski, C. (1998) Mol. Pharmacol. 53, 285–303
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Malerbe, P., Sigel, E., Baur, R., Persohn, E., Richards, J. G., and Mohler, H. (1990) J. Neurosci. 10, 2393–2397
22. Wisden, W., and Moss, S. J. (1997) Biochem. Soc. Trans. 25(3), 820–824
23. Blair, L. A., Levitan, E. S., Marshall, J., Dionne, V. E., and Barnard, E. A. (1988) Science 242, 577–579
24. Sigel, E., Baur, R., Trube, G., Mohler, H., and Malerbe, P. (1990) Nature 349, 703–711
25. Connolly, C. N., Krieshek, B. J., McDonald, B. J., Smart, T. G., and Moss, S. J. (1990) J. Biol. Chem. 271, 89–96
26. Draughn, A., Verdorn, T. A., Ewert, M., Seeburg, P. H., and Sakmann, B. (1990) Neuron 5, 781–788
27. Verdoorn, T. A., Draughn, A., Ymer, S., Seeburg, P. H., and Sakmann, B. (1990) Nature 4, 919–928
28. Perez-Velazquez, J. L., and Angeliades, K. J. (1993) Nature 361, 457–460
29. Connolly, C. N., Woolcott, J. R., Smart, T. G., and Moss, S. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9899–9904
30. Schofield, P. R., Darlington, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Reale, V., Glencorse, T. A., Seeburg, P. H., and Barnard, E. A. (1987) Nature 328, 221–227
31. Hellman, M., Maron, C., Heinemann, S. (1984) Science 193, 733–737
32. Birnbaum, R., Hastings, G. A., Kirkness, E. F., and Fraser, C. M. (1994) Mol. Pharmacol. 45, 858–865
33. Moss, S. J. and Smart, T. G. (1996) Int. Rev. Neurosci. 39, 1–52
34. Smart, T. G. (1997) Curr. Opin. Neurobiol. 7, 358–367
35. Im, W. B., Pregenzer, J. F., Binder, J. A., Dilnou, G. H., and Alberts, G. L. (1995) J. Biol. Chem. 270, 26063–26066
36. Chang, Y., Wang, R., Barot, S., and Weiss, D. S. (1996) J. Neurosci. 16, 5415–5424
37. Tretter, V., Ehy, N., Fuchs, K., and Sieghart, W. (1997) J. Neurosci. 17, 2728–2737