Identification of Residues within GABA_A Receptor α Subunits That Mediate Specific Assembly with Receptor β Subunits

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GABA_A receptors can be constructed from a range of differing subunit isoforms: α, β, γ, δ, and ϵ. Expression studies have revealed that production of GABA-gated channels is achieved after coexpression of α and β subunits. The expression of a γ subunit isoform is essential to confer benzodiazepine sensitivity on the expressed receptor. However, how the specificity of subunit interactions is controlled during receptor assembly remains unknown. Here we demonstrate that residues 58–67 within α subunit isoforms are important in the assembly of receptors comprised of $\alpha\beta$ and $\alpha\beta\gamma$ subunits. Deletion of these residues from the $\alpha1$ or $\alpha6$ subunits results in retention of either α subunit isoform in the endoplasmic reticulum on coexpression with the $\beta3$, or $\beta3$ and $\gamma2$ subunits. Immunoprecipitation revealed that residues 58–67 mediated oligomerization of the $\alpha1$ and $\beta3$ subunits, but were without affect on the production of α/γ complexes. Within this domain, glutamine 67 was of central importance in mediating the production of functional $\alpha1\beta3$ receptors. Mutation of this residue resulted in a drastic decrease in the cell surface expression of $\alpha1\beta3$ receptors and the resulting expression of $\beta3$ homomers. Sucrose density gradient centrifugation revealed that this residue was important for the production of a 9S $\alpha1\beta3$ complex representing functional GABA_A receptors.

Therefore, our studies detail residues that specify GABA_A receptor $\alpha\beta$ subunit interactions. This domain, which is conserved in all α subunit isoforms, will therefore play a critical role in the assembly of GABA_A receptors composed of $\alpha\beta$ and $\alpha\beta\gamma$ subunits.

Key words: GABA_A-receptor; assembly; cell surface expression; N-terminal; oligomerization; α subunit

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GABA_A receptors are critical mediators of fast synaptic inhibition in the brain and are also important drug targets for a range of compounds, including the benzodiazepines and barbiturates (MacDonald and Olsen, 1994; Rabow et al., 1995). GABA_A receptors are members of the ligand-gated ion channel superfamily that includes glycine, nicotinic acetylcholine (AChR), and 5-HT_3 receptors (Unwin, 1993). Molecular cloning has revealed a range of GABA_A receptor subunits that can be divided by homology into subunit classes with multiple members: $\alpha(1–6)$, $\beta(1–3)$, $\gamma(1–3)$, δ, ϵ, and π (MacDonald and Olsen, 1994; Rabow et al., 1995; Davies et al., 1997; Hedblom and Kirkness, 1997). There is considerable spatial and temporal variation in subunit expression, with many neuron types expressing multiple numbers of receptor subunits (Laurie et al., 1992; MacDonald and Olsen, 1994; Rabow et al., 1995). Clearly, to delineate the true diversity of GABA_A receptor structure in the brain, it is important to gain some insights into how these receptor subunits are assembled.

Studies using heterologous expression focusing on the receptor $\alpha1$, $\beta1$–2, and $\gamma2$ subunits, have revealed that access to the cell surface is limited to the combinations $\alpha\beta$ and $\alpha\beta\gamma2$ (Angelotti and MacDonald 1993; MacDonald and Olsen, 1994; Rabow et al., 1995; Connolly et al., 1996). Most single subunits and the $\alpha1/\gamma2$,

$\beta2/\gamma2$ combinations are largely retained in the endoplasmic reticulum (ER), where they are rapidly degraded (Connolly et al., 1996; Gorrie et al., 1997). Expression of α and β subunits produces GABA-gated currents, but coexpression with a γ subunit is essential in conferring benzodiazepine sensitivity on expressed receptors (MacDonald and Olsen, 1994; Rabow et al., 1995). Interestingly, the $\beta3$ subunit, and to a lesser extent the $\beta1$ subunit, can assemble into homomeric channels that gate spontaneously in a number of heterologous expression systems (Sigel et al., 1989; Krishek et al., 1996; Wooltorton et al., 1997). Recently, four N-terminal amino acids within the $\beta3$ subunit have been identified that control homo-oligomerization and cell surface expression of this subunit compared to $\beta2$ (Taylor et al., 1999).

To gain further insights into GABA_A receptor assembly, we have examined the functional expression of two N-terminal splice variants of the $\alpha6$ subunit (Korpi et al., 1994). These variants, termed $\alpha6$ long ($\alpha6L$) and $\alpha6$ short ($\alpha6S$), differ by the presence of amino acids 58–68, in $\alpha6L$. Here we demonstrate that residues 58–67 within both the $\alpha1$ and $\alpha6$ subunits are essential for cell surface expression with receptor β and γ subunits. Immunoprecipitation revealed that these residues were important in mediating oligomerization with the $\beta3$ subunit but did not affect oligomerization with the $\gamma2$ subunit. Sucrose density gradient centrifugation revealed that residue Q67 within this domain was of major significance in mediating the oligomerization of the $\alpha1$ subunit with $\beta3$ to produce functional receptors. Therefore, these studies identify the first residues within GABA_A receptor α subunits that mediate specific interaction with β but not γ subunits.

MATERIALS AND METHODS

Cell culture and transfection. Human Embryonic kidney 293 (A293) 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 resistance, 125 μF; Bio-Rad Gene Electroporator II) with 10 μg of DNA using equimolar ratios of expression constructs. For electrophysiology, the reporter plasmid for the S65T mutant jellyfish green fluorescent protein (Heim et al., 1995) was added to the transfection mixture. Transfected cells were maintained in culture for up to 48 hr before use.

DNA construction. The murine GABA_A receptor cDNAs encoding the α1 subunit with the 9E10 epitope (between amino acids 4 and 5) and the β2 and γ2L subunits with the FLAG epitope (between amino acids 4 and 5) in the cytomegalovirus-based pGW1 expression vector have been described previously (Connolly et al., 1996). The β3 and α6S subunit cDNAs in pGW1 were tagged with the FLAG epitope using the oligonucleotides 5′ CATGTTCCCGGGGT CCTTGTCATCGTCGTCCTTG-TAGTCGTTTACGCTCTGAG 3′ (β3) and 5′ CTTCTACTTCCGCT-GATGTTCCTGCTGCTACTGTTCTTGAAGATGAGA 3′ (α6S) by site-directed mutagenesis, as described previously (Kunkel, 1985). An α6L cDNA construct was derived from the FLAG-tagged α6S construct by site-directed mutagenesis using the oligonucleotide 5′ CTCATCAGTCC-AAGTCTGTCGAAAGAAAACATCCATTGTGTACTCCATCTC-CACATCTGA 3′.

Mutant α1 constructs were generated by site-directed mutagenesis using the oligonucleotides 5′ TTCATCCTTCCAACTCATATCGTG-GTCTGA 3′ for the ^(9E10)α1S construct, 5′ CTTCCAACTGTAGTGC-CTCAGGTAGAGGGTCATGTAAAGTCCATATCGTG 3′ for the α1/ρ1 construct, 5′ CACATCTATTGTAAAATCCATATCGTGGT 3′ for the α1^(DF) construct, 5′ TGACGGAAAAACAAAGTTATTG-TATACTCC 3′ for the α1^(TL) construct, 5′ TCCAACTTTGACGTA-AATACACATCTATTGT 3′ for the α1^(YL) construct, 5′ TTCATCCTT-TCCAATAATGACGGAAAAACA 3′ for the α1^(HY) construct, 5′ CATCCTTCCAACTATGACGGAAAAACA 3′ for the α1^(H) construct, and 5′ TTCATCCTTCCAATATTGACGGAAAAAC 3′ for α1^(Y) construct.

Immunocytochemistry. Transfected cells plated on poly-L-lysine (10 μg/ml^{−1})-coated coverslips were fixed in 3% paraformaldehyde (in PBS) 15–18 hr after transfection, and immunofluorescence was performed, as described previously (Connolly et al., 1996). When cells were permeabilized, 0.05% NP-40 was added to all solutions after fixation. The primary antibodies were applied for 1 hr at the following concentrations: anti-FLAG (DYKDDDK) M2 mouse monoclonal antibody (IBI Ltd.), 9 μg/ml; 9E10 supernatant (Connolly et al., 1996) diluted 1:2, and rabbit anti-9E10, 5 μg/ml. An affinity-purified rabbit polyclonal sera against an intracellular epitope of the β1 and β3 subunits (anti-β1/3; McDonald et al., 1998) was used at 5 μg/ml. Secondary antibodies, either fluorescein- or rhodamine-conjugated anti-mouse or anti-rabbit IgG (Pierce, Rockford, IL) at 1 μg/ml^{−1} were applied for 45 min. Fluorescence images were analyzed by confocal microscopy (MRC 1000; Bio-Rad, Hercules, CA).

Iodinated antibody binding. Affinity-purified 9E10 antibody was iodinated to a specific activity of 500 Ci/mmol using Boltan and Hunter reagents, per manufacturer's instructions (Amersham International). The iodinated antibody was titred on ^(9E10)β3-transfected A293 cells and used at saturating concentrations (10 nM) for surface binding. The affinity of the antibody for ^(9E10)β3 was determined to be 0.5 nM by Scatchard analysis, as described previously (Amato et al., 1999). Surface binding was performed by preincubation in binding medium (DMEM with 25 mM HEPES and 0.5% BSA, pH 7.4) for 1 hr on ice followed by incubation with iodinated antibody for 1 hr. Cells were washed five times in binding medium, trypsinized, and quantified by counting gamma emissions. Nonspecific binding was determined using mock-transfected cells. Significance was determined using the Student's *t* test.

Sucrose density gradient fractionation and immunoprecipitation. Expressing cells were L-methionine starved for 30 min before labeling with [³⁵S]methionine (ICN Biochemicals, Costa Mesa, CA) at 200 μCi/ml⁻¹ for 4 hr and lysed in lysis buffer (25 mM Tris-HCl, pH 7.6, 1 mM EDTA, 150 mM NaCl, 2% NP-40, 0.5% deoxycholate, 50 mM NaF, 1 mM Na₃VO₄, 0.1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml antipain, and 10 μg/ml aprotinin) either immediately, or after a 20 hr chase in normal medium. Labeled receptor subunits were subjected to sucrose density gradient fractionation on 5–20% linear sucrose density gradients in lysis buffer (Gorrie et al., 1997; Taylor et al., 1999). Before

loading, cell extracts were clarified by centrifugation (100,000 × *g* for 10 min). Gradients were calibrated by loading parallel gradients with marker proteins (1 mg/ml) of known sedimentation coefficients; BSA, 4.3 S; aldolase, 7.4 S; catalase, 11.2 S. Gradients were centrifuged in a Beckman SW55Ti rotor at 40,000 rpm for 14.14 hr at 4°C. The gradients were fractionated into 14, 350 μl fractions, and receptor subunit sedimentation was analyzed by immunoprecipitation, as described previously (Gorrie et al., 1997; Taylor et al., 1999).

Western blotting. Receptor subunits were detected in cell lysates using purified 9E10 antibody at 10 μg/ml. Western blotting was performed as described previously (Connolly et al., 1996) using an enhanced chemiluminescent substrate (Supersignal substrate; Pierce). When appropriate, levels of chemiluminescence were quantified using a Bio-Rad phosphoimager within the appropriate linear range.

Electrophysiological analysis. Whole-cell membrane currents were recorded from single A293 cells using the patch-clamp technique in conjunction with a List EPC7 amplifier. Patch electrodes (1–5 MΩ) were filled with a solution containing (in mM): 140 KCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES, 11 EGTA, and 2 adenosine triphosphate, pH 7.2 Cells were continuously superfused with a Krebs' solution containing (in mM): 140 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 HEPES, and 11 glucose, pH 7.4. Cells were used 24–48 hr after transfection and voltage-clamped at −40 mV with membrane currents. Drugs and Krebs' solution were rapidly applied (exchange rate, ∼30 msec) to single cells using a modified U-tube (Wooltorton et al., 1997). GABA equilibrium concentration–response curve data were fitted to the following equation:

$$I/I_{\text{Max}} = [1/(1 + (\text{EC}_{50}/A)^{n_{\text{H}}})] \qquad (1)$$

where I and I_{max} represent the peak GABA-activated current by a concentration, A, and by a saturating concentration of GABA, respectively. The EC₅₀ defines the GABA concentration, producing a half-maximal response. n_{H} represents the Hill coefficient. Data (mean ± SEM) were analyzed using Origin 4.1 (MicroCal) and FigP (Biosoft).

RESULTS

α6S and α6L subunits differ in their capacity to access the cell surface with receptor β subunits

To assess the possible role of the α6 subunit splice variants in mediating GABA_A receptor assembly, the α6L and α6S subunit isoforms were modified with reporter epitopes between amino acids 4 and 5 of the mature polypeptides. Previous studies have demonstrated that addition of reporter epitopes to this domain of receptor subunits is functionally silent (Connolly et al., 1996). 9E10-tagged α6L and α6S were then expressed in human embryonic kidney cells (A293) with the ^(FLAG)β3 subunit. The subcellular localization of the expressed subunits was then determined using immunofluorescence with and without membrane permeabilization. Coexpression of ^(9E10)α6L with ^(FLAG)β3 resulted in robust expression of both the ^(9E10)α6L and ^(FLAG)β3 subunits on the cell surface (Fig. 1) as defined by staining in unpermeabilized cells. In contrast, ^(9E10)α6S was unable to access the cell surface on expression with ^(FLAG)β3, as determined by the lack of signal with 9E10 antisera in unpermeabilized cells. The ^(9E10)α6S subunit could be detected in permeabilized cells and was retained in an intracellular organelle resembling the ER (Fig. 1*B*). However, the ^(FLAG)β3 subunit was clearly able to access the cell surface in the presence of α6S (Fig. 1), as defined by robust FLAG staining in unpermeabilized cells. The ER retention of α6S after coexpression with the β3 subunit is consistent with the observations of Korpi et al. (1994), who also demonstrated that coexpression of the α6S subunit with both the β2 and γ2 subunit does not produce functional GABA_A receptors.

The 10 amino acids that differ between the α6S and α6L subunits, EYTMDVFFRQ, are conserved in all α subunits (Mac-Donald and Olsen, 1994; Rabow et al., 1995). The only variant amino acid is the methionine residue present in α4 and α6 subunits that is substituted by an isoleucine in the α1, α2, α3, and
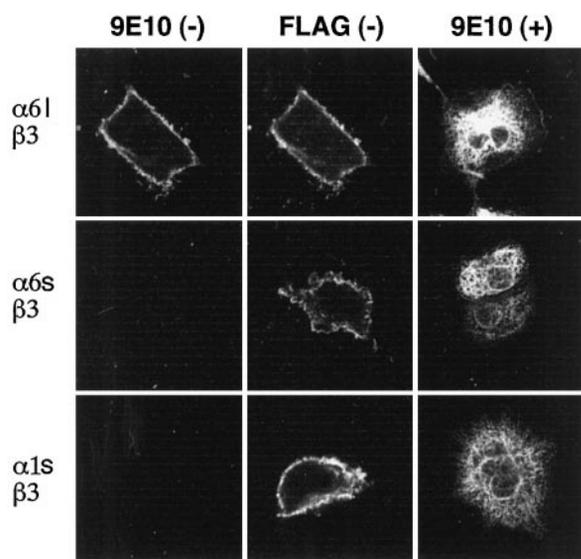
Figure 1. Coexpression of $^{(9E10)}\alpha$6L, $^{(9E10)}\alpha$6S, and $^{(9E10)}\alpha$1S with the $^{(FLAG)}\beta$3 subunit in A293 cells. The subcellular localization of receptors composed of $^{(9E10)}\alpha$6L/$^{(FLAG)}\beta$3, $^{(9E10)}\alpha$6S/$^{(FLAG)}\beta$3, and $^{(9E10)}\alpha$1S/$^{(FLAG)}\beta$3 subunits was determined by immunofluorescence on both permeabilized and nonpermeabilized A293 cells 24 hr after transfection. Coexpressing cells were stained with rabbit anti-9E10 antisera and a mouse anti-FLAG M2 monoclonal antibody in the absence (−) or presence (+) of membrane permeabilization. Subunit expression was then visualized using anti-rabbit fluorescein-conjugated secondary antibodies and anti-mouse rhodamine-conjugated antisera, respectively. Scale bar, 10 μm.

α5 subunits. To examine the potential role of these residues in controlling the assembly of other α subunit isoforms, residues 58–67 were deleted from $^{(9E10)}\alpha$1 to yield $^{(9E10)}\alpha$1S. This construct was then expressed with $^{(FLAG)}\beta$3 in A293 cells, and localization was monitored by immunofluorescence using 9E10 antisera. $^{(9E10)}\alpha$1S was unable to access the cell surface on coexpression with $^{(FLAG)}\beta$3 (Fig. 1), as defined by the absence of 9E10 staining in unpermeabilized cells (Fig. 1). The $^{(9E10)}\alpha$1S subunit could be detected in permeabilized cells and was retained within the ER (Fig. 1). The $^{(FLAG)}\beta$3 subunit was able to access the cell surface in the presence of $^{(9E10)}\alpha$1S (Fig. 1), as defined by FLAG staining in the absence of permeabilization, consistent with the results seen with $^{(9E10)}\alpha$6S and also with the ability of β3 to form functional homomeric receptors (Fig. 1; Connolly et al., 1996; Wooltorton et al., 1997, Taylor et al., 1999). In contrast, the wild-type α1 subunit can readily assemble with β3 to form functional GABA-gated channels (MacDonald and Olsen, 1994; Rabow et al., 1995). Identical ER retention of $^{(9E10)}\alpha$1S was seen on coexpression with both the β3 and γ2 subunits (data not shown). Because the α1S and α6S subunits appear to share the same defect in cell surface expression, these observations suggest a potential role for amino acids 58–67 in controlling the assembly of all α subunit isoforms.

Cells expressing the α1S and β3 subunits express functional β3 homomers

Expression of α1S and β3 subunits in A293 cells produced receptors displaying a distinctive pharmacological profile. The expressed receptors were insensitive to GABA up to 2 mM but could be activated by the allosteric modulator pentobarbitone (1 mM), which produced a desensitizing inward current with characteristic "rebound current" after application of the ligand (Fig. 2). Given that the α1S subunit is retained within the ER, the cell surface
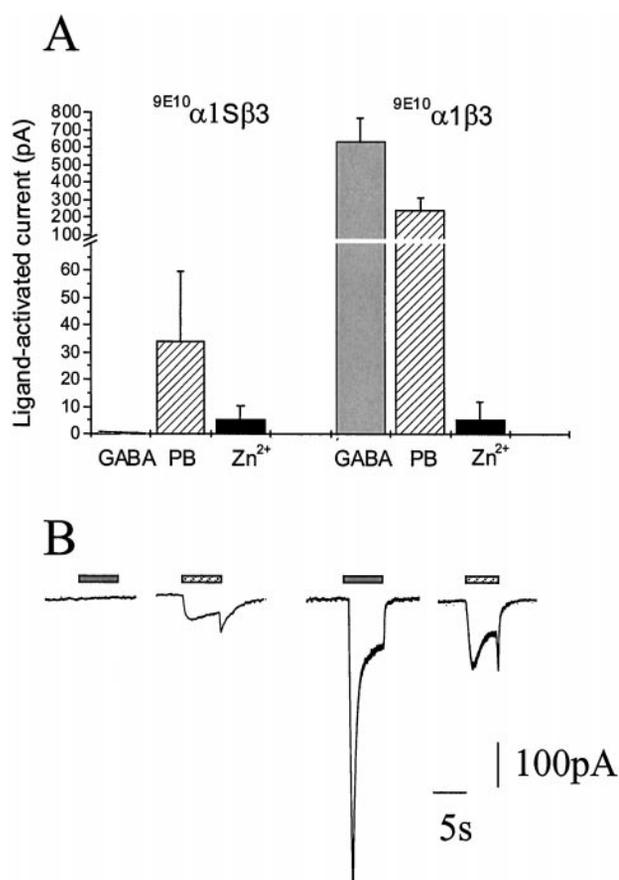

Figure 2. Functional properties of GABA$_A$ receptors produced by coexpression of α1S and β3 subunits in A293 cells. *A*, Bar graph of transfected A293 cell sensitivity to 1 mM GABA, 1 mM pentobarbitone (*PB*), and 10 μM Zn^{2+}. These concentrations produce maximal inward (*GABA* and *PB*) or outward (*Zn^{2+}*) currents for $\alpha\beta$ heteromers. The results illustrate ligand-activated currents from $n = 6$ α1Sβ3 and n = 5 α1β3 GABA$_A$ receptors. Error bars indicate the mean and SEM. Note the split ordinate axis. *B*, Membrane currents evoked by 1 mM GABA (*gray bar*), and 1 mM PB (*hatched bar*) and for α1Sβ3 (columns 1 and 2) and α1β3 (columns 3 and 4).

(Fig. 1) expressed receptors are likely to be composed of predominantly β3 subunits forming homomeric receptors (Davies et al., 1997; Wooltorton et al., 1997), which would account for the direct action of pentobarbitone (Fig. 2*B*). Homomeric β3 receptors also exhibit a degree of spontaneous gating in the absence of any ligand, which can be inhibited by Zn^{2+} (Wooltorton et al., 1997). Although in some cells ($n = 3$ of 8) 10 μM Zn^{2+} induced a small outward current, the majority of expressing cells failed to demonstrate any response to Zn^{2+} (Fig. 2*A*). Overall, the level of expression of the β3 homomers in the α1Sβ3 cDNA-transfected cells was quite low compared to control cells transfected with only β3 cDNAs (data not shown; Wooltorton et al., 1997). This may indicate why the pentobarbitone-activated currents are small and may also explain the difficulty in observing outward currents to Zn^{2+} that are indicative of spontaneous gating. It is therefore possible that the α1S subunit, while not capable of accessing the cell membrane, may hinder the functional expression of homomeric β3 subunits.

Amino acids 58–67 control the oligomerization of GABA$_A$ receptor α1 and β3 subunits

The failure of the α1S and α6S subunits to be expressed on the cell surface with the β3 subunits could result from an inability to

Figure 3. Coimmunoprecipitation of β3 and γ2L subunits with α1 and α1S subunits. Transfected A293 cells were [^{35}S]methionine-labeled and immunoprecipitated using 9E10 antibody or anti-FLAG M2 mouse monoclonal antibody coupled to protein A-Sepharose. Immune complexes were separated by SDS-PAGE using 8% gels. The molecular weights of marker proteins are indicated. *A,* Cells expressing $^{(FLAG)}\beta$3 were immunoprecipitated using FLAG-antibody, and cells expressing $^{(9E10)}\alpha$1, $^{(9E10)}\alpha$1S, $^{(FLAG)}\beta$3, $^{(9E10)}\alpha$1 + $^{(FLAG)}\beta$3, or $^{(9E10)}\alpha$1S + $^{(FLAG)}\beta$3 were immunoprecipitated using 9E10-antibody. *B,* Cells expressing $^{(FLAG)}\gamma$2L, $^{(FLAG)}\gamma$2L + $^{(9E10)}\alpha$1S, $^{(FLAG)}\gamma$2L + $^{(9E10)}\alpha$1, $^{(9E10)}\alpha$1, or $^{(9E10)}\alpha$1S were immunoprecipitated using FLAG antibody. Immunoprecipitation was also performed on untransfected methionine-labeled cells (*c*) using 9E10 or FLAG antibodies, as shown in *A* and *B*.
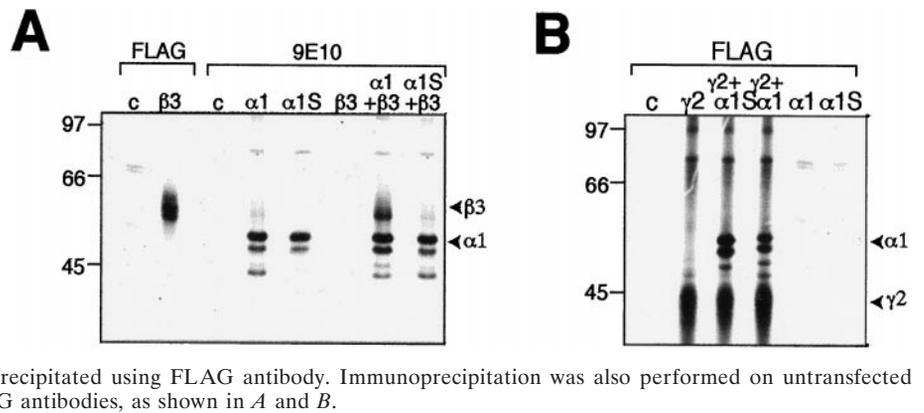
oligomerize blocking the subsequent formation of functional receptors. To address this issue, the ability of the $^{(9E10)}\alpha$1S subunit to coimmunoprecipitate $^{(FLAG)}\beta$3 was assessed after metabolic labeling with [^{35}S]methionine. Cells were labeled for 4 hr before lysis without a cold chase. This labeling period is sufficient to allow efficient oligomerization of GABA$_A$ receptor subunits to be detected (Moss et al., 1992; Krishek et al., 1994; Connolly et al., 1996; McDonald et al., 1998). $^{(FLAG)}\beta$3 was observed to migrate as two bands of 57–59 kDa, whereas $^{(9E10)}\alpha$1 migrated as three variable bands of 48–52 kDa (Fig. 3*A*; Connolly et al., 1996; Gorrie et al., 1997; McDonald et al., 1998). An additional band of 40 kDa was sometimes observed, regardless of the antibody used (Connolly et al., 1996). These three forms of $^{(9E10)}\alpha$1 differ in their levels of N-linked glycosylation, however all forms are able to oligomerize with receptor β subunits (Connolly et al., 1996; Gorrie et al., 1997). Using 9E10 antibody, the $^{(FLAG)}\beta$3 subunit coprecipitated with the $^{(9E10)}\alpha$1 subunit (Fig. 3*A*). However, smaller amounts of $^{(FLAG)}\beta$3 were seen coprecipitating with $^{(9E10)}\alpha$1S (Fig. 3*A*). This difference was quantified using a phospoimager, and ~10-fold lower amounts of $^{(FLAG)}\beta$3 were seen coprecipitating with $^{(9E10)}\alpha$1S compared to $^{(9E10)}\alpha$1. Importantly, similar amounts of $^{(9E10)}\alpha$1 and $^{(9E10)}\alpha$1S were precipitated with the 9E10 antibody (Fig. 3*A*), demonstrating that both proteins are expressed at similar levels. Similar low levels of the α1S subunit were seen coprecipitating with the β3 subunit using FLAG antibody (see Fig. 6).

To further examine the role of residues 58–67 within α1, the potential interaction with the $^{(FLAG)}\gamma$2L subunit was tested. Previous studies have revealed that although the α1 and γ2L subunits are capable of efficient oligomerization, α1/γ2 complexes are ER retained and do not produce functional cell surface receptors (Connolly et al., 1996, 1999). Immunoprecipitation of $^{(FLAG)}\gamma$2L using FLAG antibody yielded a broad smear of 42–49 kDa, as described previously (Fig. 3*B*; Connolly et al., 1996). Similar migration of the γ2 subunit has been seen previously in recombinant preparations and for the γ2 subunit expressed in neuronal membranes (Stephenson et al., 1990; Connolly et al., 1996, 1999; Tretter et al., 1997). Both $^{(9E10)}\alpha$1 and $^{(9E10)}\alpha$1S coprecipitated with $^{(FLAG)}\gamma$2L at similar levels (Fig. 3*B*). Importantly, the level of $^{(FLAG)}\gamma$2L present in each sample was comparable. This result suggests that $^{(9E10)}\alpha$1 and $^{(9E10)}\alpha$1S proteins are able to oligomerize with $^{(FLAG)}\gamma$2L at similar efficiencies (Fig. 3*B*).

Together, these observations suggest that amino acids 58–67 conserved within all α subunit isoforms are important in controlling oligomerization with receptor β subunits but not the γ2 subunit. Moreover this suggests that residues 58–67 are likely to
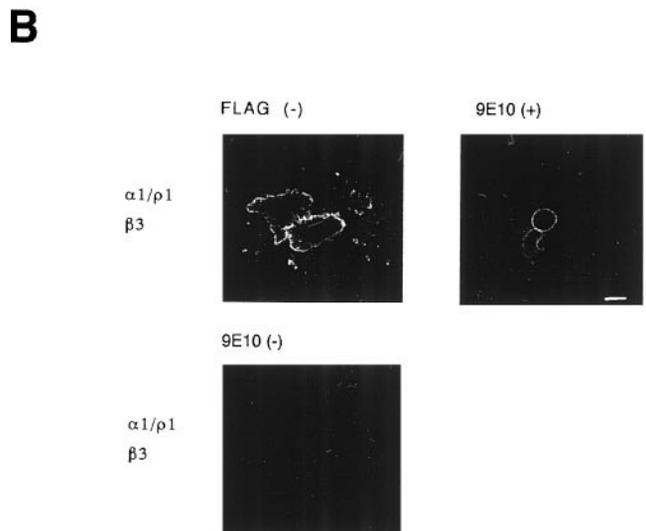

Figure 4. Production and expression of the α1/ρ1 subunit chimera. *A,* Sequence alignment of α1 and ρ1 subunits between residues 57 and 69. *B,* Cell surface of $^{(9E10)}\alpha$1/ρ1 subunit chimeras as determined by immunofluorescence. A293 cells transfected with the $^{(FLAG)}\beta$3 and $^{(9E10)}\alpha$1/ρ1 subunits were stained with either 9E10 or FLAG antibodies with (+) and without (−) membrane permeabilization.

constitute a subunit specific assembly signal rather than affecting gross subunit folding (Hammond and Helenius, 1995).

Exchange of amino acids 58–68 within the α1 subunit by the corresponding residues from ρ1 subunit prevents assembly with the β3 subunit

To further examine the role of amino acids 58–67 of the α1 subunit in mediating receptor assembly, a chimeric approach was taken. These amino acids were exchanged for the corresponding region of the GABA$_C$ receptor ρ1 subunit (Fig. 4*A*). The ρ1 subunit shares ~30% sequence identity with GABA$_A$ receptor subunits (Cutting et al., 1991; MacDonald and Olsen, 1994; Rabow et al., 1995). However, despite the coexistence of the ρ1 subunit in retinal neurons with GABA$_A$ receptor subunits (Cut-
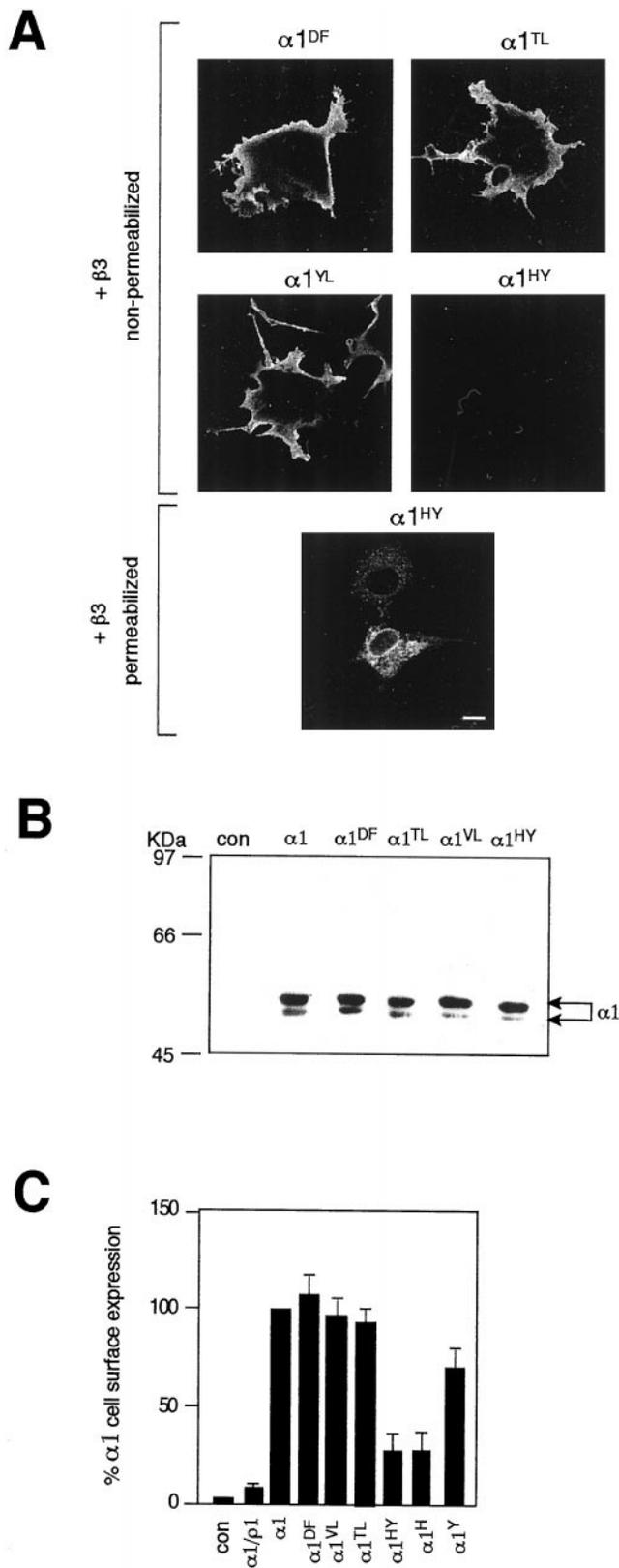
ting et al., 1991; Enz et al., 1996; Koulen et al., 1998), the $\rho1$ subunit does not appear to assemble with GABA$_A$ receptor α or β subunits (Hackam et al., 1996, 1997). Therefore, if amino acids 57–67 of $\alpha1$ are important in promoting specific association with receptor β subunits, substitution of these residues with those from the $\rho1$ subunit may be expected to disrupt the assembly of α/β receptors. To test this, residues 57–69 from the $\rho1$ subunit were exchanged for the corresponding residues in $\alpha1$ (Fig. 4*A*). This construct centered on methionine 57 and tryptophan 69, which are conserved in all GABA$_A$ and GABA$_C$ receptor subunits (Fig. 4*A*). The resulting $^{(9E10)}\alpha1/\rho1$ construct was then expressed with $^{(FLAG)}\beta3$ in A293 cells, and surface expression was measured using 9E10 and FLAG antibodies via immunofluorescence. $^{(FLAG)}\beta3$ could be detected on the cell surface of unpermeabilized cells (Fig. 4*B*). In contrast, the $^{(9E10)}\alpha1/\rho1$ construct was poorly expressed on the cell surface with the $^{(FLAG)}\beta3$ subunit in unpermeabilized cells. However, $^{(9E10)}\alpha1/\rho1$ could be readily detected in permeabilized cells with a predominant perinuclear localization, consistent with retention of this protein within the ER (Fig. 4*B*). Furthermore, the $^{(9E10)}\alpha1/\rho1$ construct was expressed at similar levels to $^{(9E10)}\alpha1$, as determined by Western blotting (data not shown).

Together, these results further support a role for residues 58–68 in promoting specific assembly of the $\alpha1$ subunit with receptor β subunits.

Residue Q67 mediates cell surface expression of the $\alpha1$ subunit with $\beta3$

To further delineate the region of $\alpha1$ subunit between residues 58 and 67 that are important for functional expression with β subunits, more refined $^{(9E10)}\alpha1/\rho1$ constructs were made. An alignment of residues 57–69 in $\alpha1$ with the same region of $\rho1$ reveals nine variant amino acids (Fig. 4*A*). The isoleucine residue at position 61 in $\alpha1$ was not mutated, because $\alpha6$ and $\rho1$ both contain methionine at position 61 (Fig. 1). Four constructs were made in which pairs of residues in $\alpha1$ were substituted for those within $\rho1$. These constructs $^{(9E10)}\alpha1^{(DF)}$, $^{(9E10)}\alpha1^{(TL)}$, $^{(9E10)}\alpha1^{(YL)}$, and $^{(9E10)}\alpha1^{(HY)}$, were then coexpressed with $\beta3$ in A293 cells, and surface expression was monitored by fluorescence using 9E10 antibody without permeabilization. $^{(9E10)}\alpha1^{(DF)}$, $^{(9E10)}\alpha1^{(TL)}$, and $^{(9E10)}\alpha1^{(YL)}$ could all be detected robustly on the cell surface. In contrast, $^{(9E10)}\alpha1^{(HY)}$ could not be detected in the majority of expressing cells, as defined by very weak signals in nonpermeabilized cells (Fig. 5*A*). However, in some experiments the occasional cell showed detectable cell surface levels of $^{(9E10)}\alpha1^{(HY)}$. In contrast, $^{(9E10)}\alpha1^{(HY)}$ could be readily detected in permeabilized cells where it appeared to be retained within the ER (Fig. 5*A*). All four $\alpha1$ subunit variants were expressed to similar levels according to Western blotting using 9E10 antibody (Fig. 5*B*). Quantification of blots using a phosphoimager within

Figure 5. Coexpression of $\alpha1$ double and single point mutants with the $\beta3$ subunit. *A,* The subcellular localization of $^{(9E10)}\alpha1^{(DF)}$, $^{(9E10)}\alpha1^{(TL)}$, $^{(9E10)}\alpha1^{(YL)}$, and $^{(9E10)}\alpha1^{(HY)}$ on coexpression with the $^{(FLAG)}\beta3$ subunit in A293 cells was determined by immunofluorescence using the 9E10 antibody with or without membrane permeabilization. *B,* The expression levels of $^{(9E10)}\alpha1$, $^{(9E10)}\alpha1^{(DF)}$, $^{(9E10)}\alpha1^{(TL)}$, $^{(9E10)}\alpha1^{(YL)}$, and $^{(9E10)}\alpha1^{(HY)}$ after coexpression with the $\beta3$ subunit were determined by Western

←

blotting with 9E10 antibody. Lysates from untransfected cells (*con*) were also included as a control. Migration of molecular weight markers is indicated. *C,* Cell surface expression levels of $^{(9E10)}\alpha1$ ($n = 4$), $^{(9E10)}\alpha1/\rho1$ ($n = 4$), $^{(9E10)}\alpha1^{(DF)}$ ($n = 4$), $^{(9E10)}\alpha1^{(YL)}$ ($n = 3$), $^{(9E10)}\alpha1^{(TL)}$ ($n = 3$), $^{(9E10)}\alpha1^{(HY)}$ ($n = 3$), $^{(9E10)}\alpha1^{(H)}$ ($n = 3$), and $^{(9E10)}\alpha1^{(Y)}$ ($n = 5$) on coexpression with $\beta3$ were determined in live cells by ^{125}I 9E10 antibody binding. 9E10 binding was also performed on untransfected cells (*con*). Cell surface 9E10 levels were then compared to cells expressing $^{(9E10)}\alpha1$ + $\beta3$, which was given a value of 100%. Significance from wild-type $^{(9E10)}\alpha1$-expressing cells ($p > 0.05$) was seen for $^{(9E10)}\alpha1/\rho1$, $^{(9E10)}\alpha1^{(HY)}$, $^{(9E10)}\alpha1^{(H)}$, and $^{(9E10)}\alpha1^{(Y)}$.

the linear range failed to demonstrate significant differences in expression between the α1 subunit mutants in three separate experiments. Furthermore, none of these $^{(9E10)}$α1 subunit variants were able to access the cell surface on homomeric expression (data not shown).

To further examine the role of residues Q67 and S68, cell surface levels of selected $^{(9E10)}$α1 constructs on expression with the β3 subunit were quantified using ^{125}I 9E10 antibody binding. Cell surface levels of 9E10 were then normalized to that for $^{(9E10)}$α1β3 receptors. Cell surface expression of $^{(9E10)}$α1$^{(HY)}$ was fourfold lower than that for α1β3 receptors (Fig. 5*C*; $p > 0.05$). However, cell surface levels of $^{(9E10)}$α1$^{(HY)}$ were still significantly higher than those observed for the α1/ρ1 chimera (Fig. 5*C*; $p > 0.05$). In contrast, the $^{(9E10)}$α1$^{(DF)}$, $^{(9E10)}$α1$^{(TL)}$, and $^{(9E10)}$α1$^{(YL)}$ constructs showed similar levels of surface expression when coexpressed with the β3 subunit, as observed with $^{(9E10)}$α1 subunit. The effect of individually mutating Q67 and S68 to the corresponding residues within the ρ1 subunits H67 and Y68, respectively, was also analyzed. The $^{(9E10)}$α1$^{(H)}$ and $^{(9E10)}$α1$^{(Y)}$ constructs were expressed with β3, and cell surface 9E10 levels were then compared to those for $^{(9E10)}$α1. Mutation of Q67 had a large effect on cell surface expression, because surface levels of $^{(9E10)}$α1$^{(H)}$ were reduced approximately fourfold compared to $^{(9E10)}$α1 (Fig. 5*C*; $p > 0.05$). Interestingly, the values for surface expression of $^{(9E10)}$α1$^{(H)}$ were not significantly different from those seen for $^{(9E10)}$α1$^{(HY)}$ (Fig. 5*C*; $p > 0.05$). This results suggests that Q67 is of more significance for assembly of the α1 subunit with β3 than S68. In agreement with this observation, mutation of S68 alone had a much smaller effect on cell surface expression of the $^{(9E10)}$α1 subunit.

Therefore, together our observations suggest a major role for residue Q67 within the α1 subunit in mediating cell surface expression with the β3 subunit.

Reduced oligomerization of $^{(9E10)}$α1$^{(HY)}$ with the β3 subunit

To further analyze the role of Q67 and S68 in promoting GABA$_A$ receptor assembly, selected $^{(9E10)}$α1 constructs were coexpressed with $^{(FLAG)}$β3, expressing cells were labeled with [^{35}S]methionine, chased for 4 hr, and lysed immediately or chased for 20 hr. Detergent-soluble cell extracts were then fractionated on sucrose density gradients. Receptor subunits were isolated from gradient fractions by immunoprecipitation. Each gradient fraction was divided into two samples that were separately immunoprecipitated with either 9E10 or FLAG antibodies against either $^{(9E10)}$α1 or $^{(FLAG)}$β3. Precipitated material was then resolved by SDS-PAGE; the distinct migration of ^{9E10}α1 and $^{(FLAG)}$β3 (Fig. 3) allows coimmunoprecipitation to be easily assessed. After a 4 hr labeling period, $^{(9E10)}$α1 (52 kDa) and $^{(FLAG)}$β3 (57 kDa) subunits could be seen coimmunoprecipitating using either antibody in gradient fractions 7–10 (Fig. 6*A*). The levels of $^{(9E10)}$α1 and $^{(FLAG)}$β3 proteins in each gradient fraction precipitated using FLAG antibody were quantified using a phosphoimager (Fig. 6*A,B*). Quantitation of the gradients revealed that both proteins exhibited 9 S sedimentation coefficients, as previously described for functional GABA$_A$ receptors composed of αβ or αβγ subunits (Fig. 6*A,B*; Mamalaki et al., 1987, 1989; Hadingham et al., 1992; Gorrie et al., 1997; Tretter et al., 1997). In contrast, unassembled α or β subunits have 5 S sedimentation coefficients (Gorrie et al., 1997; Tretter et al., 1997). To assess the stability of the 9 S α1β3 complexes, expressing cells were labeled with [^{35}S]methionine and chased for 20 hr before fractionation on

sucrose density gradients. At 20 hr, coimmunoprecipitation of $^{(9E10)}$α1 and $^{(FLAG)}$β3 was still evident using either antisera (Fig. 6*A–C*). Both subunits exhibited 9 S sedimentation coefficients, as seen at 0 hr (Fig. 6*B,C*). Quantification of the levels of $^{(9E10)}$α1 and $^{(FLAG)}$β3 precipitating with FLAG antibody (Fig. 6*B,C*) revealed that over this 20 hr period ~50% of the $^{(9E10)}$α1 and $^{(FLAG)}$β3 subunits were degraded (Fig. 7*B*). This suggests a half life of 20 hr for α1β3 receptors in good agreement with that reported for α1/β2 receptors (24 hr, Gorrie et al., 1997). Similar association and stability of the α1 and β3 subunits was seen in two other experiments.

The sedimentation of $^{(9E10)}$α1$^{(HY)}$ on coexpression with $^{(FLAG)}$β3 was also assessed via immunoprecipitation with both 9E10 and FLAG antibodies (Fig. 6*D*). After a 4 hr labeling period, precipitation with FLAG antibody revealed large amounts of the $^{(FLAG)}$β3 subunit present in gradient fractions 8–14 (Fig. 6*D*). However, only small amounts of α1$^{(HY)}$ could be detected coprecipitating with $^{(FLAG)}$β3, which is in contrast to the results seen with $^{(9E10)}$α1 (Fig. 6*A*).

Quantification of the material precipitated with FLAG antibody (Fig. 6*E*) revealed only low levels of $^{(9E10)}$α1$^{(HY)}$ coprecipitating with $^{(FLAG)}$β3. Furthermore, $^{(9E10)}$α1$^{(HY)}$ was found uniformly distributed throughout the gradient and did not exhibit a 9 S sedimentation coefficient, as seen for $^{(9E10)}$α1 (Fig. 6*E*). This distribution may reflect nonspecific aggregation, or $^{(9E10)}$α1$^{(HY)}$ may be interacting with chaperone molecules such as BiP and calnexin that participate in GABA$_A$ receptor assembly (Connolly et al., 1996). Interestingly, $^{(FLAG)}$β3 exhibited two distinct sedimentation coefficients of 9 S and 11 S (Fig. 6*E*). These peaks must predominately represent $^{(FLAG)}$β3 homomers that have 9 S sedimentation coefficients (Taylor et al., 1999), given the low levels of the $^{(9E10)}$α1$^{(HY)}$ subunit that coprecipitated with $^{(FLAG)}$β3. The 11 S peak may possibly represent nonspecific aggregates of the $^{(FLAG)}$β3 subunit. After a 20 hr chase period, very low levels of $^{(FLAG)}$β3 could be detected via precipitation with FLAG antisera, and trace levels of $^{(9E10)}$α1$^{(HY)}$ could be detected associating with this protein on long exposures (Fig. 6*D,E*). $^{(FLAG)}$β3 exhibited a predominant 9 S sedimentation coefficient after a 20 hr chase period, however the 11 S species was still evident. In contrast, no $^{(9E10)}$α1$^{(HY)}$ protein was detectable in any gradient fractions using precipitation with 9E10 antibody after a 20 hr chase (Fig. 6*F*). Identical behavior of the $^{(9E10)}$α1$^{(HY)}$ and $^{(FLAG)}$β3 subunits was seen in two other separate experiments.

Together, these results demonstrate that Q67 and S68 within the α1 subunit are critical in mediating assembly with β3 to form 9S complexes, representing functional cell surface receptors (Mamalaki et al., 1987, 1989; Hadingham et al., 1992; Gorrie et al., 1997; Tretter et al., 1997). $^{(9E10)}$α1$^{(HY)}$ subunits appear to oligomerize less efficiently with $^{(FLAG)}$β3 compared to $^{(9E10)}$α1 and are rapidly degraded as previously described for unassembled wild-type α1 subunits (Gorrie et al., 1997).

Functional properties of α1$^{(HY)}$/β3, α1$^{(H)}$/β3, and α1$^{(Y)}$/β3 receptors

Expression of α1$^{(HY)}$β3 subunit GABA$_A$ receptors in A293 cells resulted in a range of sensitivities to GABA, pentobarbitone, and Zn^{2+} that were used to assess the expression of αβ heteromers or β3 homomers. Most expressing cells ($n = 17$ of 20) exhibited limited sensitivity to GABA (0.01–1000 μM). In comparison to GABA, the sensitivity to pentobarbitone was far higher with 1 mM pentobarbitone (maximally effective concentration) producing almost 20- to 30-fold larger currents than a maximal concen-
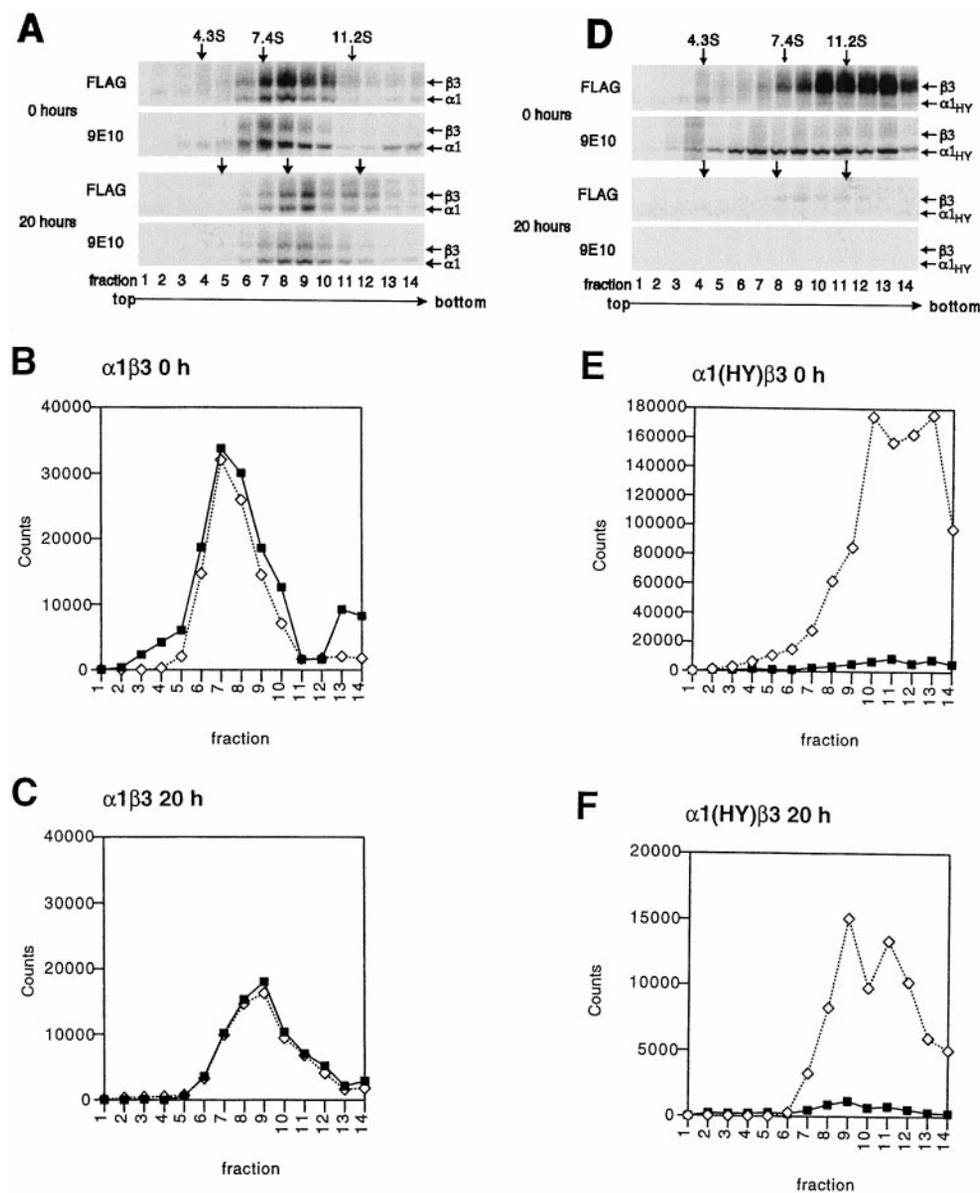
Figure 6. Sucrose gradient analysis of $^{(9E10)}\alpha1$ $^{(FLAG)}\beta3$ and $^{(9E10)}\alpha1$ $^{(HY)}$ $^{(FLAG)}\beta3$ receptor complexes. Lysates from [^{35}S]methionine-labeled A293 cells, cotransfected with $^{(9E10)}\alpha1$ and $^{(FLAG)}\beta3$ subunits (*A*) or $^{(9E10)}\alpha1$ $^{(HY)}$ and $^{(FLAG)}\beta3$ subunits (*B*) were separated on 5–20% linear sucrose gradients and fractionated into 14 equal fractions. $^{(9E10)}\alpha1$ (*A*) or $^{(9E10)}\alpha1$ $^{(HY)}$ (*D*) subunits were immunoprecipitated from half of each fraction using the 9E10 antibody, and the $^{(FLAG)}\beta3$ subunit was precipitated from the other half of each fraction using anti-FLAG. The precipitated proteins were resolved on 8% polyacrylamide gels and detected using autoradiography. For the 0 time point, cells were lysed immediately after a 4 hr labeling period, and for the 20 hr time point, labeled cells were incubated in normal medium for 20 hr before being lysed. The migration of the $^{(FLAG)}\beta3$ subunit (57–59 kDa) and the $^{(9E10)}\alpha1$ and $^{(9E10)}\alpha1$ $^{(HY)}$ subunits (50 kDa) are indicated. The *vertical arrows* represent the migration of standard proteins: from *left* to *right*, BSA (4.3 S), Aldolase (7.4 S), and Catalase (11.2 S), respectively. The levels of $^{(9E10)}\alpha1$ and $^{(FLAG)}\beta3$ subunits precipitated with FLAG antibody were quantified from gradient fractions after a 4 hr labeling period (*B*) or a 4 hr labeling period followed by a 20 hr chase (*C*) were determined using a Bio-Rad phosphoimager. Background was subtracted using the same volume that was used to integrate the subunit signals; □, $^{(FLAG)}\beta3$; ■, $^{(9E10)}\alpha1$. The levels of $^{9E10}\alpha1$ and $^{(FLAG)}\beta3$ precipitating with FLAG antibody were also analyzed after a 4 hr labeling period (*E*), or a 4 hr labeling period with a 20 hr Chase (*F*); □, $^{(FLAG)}\beta3$; ■, $^{(9E10)}\alpha1$ $^{(HY)}$. Quantification of subunit levels was as above. The *arrows* in *B, C, E,* and *F* represent the sedimentation of protein standards from *right* to *left*; BSA (4.3 S), Aldolase (7.4 S), and Calalase (11.2 S), respectively.

tration of GABA (1 mM; Fig. 7). Furthermore, the size of the pentobarbitone currents suggested that receptor expression was not compromised by the $\alpha1^{(HY)}$ mutation. Thus, the expressed receptors also exhibited clear sensitivity to Zn^{2+}, resulting in outward currents in accordance with some spontaneous gating of these receptors. The limited sensitivity to GABA and clear effects of pentobarbitone and Zn^{2+} all indicated the likely presence of a small number of $\alpha\beta$ heteromers and a larger population of $\beta3$ homomers. The presence of large rebound currents after application of pentobarbitone was also indicative of the presence of $\beta3$ homomeric receptors. In comparison, $\alpha1\beta3$ wild-type GABA$_A$ receptors exhibited larger currents to 1 mM GABA compared to 1 mM pentobarbitone and virtually zero sensitivity to Zn^{2+}, as expected of a population of predominantly $\alpha\beta$ heteromers (Fig. 7).

Analysis of the GABA concentration–response curve for the $\alpha1^{(HY)}\beta3$ subunit receptor revealed a GABA EC$_{50}$ of 5.1 ± 0.94 μM and Hill coefficient of 0.8 ± 0.1 ($n = 3$; Fig. 8). These values are in accordance with a typical $\alpha1\beta3$ receptor profile (Yemer et al., 1989) and suggest that the mutation HY does not interfere per se with the ability of GABA to bind to the receptor and activate

the ion channel. It therefore appears likely that the reduced responsiveness to GABA is a result of limited numbers of functional cell surface $\alpha\beta$ heteromers. This is consistent with the reduced surface levels of the $\alpha1^{(HY)}$ construct on coexpression with the $\beta3$ subunit compared to $\alpha1\beta3$ receptors (Fig. 5). Furthermore, the presence of $\beta3$ homomers on expression with $\alpha1^{(HY)}$ is in agreement with the low levels of oligomerization seen for $\beta3$ and $\alpha1^{(HY)}$ subunits, as revealed by sucrose density gradient centrifugation (Fig. 6).

Sequential mutation of the $\alpha1$ subunit yielded two discrete forms, $\alpha1^{(H)}$ and $\alpha1^{(Y)}$. Expression of the $\alpha1^{(H)}$ subunit with wild-type $\beta3$ subunits yielded receptors with limited sensitivity to 1 mM GABA, reduced sensitivity to 1 mM pentobarbitone, and variable sensitivity to 10 μM Zn^{2+}. The pharmacological profile suggested that $\alpha1^{(H)}\beta3$ heteromers were not forming efficiently, limiting the ability of GABA to activate the channel. The low sensitivity to pentobarbitone also suggested hindered expression of $\beta3$ homomers, and this would make resolution of spontaneous gating via the action of Zn^{2+} more difficult (Fig. 8). In contrast, expression of $\alpha1^{(Y)}\beta3$ subunits resulted in clear activation by
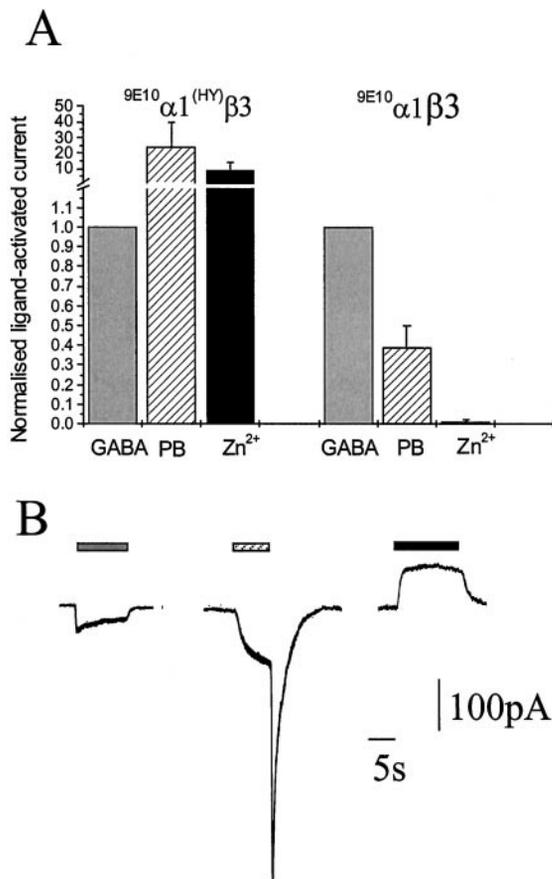
Figure 7. Functional properties of $^{(9E10)}\alpha 1^{(HY)}\beta 3$ GABA$_A$ receptors. *A,* Bar graph illustrating the sensitivity to 1 mM GABA, 1 mM pentobarbitone (*PB*), and 10 μM Zn^{2+} in A293 cells transfected with $^{(9E10)}\alpha 1^{(HY)}\beta 3$ (*left*) or $^{(9E10)}\alpha 1^{(HY)}\beta 3$ (*right*) cDNAs. The ligand-activated currents have been normalized to the response evoked by 1 mM GABA for each receptor construct (1), and Zn^{2+} induces outward currents in $^{(9E10)}\alpha 1^{(HY)}\beta 3$ GABA$_A$ receptors. Note the split ordinate axis. *B,* Whole-cell membrane currents recorded from a A293 cell expressing $^{(9E10)}\alpha 1^{(HY)}\beta 3$ and superfused with 1 mM GABA (*gray bar*), 1 mM PB (*hatched bar*), and 10 μM Zn^{2+} (*black bar*).

GABA, large pentobarbitone-activated currents, and no sensitivity to Zn^{2+} (Fig. 8). The properties of the $\alpha 1^{(Y)}\beta 3$ heteromer was virtually indistinguishable from the $\alpha 1\beta 3$ wild-type GABA$_A$ receptors. These results are consistent with a minor role for S68 compared to Q67 in controlling assembly of the $\alpha 1$ subunit with the $\beta 3$ confirming our cell biological observations (Fig. 5).

GABA concentration–response curve analysis for the $\alpha 1^{(H)}\beta 3$ receptor produced an EC$_{50}$ of 5.6 $\pm$ 1.38 μM and Hill coefficient of 0.86 $\pm$ 0.15 (*n* = 4). In comparison, the EC$_{50}$ for GABA activation of the $\alpha 1^{(Y)}\beta 3$ receptor was 2.18 $\pm$ 0.2 μM with a Hill coefficient of 0.85 $\pm$ 0.1 (*n* = 3). As for the $\alpha 1^{(HY)}$ mutation, nether the $\alpha 1^{(H)}$ nor $\alpha 1^{(Y)}$ mutations appeared to have dramatic effects on the ability of GABA to bind and/or activate these mutant ion channels (Fig. 8).

DISCUSSION

GABA$_A$ receptors can be assembled from six subunit classes with multiple members: $\alpha(1–6)$, $\beta(1–3)$, $\gamma(1–3)$, δ, ϵ, and π (MacDonald and Olsen, 1994; Rabow et al., 1995; Davies et al., 1997; Hedblom and Kirkness 1997), generating the potential for extensive receptor heterogeneity. To fully understand the diversity of
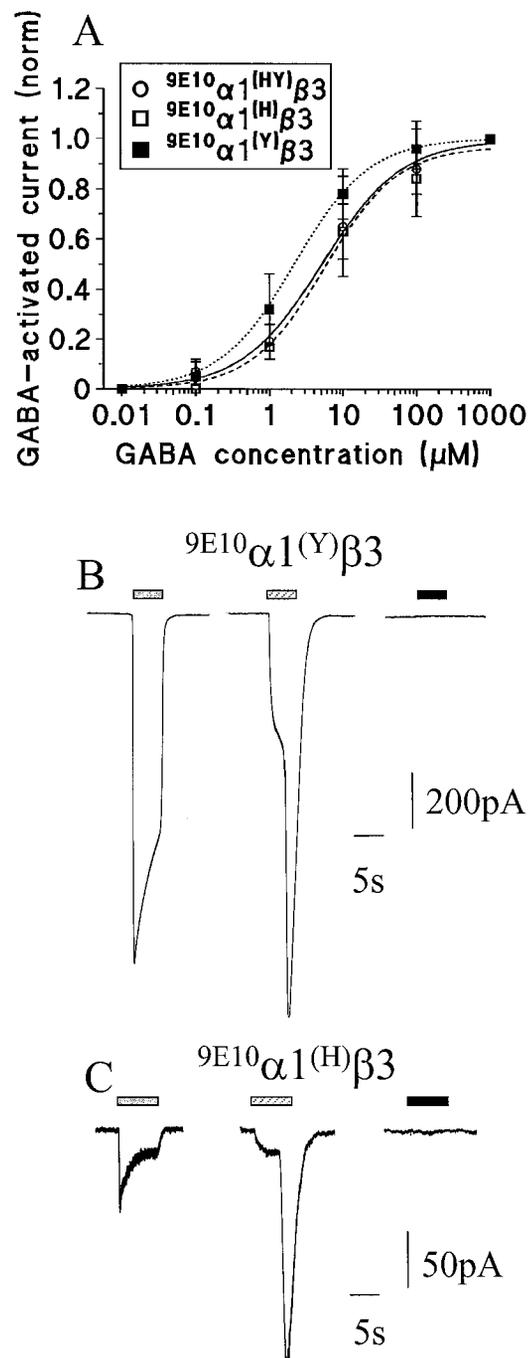

Figure 8. Functional properties of $\alpha 1^{(H)}$ and $\alpha 1^{(Y)}$ mutant GABA$_A$ receptors. Equilibrium concentration–response curves for GABA constructed for $^{(9E10)}\alpha 1^{(HY)}\beta 3$ (○), $^{(9E10)}\alpha 1^{(H)}\beta 3$ (□), and $^{(9E10)}\alpha 1^{(Y)}\beta 3$ (■) receptor constructs are shown in *A*. All points represent the mean $\pm$ SEM and were normalized to the response to 1 mM GABA for each construct. The points were fitted as described in Materials and Methods (*n* = 3–8). *B* and *C* show whole-cell currents activated by 1 mM GABA (*gray bar*), 1 mM pentobarbitone (*hatched bar*), and 10 μM Zn^{2+} (*black bar*) on $^{(9E10)}\alpha 1^{(H)}\beta 3$ (*B*) or $^{(9E10)}\alpha 1^{(Y)}\beta 3$ GABA$_A$ receptor constructs (*C*) recorded at a holding potential of -40 mV. Note the different current calibrations in *B* and *C*.

GABA$_A$ receptor structure in the brain, it is therefore of importance to understand how these receptors are assembled.

Here, we have examined the role of residues 58–67 conserved within all in GABA$_A$ receptor α subunits in controlling the

assembly of receptors composed of α, β, and γ subunits. Our studies were instigated by two naturally occurring splice variants of the α6 subunit termed α6S and α6L (Korpi et al., 1994) that differ by the presence of residues 58–67 in the α6L subunit. Deletion of these residues from the α6 subunit prevented functional cell surface expression with the β3 subunit. Similar disruption of cell surface expression was seen on deletion of residues 58–67 from the α1 subunit on expression with the β3 or the β3 and γ2 subunits. Both the α1S and α6S subunits were ER retained, suggesting that residues 58–67, which are conserved within all receptor α subunits, may be of importance in mediating GABA$_A$ receptor assembly. Electrophysiological studies revealed the presence of functional β3 homomers in cells coexpressing α1S and the β3 subunit (Wooltorton et al., 1997; Taylor et al., 1999), further demonstrating that residues 58–67 are critical for the production of functional α1β3 receptors. The role of residues 58–67 in mediating the oligomerization of α1 with the β3 and γ2 subunits was investigated using immunoprecipitation. Interestingly, these residues appeared to be of importance for selective oligomerization with the β3 subunit without affecting oligomerization with γ2. Given that the three-dimensional structure of GABA$_A$ receptors remains unknown, and the precise mechanisms of receptor assembly have not been fully elucidated, it is possible that these mutations are interfering with subunit folding. However, the selective effect of deleting residues 58–67 on α1 subunit oligomerization is of significance, because it suggests that removal of these residues does not cause general α subunit misfolding (Hammond and Helenius 1995). Furthermore, these results also strongly suggest that β3 and γ2 subunits interact with distinct domains of the α1 subunit.

To further identify the specific residues that mediate assembly of α1 with the β3 subunit, residues 58–68 within α1 were substituted with the corresponding residues from the ρ1 subunit (Hackam et al., 1996, 1997). This domain was chosen because the ρ1 subunit does not coassemble with GABA$_A$ receptor α or β subunits (Cutting et al., 1991; Enz et al., 1996; Koulen et al., 1998). Coexpression of this α1/ρ1 mutant blocked assembly with receptor β subunits in a similar manner, as observed on deletion of residues 58–67. Mutation of residues Q67 and S68 led to a fourfold reduction in cell surface expression of the α1 subunit with β3, whereas pairwise mutation of the other residues did not have significant effects on receptor cell surface expression. Of these two residues, mutation of Q67 had a much larger effect on cell surface expression, suggesting that this residue is of major importance in mediating assembly of the α1 subunit with β3. However, the reduction of cell surface expression on mutation of Q67 was not as drastic as the substitution of residues 57–69 of the α1 subunit with the corresponding region of the ρ1 subunit. This result suggests Q67 and S68 may interact with other as yet undefined amino acids between residues 58–67 of the α1 subunit to enhance assembly with the β3 subunit. Precisely how these residues affect the interaction of the α1 and β3 subunit remains unknown. They could directly mediate the interaction of subunits or may alternatively be important in controlling subunit structure, allowing interaction with β3 subunit. These issue can only be resolved when the tertiary structure of GABA$_A$ receptors has been resolved at high resolution.

To further analyze the role of Q67 in mediating assembly of α1 with the β3 subunit, sucrose density gradient centrifugation was used. α1β3 receptors migrated as a 9 S complex that was stable, exhibiting a half life in excess of 20 hr. Functional GABA$_A$ receptors composed of α1β2 and α1β3γ2 subunits exhibit similar

sedimentation coefficients and half lives (Gorrie et al., 1997; Tretter et al., 1997). In contrast, oligomerization of α1$^{(HY)}$ with the β3 subunit was greatly reduced. In addition, α1$^{(HY)}$ did not exhibit a 9 S sedimentation coefficient after coexpression with β3 and was rapidly degraded, similar to unassembled α1 subunits (Gorrie et al., 1997). The β3 subunit however, was able to form a 9 S complex on coexpression with α1$^{(HY)}$, which predominantly represents β3 homomers caused by the low levels of coprecipitation of α1$^{(HY)}$ (Wooltorton et al., 1997; Taylor et al., 1999). In agreement with this, the presence of spontaneously gating β3 homomers (Wooltorton et al., 1997; Taylor et al., 1999) was detected in cells coexpressing α1$^{(HY)}$β3 and α1$^{(H)}$β3 subunits. In contrast, coexpression of wild-type α1/β3 leads to the production of GABA-gated chloride channels (MacDonald and Olsen, 1994; Rabow et al., 1995). Given that Q67 is conserved in all receptor α subunits, our results suggest a critical role for this residue in mediating specific oligomerization of receptor α and β subunits. Interestingly, the α6S subunit that has residues 58–67 deleted is highly expressed in granule cells within the cerebellum (Korpi et al., 1994). Given that these residues are critical in mediating oligomerization with receptor β subunit without affecting oligomerization with the γ2 subunit, this may allow α6S to act as a "sink" for free γ2 subunits. This may be of importance, given that the γ2S subunit has the capacity to access the cell surface on homomeric expression (Connolly et al., 1999).

Mutagenesis studies have identified amino acids within the N-terminal domains of both GABA$_A$ receptor α and β subunits that are involved in the formation of GABA-binding sites, leading to the hypothesis that the GABA-binding site is located at the interface between the α and β subunits (Amin and Weiss 1993; Smith and Olsen, 1995). Interestingly, residues between 57 and 69 within α1 have previously been implicated in the binding of receptor agonists (Smith and Olsen, 1995). Recent experimental evidence using the cysteine accessibility method has suggested that this domain of the α1 subunit is a β-strand (Boileau et al., 1999). Residue F64 within α1 is of special significance because it is photoaffinity-labeled by muscimol, a GABA agonist (Smith and Olsen, 1994). Furthermore, mutation of this residue greatly reduces GABA affinity (Sigel et al., 1992). However, from our studies it is evident that mutation of F64 does not significantly affect receptor assembly. Conversely, mutation of Q67 or S68 did not have large effects on agonist affinity. Together, these observations suggests that distinct but closely linked amino acids mediate subunit interactions and the production of agonist binding sites in the case of GABA$_A$ receptors. The presence of an assembly signal in close proximity to sites for agonist binding is attractive, because the assembly signal will bring the α and β subunits into close contact during the assembly process within the ER. This may facilitate the production of high-affinity agonist-binding sites at the subunit interfaces, using distinct residues from both subunits. Although our studies identify that residue Q67 plays an important role in mediating assembly, other adjacent residues, most notably W69 and 94 within the α1 subunit, are also of importance in this process, because mutation of these residues blocks expression of α/β receptors (Srinivasan et al., 1999). Interestingly, Q67 is conserved in GABA$_A$, 5-HT3, and glycine receptor subunits, in addition to the α subunits of the AChR (Unwin, 1993). Together these observations suggest that this conserved residue may play a role in the assembly of all ligand-gated ion channels.

Interestingly, the residues that determine the specificity of AChR α/γ and α/δ subunit oligomerization are adjacent to or identical to the residues that actually form the ligand-binding site

(Gu et al., 1991; Kreienkamp et al., 1995; Green and Millar 1995). However, other distinct domains of the receptor α and γ subunits are also important for heteromeric receptor assembly (Green and Wanamaker, 1998; Eertmoed and Green, 1999).

Finally, our studies for the first time identify residues conserved within all GABA$_A$ receptor α subunits that mediate the selective oligomerization with receptor β subunits without affecting interaction with the $\gamma2$ subunit. Given that the majority of GABA$_A$ receptors in the brain are believed to be composed of α, β, and γ subunits, this domain of the receptor α subunits will play a critical role in mediating GABA$_A$ receptor assembly.

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