The C-Terminal Domains of the $GABA_B$ Receptor Subunits Mediate Intracellular Trafficking But Are Not Required for Receptor Signaling

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$GABA_B$ receptors are G-protein-coupled receptors that mediate slow synaptic inhibition in the brain and spinal cord. These receptors are heterodimers assembled from $GABA_{B1}$ and $GABA_{B2}$ subunits, neither of which is capable of producing functional $GABA_B$ receptors on homomeric expression. $GABA_{B1}$, although able to bind GABA, is retained within the endoplasmic reticulum (ER) when expressed alone. In contrast, $GABA_{B2}$ is able to access the cell surface when expressed alone but does not couple efficiently to the appropriate effector systems or produce any detectable GABA-binding sites. In the present study, we have constructed chimeric and truncated $GABA_{B1}$ and $GABA_{B2}$ subunits to explore further $GABA_B$ receptor signaling and assembly. Removal of the entire C-terminal intracellular domain of $GABA_{B1}$ results in plasma membrane expression without the production of a functional $GABA_B$ receptor. However, coexpression of this truncated $GABA_{B1}$ subunit with either $GABA_{B2}$ or a truncated $GABA_{B2}$ subunit in which the C terminal has also been removed is capable of functional signaling via G-proteins. In contrast, transferring the entire C-terminal tail of $GABA_{B1}$ to $GABA_{B2}$ leads to the ER retention of the $GABA_{B2}$ subunit when expressed alone. These results indicate that the C terminal of $GABA_{B1}$ mediates the ER retention of this protein and that neither of the C-terminal tails of $GABA_{B1}$ or $GABA_{B2}$ is an absolute requirement for functional coupling of heteromeric receptors. Furthermore although $GABA_{B1}$ is capable of producing GABA-binding sites, $GABA_{B2}$ is of central importance in the functional coupling of heteromeric $GABA_B$ receptors to G-proteins and the subsequent activation of effector systems.

Key words: $GABA_B$; GPCR; trafficking; signaling; intracellular retention; G-protein coupling; chimeras; receptor subunits

GABA is the most widely expressed inhibitory neurotransmitter in the mammalian CNS and mediates its actions via both ionotropic ($GABA_{A/C}$) and metabotropic ($GABA_B$) receptors (Bowery, 1993; Mott and Lewis, 1994; Rabow et al., 1995). $GABA_B$ receptors are members of the group 3 (C) family of G-protein-coupled receptors (GPCRs) (for review, see Couve et al., 2000), and the modulation of $GABA_B$ receptors is thought to be involved in a number of physiological and disease processes, including nociception, cognitive impairment, epilepsy, and spasticity, and also in the etiology of drug addiction (Bettler et al., 1998). $GABA_B$ receptors are unique among the group 3 GPCRs in that they are believed to be heterodimers of $GABA_{B1}$ and $GABA_{B2}$ subunits, each of which is unable to form a functional $GABA_B$ receptor in its own right (for review, see Marshall et al., 1999). The heterodimerization of $GABA_{B1b}$ and $GABA_{B2}$ has been shown to be mediated, at least in part, by interactions between two homologous α-helical coiled-coil domains present in the intracellular C terminals of both $GABA_{B1b}$ and $GABA_{B2}$ (Kammerer et al., 1999; Kuner et al., 1999). Although the initial papers describing the cloning and expression of $GABA_{B1}$ reported some functional activity for $GABA_{B1}$ when expressed alone in mammalian cells (Kaupmann et al., 1997, 1998a), a number of subsequent studies have reported that, when expressed alone, $GABA_{B1}$ is not able to inhibit adenylate cyclase activity effectively (White et al., 1998; Kuner et al., 1999; Ng et al., 1999), nor can it efficiently couple to K^+ channels in either *Xenopus* oocytes (Jones et al., 1998; Kaupmann et al., 1998b; Ng et al., 1999) or human embryonic kidney (HEK)-293 cells (Jones et al., 1998; Kuner et al., 1999). Furthermore, $GABA_{B1}$ is unable to inhibit calcium channel activity when injected alone into sympathetic neurons (Couve et al., 1998; Filippov et al., 2000). These findings are at least partly explained by the fact that, when expressed alone in heterologous systems, $GABA_{B1}$ is not expressed on the cell surface but is retained within intracellular membranes (Couve et al., 1998), and although it is able to bind $GABA_B$ ligands, its pharmacological profile with respect to agonist binding is different from that of endogenous receptors (Kaupmann et al., 1997). However, coexpression of $GABA_{B1}$ with $GABA_{B2}$ results in the correct trafficking of both subunits to the cell surface as heterodimers and the formation of functional receptors with pharmacology similar to that of $GABA_B$ receptors *in vivo* (Jones et al., 1998; Kaupmann et al., 1998b; White et al., 1998; Kuner et al.,

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Table 1. Forward and reverse primers used to amplify truncated and chimeric $GABA_B$ receptor subunits

PCR product	Forward primer (5′–3′)	Reverse primer (5′–3′)
$GABA_{B1b}\Delta806$	CCCGAATTCATGGGGC CCGGGGCC	TTAGAGCTGCTGCCGAGA CTGGAGTTG
$GABA_{B1b}\Delta771$	CCCGAATTCATGGGGC CCGGGGCC	TTACTTCTCCTCCTCGTTG TTGTTGGTC
$GABA_{B1b}\Delta747$	CCCGAATTCATGGGGC CCGGGGCC	CCCAAGCTTCCTCGGGTG ATCAGCCTG
$GABA_{B1b}(750–844)$	CCCAAGCTTGCAGTCG GAGGCGCAGG	CCCCTCGAGTCACTTATA AAGCAAATGCACTCGAC
$GABA_{B2}\Delta748$	CCCGAATTCATGGCTTC CCCGCGGAGC	CCCAAGCTTCTCAGGGTG ATGAGCTTCGGC
$GABA_{B2}(751–941)$	CCCAAGCTTCCCAGAT GCAGCAACGCAGAAC	CCCCTCGAGTTACAGGCC CGAGACCATGACTC

1999; Ng et al., 1999). Thus the dimerization of $GABA_{B2}$ with $GABA_{B1}$ results in an increase in the affinity of the receptor for $GABA_B$ agonists, despite the fact that agonists are thought to bind specifically to $GABA_{B1}$, showing that there is some form of cooperativity in ligand binding between $GABA_{B1}$ and $GABA_{B2}$ (for review, see Bowery and Enna, 2000).

In this study we have generated a number of C-terminal truncations of $GABA_{B1}$ and chimeric subunits between $GABA_{B1}$ and $GABA_{B2}$ and used these molecules to investigate the roles of the two subunits in the intracellular trafficking and downstream signaling of $GABA_B$ receptors.

MATERIALS AND METHODS

Construction of truncated and chimeric $GABA_B$ receptor subunits. A full-length human $GABA_{B1b}$ cDNA was tagged by site-directed mutagenesis (Transformer SDM kit; Clontech, Cambridge, UK) with the *c-myc* epitope (EQKLISEEDL) recognized by the 9E10 mouse monoclonal antibody (Roche Diagnostics). The tag was introduced after amino acid 35 of the nascent protein. $GABA_{B2}$ was hemagglutinin (HA)-tagged (AAAYPYDVPDYA; recognized by 3F10 rat monoclonal antibody; Roche Diagnostics) after amino acid 42 of the nascent protein. Both full-length cDNAs were cloned into pcDNA3.1 (Invitrogen, San Diego, CA). $GABA_{B1b}$ and $GABA_{B2}$ deletion mutants and chimeras were generated by PCR from these full-length, tagged subunit cDNAs. All the PCR primers used had *Hin*dIII restriction enzyme sites engineered into them to facilitate cloning and are described in Table 1. $GABA_{B1b}\Delta806$ and $GABA_{B1b}\Delta771$ were amplified in single PCR reactions and cloned into pcDNA3.1/V5-His. $GABA_{B1b}\Delta747$ and $GABA_{B2}\Delta748$ were generated by PCR and cloned into pCMV-5 (Stratagene, La Jolla, CA). $GABA_{B2/1bC}$ was generated by ligation of $GABA_{B2}\Delta748$ and $GABA_{B1b}(750–844)$ and cloned into pcDNA3.1 (Invitrogen). $GABA_{B1b/2C}$ was generated by ligation of $GABA_{B1}\Delta747$ and $GABA_{B2}(751–941)$ and cloned into pcDNA3.1. The truncated and chimeric receptor subunits are all shown schematically in Figure 1. All experiments were performed using epitope-tagged wild-type, deletion, and chimeric receptor subunits unless otherwise stated.

Culture and transfection of HEK-293 cells. All cell culture reagents were obtained from Life Technologies, Paisley, UK. HEK-293 cells were maintained in DMEM supplemented with 10% fetal calf serum and 1% nonessential amino acids. Exponentially growing cells were transfected using Lipofectamine Plus (Life Technologies) according to the manufacturer's instructions and then incubated for 24 hr to allow for protein expression before analysis.

Antibodies and immunofluorescence. A rabbit antiserum specific for $GABA_{B1b}$ was raised against the peptide CHSPHLPRPHPRVPPHPS (amino acids 31–47) and affinity purified as described previously (Calver et al., 2000). The rabbit antiserum specific for $GABA_{B2}$ was raised against a GST fusion protein, which contained the entire intracellular C terminal of the rat $GABA_{B2}$ protein (amino acids 745–941), and has been described previously (Calver et al., 2000).

For immunocytochemistry, cells on glass coverslips were fixed with 4% paraformaldehyde for 5 min and then either permeabilized with 0.1% Triton X-100 for 10 min or washed with PBS. Cells were incubated in primary antibody (anti c-myc or anti-HA; 1:5000 in PBS; 60 min), washed in PBS, and then incubated in goat anti-mouse IgG-FITC (for anti *c-myc*) or goat anti-rat IgG-FITC (for anti-HA; both obtained from Sigma, Poole, UK, and used at 1:100 in PBS; 45 min). Cells were then washed in PBS, mounted in Citifluor (Citifluor, London, UK), and viewed using a Leica laser-scanning confocal microscope.

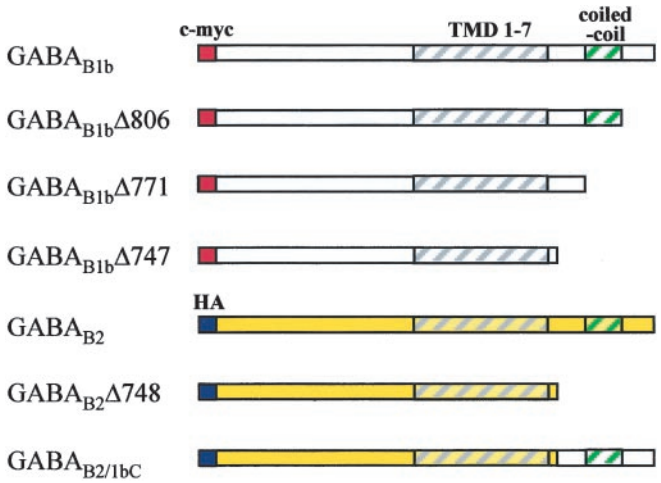

Figure 1. $GABA_B$ receptor subunit truncations and chimeras used in transfection experiments. c-myc and HA are epitope tags for immunocytochemistry and Western blotting. *TMD* 1–7 are the seven transmembrane domains; coiled-coil is the C-terminal domain implicated in the interaction between $GABA_{B1b}$ and $GABA_{B2}$.

Immunoprecipitation and Western blotting. Crude membranes from rat whole brain were prepared as described previously (Benke et al., 1999). Transiently transfected HEK-293 cells were lysed with ice-cold 1% (v/v) Triton X-100 including protease inhibitors (protease inhibitor cocktail tablets; Roche Diagnostics). After centrifugation the lysate supernatants were precleared with normal rabbit serum and then incubated overnight with anti-$GABA_{B1b}$ antibody (5 μg). Antigen–antibody complexes were immunoprecipitated with Protein A–Sepharose, washed extensively in PBS containing Triton X-100, and then resuspended in sample buffer containing 2% (v/v) 2-mercaptoethanol and boiled for 5 min. Eluted proteins were resolved by discontinuous SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a semidry transfer system (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat milk in PBS and 0.05% Tween 20 and then incubated overnight with anti-$GABA_{B2}$ antibody at 0.1 μg/ml in blocking solution. Immunoreactive bands were detected with a goat anti-rabbit antibody conjugated to horseradish peroxidase followed by chemiluminescence detection (ECL; Amersham).

Radioligand binding. Transfected cells were homogenized in ice-cold

50 mM Tris-HCl and 2.5 mM $MgCl_2$ buffer, pH 7.4, using a Kinematic Ultra-Turrax homogenizer. The homogenates were then centrifuged at 35,000 × *g* for 15 min at 4°C. Membrane pellets were resuspended in the buffer and homogenized and centrifuged as before. The final membrane pellet was resuspended in buffer and stored at −80°C until required. Binding assays consisted of 50 μl of displacing compound or buffer, 400 μl of membrane suspension (corresponding to ~30 μg of protein/well), and 50 μl of [^{3}H]CGP-54626 (specific activity, 40 Ci/mmol). In competition binding experiments, 10 concentrations of the competing ligands were tested, at a final [^{3}H]CGP-54626 concentration of 2 nM. Nonspecific binding was defined using 1 mM GABA or 10 μM CGP-62349. The experiments were terminated by rapid filtration over Whatman GF/B glass fiber filters, presoaked with 0.3% (v/v) polyethyleneimine, and washed with 6 ml of ice-cold 50 mM Tris-HCl buffer. Radioactivity was determined by liquid scintillation spectrometry using a Packard 2700 liquid scintillation counter. The concentration of GABA inhibiting specific [^{3}H]CGP-54626 binding by 50% (IC_{50}) was determined by iterative curve fitting using a four-parameter logistic fit (Grafit, Erithacus Software). p*Ki* values (−log of the inhibition constant) were then calculated from the IC_{50} values by the method described by Cheng and Prusoff (1973); the K_D had been determined previously in the present system and was 4.2 ± 0.8 nM (data not shown).

Calcium mobilization assay. Transfected cells were seeded into black-walled 96-well plates (Corning Costar Ltd.) at a density of 30,000 cells/well and incubated at 37°C in 5% CO_2 for 24 hr before use. Cells were loaded with media containing 4 μM Fluo-3 (Molecular Probes, Eugene, OR), a Ca^{2+}-sensitive dye, in the presence of 2.5 mM probenecid and incubated for 60 min at 37°C in 5% CO_2. Cells were then washed four times with 125 μl of modified Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose, 1.2 mM $MgCl_2$, 2.5 mM probenecid, and 0.15 mM $CaCl_2$) and then incubated in 150 μl of the same buffer for 20 min at 37°C in 5% CO_2. Agonist was added, and the resulting intracellular calcium mobilization was recorded using a fluorimetric-imaging plate reader (FLIPR; Molecular Devices, Palo Alto, CA). Peak fluorescence was determined for each agonist addition, and the data were iteratively curve-fitted using a four-parameter logistic model (Bowen and Jerman, 1995). In addition to HEK-293 cells, all of the functional data presented here have been repeated and confirmed in another cell line, Chinese hamster ovary (CHO)-K1 cells (data not shown).

RESULTS

Intracellular retention of $GABA_{B1}$ is mediated by the coiled-coil motif within the C terminal of the protein

Using PCR amplification from a full-length, *myc*-tagged $GABA_{B1}$ cDNA, we constructed a number of truncated coding sequences for this subunit and subcloned them into mammalian expression vectors, as described in Materials and Methods and Table 1. The first of these, $GABA_{B1b}\Delta806$, was a truncation removing the most distal part of the C terminal, up to the end of the coiled-coil domain shown previously to be involved in the interaction with $GABA_{B2}$ (Kammerer et al., 1999; Kuner et al., 1999). The second truncation ($GABA_{B1b}\Delta771$) was similar but removed an additional 35 amino acids covering the $GABA_{B2}$-interacting stretch of the coiled-coil domain. The final truncation of $GABA_{B1b}$ ($GABA_{B1b}\Delta747$) removed the entire intracellular C terminal except the four amino acids downstream of the putative seventh transmembrane domain (Fig. 1). As expected, when transfected into HEK-293 cells in isolation, $GABA_{B1b}$ was not detected by immunocytochemistry on the cell surface but could only be visualized after permeabilization of the cells with detergent (Fig. 2*A,B*) (Couve et al., 1998). Similarly, $GABA_{B1b}\Delta806$ was not expressed on the cell surface but could be detected after membrane disruption with Triton X-100 (Fig. 2*C,D*). In contrast however, $GABA_{B1b}\Delta771$, which lacks the coiled-coil domain shown previously to interact with $GABA_{B2}$, was readily detectable by immunofluorescence on the cell surface of intact transfected cells, as well as after permeabilization (Fig. 2*E,F*). In addition, the truncated $GABA_{B1b}$ subunit lacking the entire C terminal, $GABA_{B1b}\Delta747$, was also expressed on the cell surface at levels

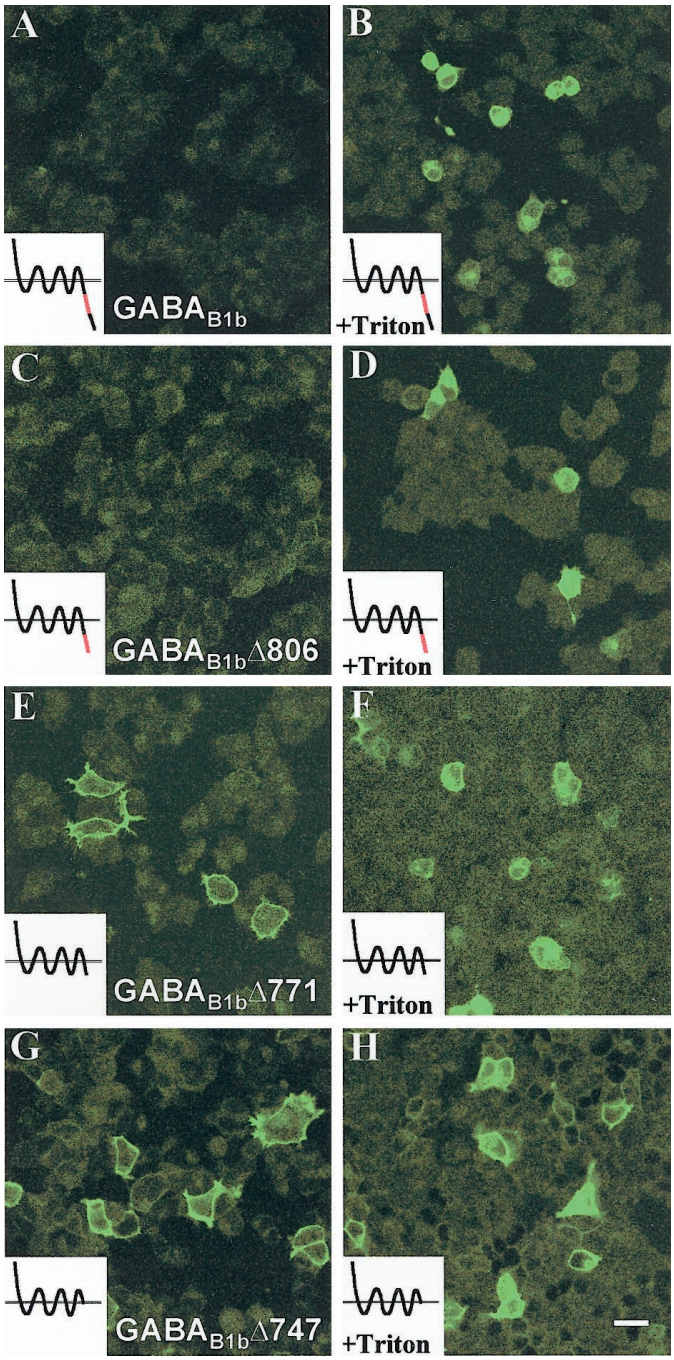

Figure 2. Intracellular retention of $GABA_{B1b}$ is mediated by the coiled-coil motif within the C terminal of the protein. *A–H*, HEK-293 cells were transiently transfected with constructs encoding either the full-length $GABA_{B1b}$ (*A, B*) or progressive deletions of $GABA_{B1b}$ (*C–H*). All of the full-length and truncated subunits were tagged with a c-myc epitope recognized by the 9E10 antibody. Transfected cells were examined after 24 hr by immunofluorescence using the 9E10 antibody either with (*B, D, F, H*) or without (*A, C, E, G*) permeabilization. $GABA_{B1b}$ and $GABA_{B1b}\Delta806$ are both retained intracellularly (*A–D*), whereas $GABA_{B1b}\Delta771$ and $GABA_{B1b}\Delta747$ are both expressed on the cell surface (*E–H*). *Insets*, Diagrams of the truncations are shown, with the coiled-coil region necessary for receptor dimerization indicated in *red*. All of the transfections were performed and analyzed at least three times, and the results with each construct were consistent. Scale bar, 10 μm.

similar to those obtained in cotransfection experiments with $GABA_{B2}$ (Fig. 2*G,H*). Thus the normal intracellular retention of $GABA_{B1b}$ in the absence of $GABA_{B2}$ is mediated via the putative α-helical coiled-coil motif in the same region in which the $GABA_{B1b}$–$GABA_{B2}$ interaction occurs.

The C-terminal domain of $GABA_{B1}$ is sufficient to sequester $GABA_{B2}$ within the cell

We were therefore interested in whether the intracellular C-terminal tail of $GABA_{B1b}$ was able to redirect the normally cell surface-expressed $GABA_{B2}$ subunit to intracellular membranes. To investigate this, we replaced the entire intracellular tail of $GABA_{B2}$ (amino acids 751–941) with the equivalent domain of $GABA_{B1b}$ (amino acids 750–844) to generate the chimeric subunit $GABA_{B2/1bC}$ (Fig. 1). As a control, we also generated a truncated $GABA_{B2}$ subunit that lacked the entire intracellular tail ($GABA_{B2}\Delta748$; Fig. 1). When expressed alone in transient transfections, full-length $GABA_{B2}$ is transported to and detectable by immunocytochemistry both on the cell surface (Fig. 3*A*) and intracellularly (Fig. 3*B*), as has been described previously (Martin et al., 1999). Similarly, our deletion mutant $GABA_{B2}\Delta748$ could be detected readily in transfected cells both with and without detergent permeabilization and with the same cellular distribution as its full-length counterpart (Fig. 3*C,D*). However, when the chimeric receptor subunit $GABA_{B2/1bC}$ was expressed transiently in HEK-293 cells, although intracellular expression was seen after permeabilization, there was no evidence of transport of the subunit to the plasma membrane (Fig. 3*E,F*). When this chimera was coexpressed transiently with wild-type $GABA_{B2}$, however, this intracellular retention was overcome, and the chimeric $GABA_{B2/1bC}$ was transported to the cell surface (Fig. 3*G,H*). The full-length $GABA_{B2}$ used for this cotransfection was not tagged with the HA epitope, so the immunofluorescence seen using the anti-HA antibody 3F10 on unpermeabilized cells was specific to the HA-tagged $GABA_{B2/1bC}$.

C-terminally truncated $GABA_{B1b}$ subunits are able to bind GABA

We investigated the effect of C-terminal truncation and subsequent cell surface localization of the $GABA_{B1b}\Delta747$ subunit on the ability of the subunit to bind GABA. Removal of the intracellular C terminal of $GABA_{B1b}$ had no effect on the ability of GABA to displace the antagonist CGP-54626 in competition binding assays. Furthermore, the potency of GABA at this truncated receptor subunit was not significantly different from its potency at the full-length $GABA_{B1b}$ subunit (Fig. 4) ($p < 0.001$, one-way ANOVA with *post hoc t* test). As has been reported previously (for review, see Bowery and Enna, 2000), coexpression of $GABA_{B1b}$ with $GABA_{B2}$ resulted in a significant increase in the potency of GABA binding compared with GABA binding at $GABA_{B1b}$ alone, and such a shift in potency was also observed when the truncated $GABA_{B1b}$ subunit was coexpressed with $GABA_{B2}$ (Fig. 4) ($p < 0.001$, one-way ANOVA with *post hoc t* test).

C-terminally truncated $GABA_{B1b}$ subunits are nonfunctional when expressed alone but couple to G-proteins when coexpressed with $GABA_{B2}$

To determine whether cell surface expression of the $GABA_{B1b}$ subunit was sufficient to form a functional $GABA_B$ receptor, we tested the ability of C-terminally truncated $GABA_{B1b}$ to activate

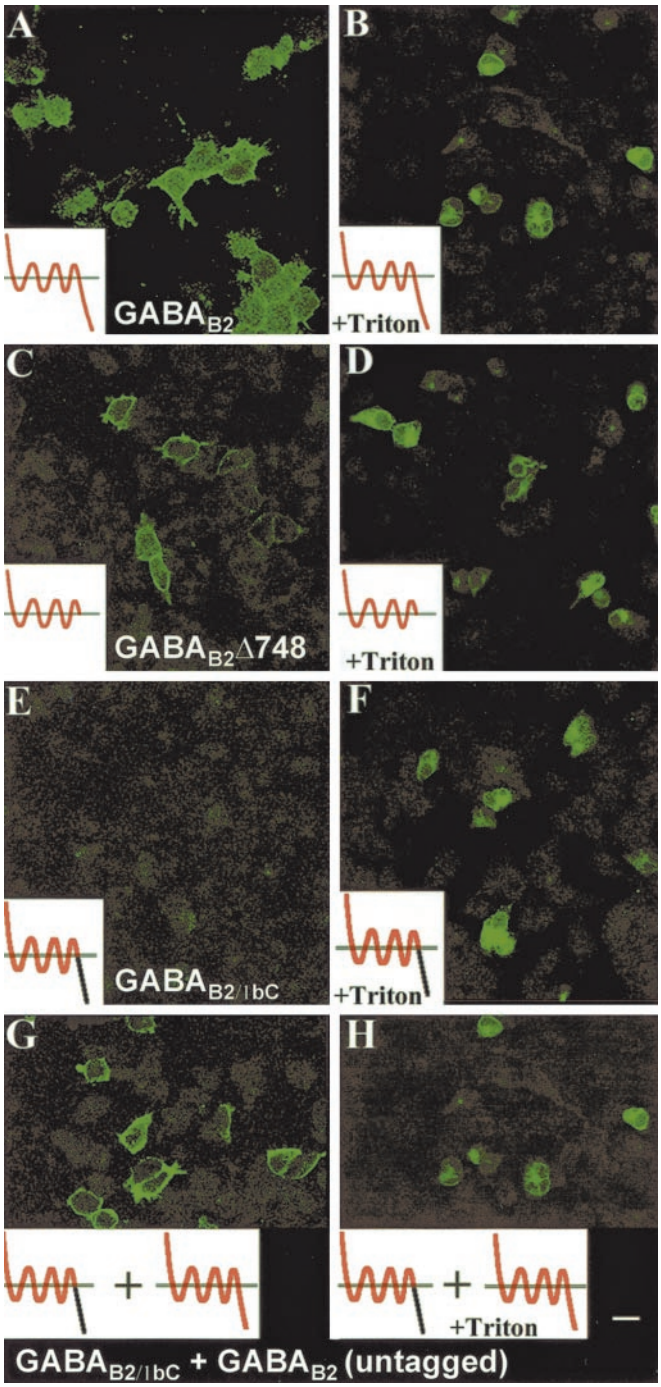

Figure 3. The C-terminal domain of $GABA_{B1b}$ is sufficient to sequester $GABA_{B2}$ within the cell. *A–H*, HEK-293 cells were transfected with constructs encoding either the full-length $GABA_{B2}$ (*A, B*), $GABA_{B2}\Delta748$ (*C, D*), $GABA_{B2/1bC}$ (*E, F*), or $GABA_{B2/1bC}$ + $GABA_{B2}$ (*G, H*). All of the full-length and truncated subunits were tagged with an HA epitope recognized by the 3F10 antibody, except for the full-length $GABA_{B2}$ used in the cotransfection (*G, H*) that was not epitope-tagged. Transfected cells were examined after 24 hr by immunofluorescence using the 3F10 antibody either with (*B, D, F, H*) or without (*A, C, E, G*) permeabilization. Both full-length $GABA_{B2}$ and $GABA_{B2}\Delta748$ when transfected alone are expressed on the cell surface (*A–D*), whereas $GABA_{B2/1bC}$ is retained within the cell (*E, F*). The HA-tagged $GABA_{B2/1bC}$ is able to reach the cell surface and be detected by anti-HA, however, when coexpressed with the untagged $GABA_{B2}$ (*G, H*). *Insets*, Diagrams of the truncations and chimeras are shown; $GABA_{B2}$ sequences are shown in *red*, whereas $GABA_{B1b}$ sequences are shown in *black*. All of the transfections were performed and analyzed at least three times, and the results with each construct were consistent. Scale bar, 10 μm.

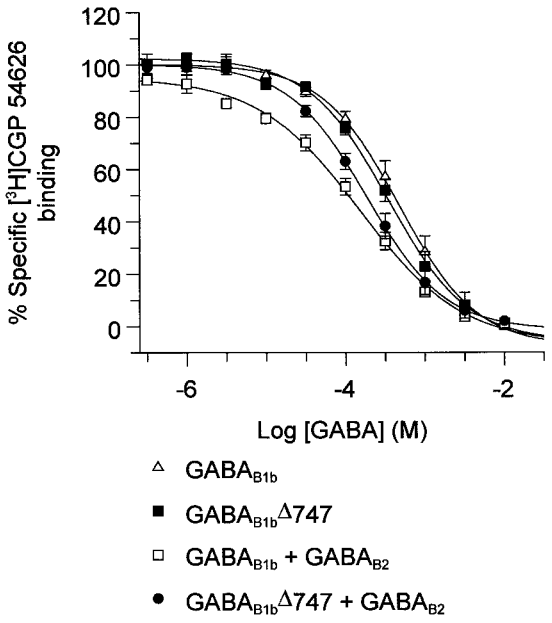

Figure 4. Removal of the C terminal of $GABA_{B1b}$ does not affect ligand binding. Cells transiently transfected with either $GABA_{B1b}$ alone, $GABA_{B1b}$ + $GABA_{B2}$, $GABA_{B1b}\Delta747$ alone, or $GABA_{B1b}\Delta747$ + $GABA_{B2}$ specifically bound [^{3}H]CGP-54626, and this could be completely displaced by GABA (10 mM), with p*Ki* values of 3.60 ± 0.13, 4.14 ± 0.05, 3.70 ± 0.10, and 4.00 ± 0.09, respectively. Data are expressed as means ± SEM (n = 4–6). All receptor subunits were epitope-tagged as described in Materials and Methods.

G-proteins in response to GABA. Although $GABA_B$ receptors are known to inhibit adenylate cyclase via their interaction with G_i, we cotransfected the chimeric G-protein G_{qi5} (Conklin et al., 1993) in transient transfections so that stimulation of a $GABA_B$ receptor would activate the phospholipase C pathway, resulting in mobilization of intracellular calcium, which could then be measured on a FLIPR. Coexpression of $GABA_{B1b}$ and $GABA_{B2}$ together with Gqi5 in HEK-293 cells resulted in robust calcium mobilization in response to agonist, whereas coexpression of $GABA_{B1b}$ and Gqi5 gave no response in this functional assay, even at a GABA concentration of 0.1 mM (Fig. 5*A*). When we tested the cell surface-expressed C-terminally truncated $GABA_{B1b}\Delta747$ with Gqi5 in this system, it also exhibited no functional coupling to the G-protein when expressed on its own (Fig. 5*A*).

We next tested the effect of coexpression of the $GABA_{B2}$ subunit with the C-terminally truncated $GABA_{B1b}$ on the coupling of the receptor to the chimeric G-protein Gqi5. Both the full-length $GABA_{B1b}$ subunit and the C-terminally truncated $GABA_{B1b}\Delta747$, when coexpressed with $GABA_{B2}$, were capable of activating Gqi5 and activating the downstream phospholipase C pathway (Fig. 5*B*). The functional response of the truncated $GABA_{B1b}$ with $GABA_{B2}$, as measured by the EC_{50} in response to GABA, was not significantly different from that of the wild-type $GABA_{B1b}$ subunit expressed with $GABA_{B2}$ [pEC_{50} ($GABA_{B1b}$ + $GABA_{B2}$) = 7.08 ± 0.02; pEC_{50} ($GABA_{B1b}\Delta747$ + $GABA_{B2}$) = 6.93 ± 0.05; n = 4–6]. In addition to HEK-293 cells, all of the functional experiments presented here have also been performed in another cell line, CHO-K1 cells, with qualitatively identical results.

Functional coupling of the $GABA_B$ receptor to G-proteins requires neither the C-terminal of $GABA_{B1b}$ nor the C-terminal of $GABA_{B2}$

Because we had shown that the C-terminal domain of $GABA_{B1b}$ was not necessary for $GABA_B$ receptor heterodimers to couple functionally to Gqi5, we investigated the importance of the C-terminal of $GABA_{B2}$ with respect to the correct functionality of the receptor. We again performed the calcium mobilization assay experiments on the FLIPR, using cells transiently transfected with the C-terminally truncated $GABA_{B1b}\Delta747$, a C-terminally truncated $GABA_{B2}$ subunit ($GABA_{B2}\Delta748$), and Gqi5. This also resulted in the expression of a receptor complex capable of coupling to Gqi5 (Fig. 5*C*). The EC_{50} of this response was significantly lower than that for the wild-type receptor ($p < 0.001$, F test), although it is unclear whether this reflects genuine differences in the ability of the mutant receptor subunits to couple to G-proteins in a nontransient system [pEC_{50} ($GABA_{B1b}\Delta747$ + $GABA_{B2}\Delta748$) = 6.34 ± 0.01].

The C-terminal interaction between $GABA_{B1}$ and $GABA_{B2}$ is not necessary for the formation of heterodimers

It has been well documented that $GABA_{B1b}$ and $GABA_{B2}$ form heterodimers, both in transfected cells and in native tissues, and that the only reported region of dimerization is between the α-helical coiled-coil motifs present in the C terminals of the two subunits (Kammerer et al., 1999; Kuner et al., 1999). However we have demonstrated here that this interaction is not necessary for a functional response of $GABA_B$ receptor heterodimers. We performed immunoprecipitation experiments to investigate further whether this coiled-coil interaction was indeed necessary for heterodimerization of $GABA_{B1b}$ and $GABA_{B2}$ subunits. We raised antibodies in rabbits against an N-terminal peptide of $GABA_{B1b}$ (see Materials and Methods) that recognized single bands on Western blots both with cells transfected with $GABA_{B1b}$ and with brain membranes (Fig. 6*A*). We did consistently observe a small difference in size between the human tagged recombinant $GABA_{B1b}$ and the rat brain-derived $GABA_{B1b}$, but we would suggest that this may reflect differences in expression and post-translational modification between the recombinant human and endogenous rat receptor subunits. Both these bands could be specifically blocked by preincubation of the antiserum with the immunizing peptide (data not shown). After immunoprecipitation with anti-$GABA_{B1b}$ and immunodetection on Western blots with anti-$GABA_{B2}$ (Calver et al., 2000), heterodimers could be readily detected in membrane preparations from cells transfected with both full-length subunits together but not in cells transfected with either $GABA_{B1b}\Delta748$ or $GABA_{B2}$ alone (Fig. 6*B*). However, when $GABA_{B2}$ was transiently coexpressed with $GABA_{B1b}$ lacking a C terminal, a band corresponding to $GABA_{B2}$ was detected after immunoprecipitation with anti-$GABA_{B1b}$ and immunoblotting with anti-$GABA_{B2}$ antibodies (Fig. 6*B*). Although this band is less intense than that observed after coexpression of the two full-length subunits, it nevertheless indicates that $GABA_{B1b}$ and $GABA_{B2}$ are capable of forming heterodimers in the absence of the C-terminal coiled-coil interaction. Other bands were also observed in all lanes above and below those corresponding to $GABA_{B2}$, but these were also observed in untransfected cells (Fig. 6*B*, *lane 1*) and thus represent nonspecific bands unrelated to the transfected $GABA_B$ cDNAs.

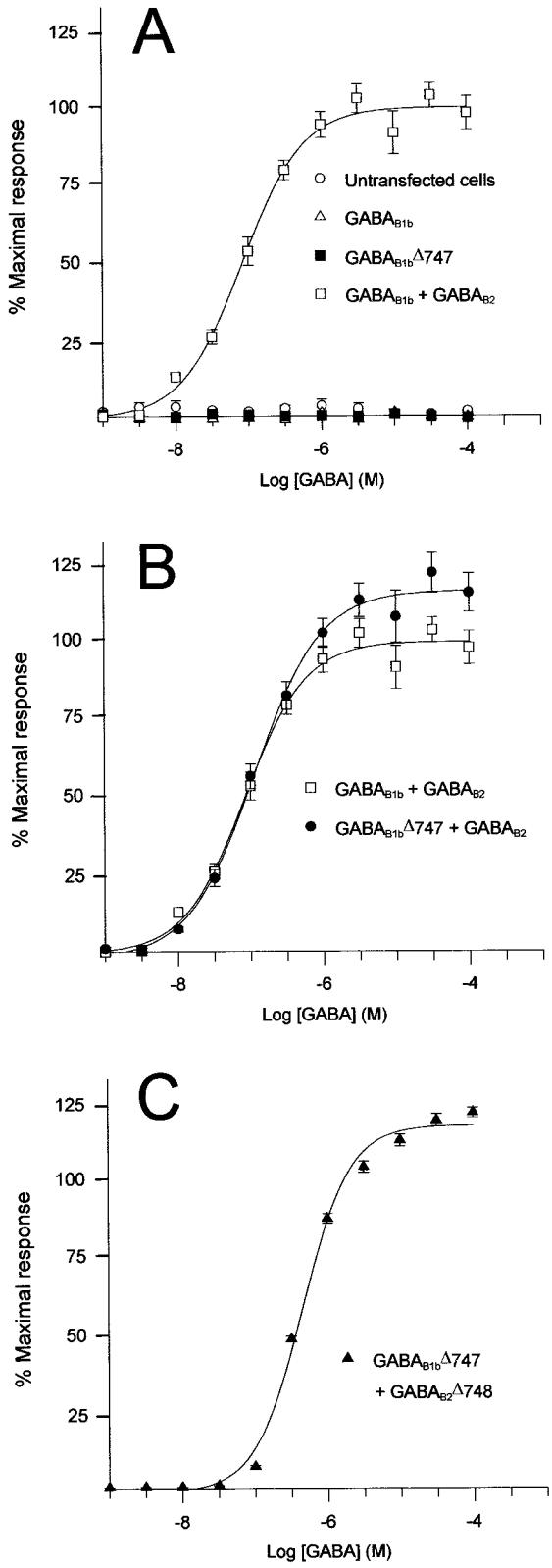

Figure 5. $GABA_{B1b}$ subunits lacking the intracellular C terminal are nonfunctional, whereas heterodimers formed between the C-terminally truncated $GABA_{B1}$ and either full-length or C-terminally truncated $GABA_{B2}$ signal via G-proteins. HEK-293 cells were cotransfected with the expression constructs shown together with the chimeric G-protein Gqi5 and assayed for intracellular Ca^{2+} mobilization in response to GABA stimulation in a FLIPR. *A*, No response was observed from mock-transfected cells or from cells transfected with $GABA_{B1b}$ or $GABA_{B1b}\Delta747$ on their own, whereas a robust functional response was seen when $GABA_{B1b}$ was cotransfected with $GABA_{B2}$ (pEC_{50} = 7.08 ± 0.02). *B*, A similar response was seen when $GABA_{B2}$ was cotransfected with $GABA_{B1b}\Delta747$ or when $GABA_{B2}$ was cotransfected with $GABA_{B1b}$ [pEC_{50} ($GABA_{B1b}$ + $GABA_{B2}$) = 7.08 ± 0.02; pEC_{50} ($GABA_{B1b}\Delta747$ + $GABA_{B2}$) = 6.93 ± 0.05]. *C*, A functional $GABA_B$ receptor was also detected in FLIPR when $GABA_{B1b}\Delta747$ was cotransfected with $GABA_{B2}\Delta748$ and Gqi5 (pEC_{50} = 6.34 ± 0.01). The data in *A–C* are taken from a single representative experiment. All receptor subunits were epitope-tagged as described in Materials and Methods.

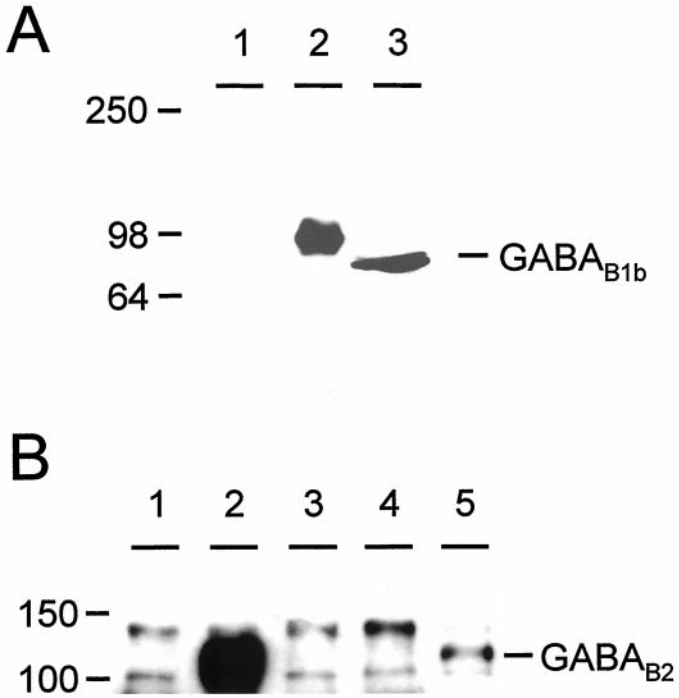

Figure 6. Heterodimers between $GABA_{B1b}$ and $GABA_{B2}$ form in the absence of the C-terminal coiled-coil interaction. *A*, Western blot to show specificity of anti-$GABA_{B1b}$ antibody. *Single bands* are observed in *lanes* containing either rat brain membranes (*lane 3*) or cells transfected with $GABA_{B1b}$ (*lane 2*); no bands are observed in untransfected cells (*lane 1*). *B*, Immunoprecipitation from transfected cell membranes with anti-$GABA_{B1b}$ antibody followed by Western blotting with anti-$GABA_{B2}$. HEK-293 cell membranes were prepared from mock-transfected cells (*lane 1*) and from cells transiently transfected with $GABA_{B1b}$ + $GABA_{B2}$ (*lane 2*), $GABA_{B1b}\Delta748$ (*lane 3*), $GABA_{B2}$ (*lane 4*), or $GABA_{B1b}\Delta748$ + $GABA_{B2}$ (*lane 5*). This experiment clearly demonstrates an interaction between $GABA_{B1b}\Delta748$ and $GABA_{B2}$ (*lane 5*), in addition to the strong interaction between $GABA_{B1b}$ and $GABA_{B2}$ (*lane 2*). *Numbers* on the *left* indicate the position of molecular weight markers, expressed in kilodaltons.

DISCUSSION

We have shown in this study that the C-terminal intracellular domain of the $GABA_{B1b}$ receptor subunit is responsible for the retention of the subunit within the cell when expressed in the absence of $GABA_{B2}$. When this domain is removed from the $GABA_{B1b}$ subunit, the truncated $GABA_{B1b}$ is trafficked to the cell surface independently of $GABA_{B2}$. We have also shown that the C-terminal stretch of 35 amino acids responsible for intracellular retention is the same domain that mediates the interaction etween $GABA_{B1b}$ and $GABA_{B2}$ and is situated within the putative "coiled-coil" domain between amino acids 771 and 806 of $GABA_{B1b}$. In addition, the C-terminal domain of $GABA_{B1b}$ is sufficient to cause the normally cell surface-

expressed $GABA_{B2}$ to be retained within the cell, when it is exchanged for the equivalent region of the native $GABA_{B2}$ (chimera $GABA_{B2/1bC}$). There are a number of other examples in which the C terminal of a receptor or ion channel is responsible for its intracellular retention in the absence of accessory molecules (McIlhinney et al., 1998; Zerangue et al., 1999). For example, the ionotropic NMDA receptor subunit NR1 is retained within cells in the absence of the NR2 subunit (McIlhinney et al., 1998). This intracellular retention appears to be mediated by the C terminal of the NR1 protein, because splice variants in this region are able to reach the cell surface in the absence of NR2 (Okabe et al., 1999). In addition, GPCRs for which the C terminal appears to be important for targeting to the plasma membrane include the metabotropic glutamate receptors (Stowell and Craig, 1999; Chan et al., 2000) and the serotonin receptor 5-HT_{1B} (Jolimay et al., 2000).

The intracellular retention of the chimeric $GABA_{B2/1bC}$ can be overcome by the coexpression of $GABA_{B2/1bC}$ with the full-length $GABA_{B2}$, presumably as a result of the interaction between the two C terminals. These data suggest that the coiled-coil interaction between $GABA_{B2}$ and $GABA_{B1b}$ competes with a similar coiled-coil interaction between $GABA_{B1b}$ and another unknown, presumably ER-associated protein, which in the absence of $GABA_{B2}$ mediates the retention of the $GABA_{B1b}$ subunit within the ER. This would not be surprising because coiled-coil motifs are secondary structures present in a large number of proteins and have been implicated in the formation of several multimeric protein complexes (Lupas, 1996).

It is known that expression of $GABA_{B1b}$ alone results in the formation of nonfunctional $GABA_B$ receptors, at least in part because of the fact that when expressed alone it is not present on the cell surface (Couve et al., 1998). However, cell surface expression of $GABA_{B1}$ alone is not sufficient to form a functional $GABA_B$ receptor, as demonstrated by a recent study in which coexpression of metabotropic glutamate receptor 4 (mGluR4) in *Xenopus* oocytes resulted in cell surface expression of $GABA_{B1}$, although not as a $GABA_{B1}$–mGluR4 heterodimer (Sullivan et al., 2000). In this system the $GABA_{B1}$ subunit was unable to couple to members of the Kir3.0 family of potassium channels or to couple negatively to adenylate cyclase. The results we present here support these findings, because the C-terminally truncated cell surface-expressed $GABA_{B1b}$ subunit, when expressed alone, binds GABA but is unable to couple functionally to the chimeric G-protein Gqi5. It is only by coexpressing the truncated $GABA_{B1b}$ with $GABA_{B2}$ that we were able to reconstitute a functional G-protein-coupled receptor. Interestingly we also detected a functional receptor when we coexpressed the C-terminally truncated $GABA_{B1b}$ with C-terminally truncated $GABA_{B2}$. This suggests that neither of the intracellular C terminals of $GABA_{B1b}$ or $GABA_{B2}$ is necessary for the coupling of the $GABA_B$ receptor to its second messenger system, although we cannot exclude the possibility that the functionality of the receptor is altered in a more subtle manner by such deletions. This is consistent with the findings of Gomeza et al. (1996), who demonstrated that although all of the intracellular domains of mGluR1 play a role in G-protein coupling, none of them apart from intracellular loop two is absolutely required for the activation and downstream signaling of this receptor.

The data presented here also suggest that it may be the $GABA_{B2}$ subunit that binds to G-proteins and subsequently initiates the downstream signaling cascades. Alternatively, it may be that the intracellular loops of $GABA_{B1b}$ are in fact the important regions for G-protein coupling and that the presence of $GABA_{B2}$ is required for $GABA_{B1b}$ to assume the correct tertiary structure to mediate this interaction. However, when one compares the sequences of the intracellular loops of $GABA_{B2}$ with those of $GABA_{B1}$ and the other seven transmembrane receptors that are known to bind and couple to G-proteins, it is not unreasonable to suggest that the amino acids present in the $GABA_{B2}$ subunit intracellular loops render it a more attractive candidate for G-protein coupling than do the corresponding residues present in $GABA_{B1}$.

As well as trafficking and G-protein coupling, our data have novel implications for the heterodimerization of $GABA_B$ receptors, which until now has been thought to be solely mediated by the C-terminal coiled-coil interaction between $GABA_{B1b}$ and $GABA_{B2}$. In this study, we have shown that when the intracellular tail of $GABA_{B1b}$ is removed, the resulting truncated subunit is still capable of forming functional heterodimers with $GABA_{B2}$, although the efficiency of the heterodimerization is reduced. This demonstrates that other sequences exist within the two $GABA_B$ subunits that must be capable of interacting with each other. It is unclear from the data presented here whether this interaction occurs within the extracellular N terminals of the subunits or alternatively within the transmembrane domains, but further truncation and chimera experiments are in progress to identify such interactions. Other GPCRs have been shown to heterodimerize, such as the κ and δ opioid receptors (Jordan and Devi, 1999) and the somatostatin (SST5) and dopamine (D_2) receptors (Rocheville et al., 2000), in the absence of C-terminal coiled-coil domains. Such receptors must therefore use other protein–protein interactions to form dimers. Interestingly, the interaction between SST5 and D_2 was demonstrated recently by the functional rescue of an inactive C-terminally truncated SST5 receptor by a full-length D_2 receptor, demonstrating that a C-terminal interaction between these two receptors is not necessary for the formation of heterodimers (Rocheville et al., 2000).

In summary, our data support a model in which the $GABA_{B1b}$ subunit, when expressed alone, is retained within the cell by protein–protein interactions between its C-terminal coiled-coil domain and, presumably, components of the ER. In the presence of $GABA_{B2}$, the equivalent coiled-coil domain in the C terminal of $GABA_{B2}$ competes for these interactions, and thus the intracellular retention of $GABA_{B1b}$ is overcome, and the subunit is able to reach the cell surface. In addition, the elements of the $GABA_B$ receptor responsible for G-protein coupling are probably not present within the C terminal of either the $GABA_{B1b}$ or the $GABA_{B2}$ subunit and may indeed lie within the intracellular domains of $GABA_{B2}$.

Notes added in proof. Since the submission of this manuscript, two papers have appeared in press that confirm a number of our observations. First, Margeta-Mitrovic et al. (2000) have reported the presence of an intracellular retention motif in the C terminal of $GABA_{B1}$, although, in contrast to the data shown in our paper, they also report that the C-terminal coiled-coil interaction between $GABA_{B1}$ and $GABA_{B2}$ is necessary to form a functional $GABA_B$ receptor. Second, Schwartz et al. (2000) have identified a splice variant of $GABA_{B1}$, termed $GABA_{B1e}$, consisting of just the extracellular N terminal of $GABA_{B1}$. This subunit is capable of forming (nonfunctional) heterodimers with $GABA_{B2}$, confirming that the coiled-coil interaction is not necessary for the heterodimerization of $GABA_B$ receptors. The data presented here both confirm and extend these observations.

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