C-Terminal Interaction Is Essential for Surface Trafficking But Not for Heteromeric Assembly of GABA_B Receptors

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Assembly of fully functional GABA_B receptors requires heteromerization of the GABA_{B(1)} and GABA_{B(2)} subunits. It is thought that GABA_{B(1)} and GABA_{B(2)} undergo coiled-coil dimerization in their cytoplasmic C termini and that assembly is necessary to overcome GABA_{B(1)} retention in the endoplasmic reticulum (ER). We investigated the mechanism underlying GABA_{B(1)} trafficking to the cell surface. We identified a signal, RSRR, proximal to the coiled-coil domain of GABA_{B(1)} that when deleted or mutagenized allows for surface delivery in the absence of GABA_{B(2)}. A similar motif, RXR, was recently shown to function as an ER retention/retrieval (ERR/R) signal in K_{ATP} channels, demonstrating that G-protein-coupled receptors (GPCRs) and ion channels use common mechanisms to control surface trafficking. A C-terminal fragment of GABA_{B(2)} is able to mask the RSRR signal and to direct the GABA_{B(1)} monomer to the cell

surface, where it is functionally inert. This indicates that in the heteromer, GABA_{B(2)} participates in coupling to the G-protein. Mutagenesis of the C-terminal coiled-coil domains in GABA_{B(1)} and GABA_{B(2)} supports the possibility that their interaction is involved in shielding the ERR/R signal. However, assembly of heteromeric GABA_B receptors is possible in the absence of the C-terminal domains, indicating that coiled-coil interaction is not necessary for function. Rather than guaranteeing heterodimerization, as previously assumed, the coiled-coil structure appears to be important for export of the receptor complex from the secretory apparatus.

Key words: GABA-B receptor; G-protein; metabotropic; heterodimer; endoplasmic reticulum; coiled-coil α -helices; leucine zippers

GABA_B receptors are G-protein-coupled receptors (GPCRs) that, by activating second messenger cascades and modulating ion channel activity, control neurotransmitter release and postsynaptic silencing of excitatory neurotransmission (Marshall et al., 1999). They have been implicated in hippocampal long-term potentiation, short-wave sleep, muscle relaxation, and antinociception (Kim et al., 1997; Bettler et al., 1998). GABA_B receptors belong to the family 3 GPCRs and as such share homology with metabotropic glutamate (mGlu), Ca²⁺-sensing, pheromone, and taste receptors. Three prominent GABA_B proteins, GABA_{B(1a)}, GABA_{B(1b)}, and GABA_{B(2)} have been identified. GABA_{B(1a)} and GABA_{B(1b)} derive from the same gene by N-terminal alternative splicing. GABA_B receptors are expressed on the cell surface as a heteromeric complex of GABA_{B(1a,2)} or GABA_{B(1b,2)}. Available ligands, such as the photoaffinity antagonist [¹²⁵I]CGP71872, bind to the N-terminal extracellular region that is conserved

between the GABA_{B(1a)} and GABA_{B(1b)} subunits (Galvez et al., 1999; Malitschek et al., 1999).

Although GPCRs were believed to be monomeric entities, a growing body of evidence indicates that they can form dimers (Salahpour et al., 2000). The implications of GPCR dimerization have not been firmly established. GABA_B receptors are the first GPCRs where not only assembly into heteromeric complexes was demonstrated, but where dimerization also had clear functional consequences (Marshall et al., 1999). Unlike other dimeric GPCRs, where the individual subunits can form receptors in their own right, GABA_{B(1)} and GABA_{B(2)} are functionally impaired when expressed alone in heterologous cells. Experiments with transfected sympathetic neurons further support the possibility that heteromeric assemblies of GABA_{B(1,2)} are essential to mediate functional responses (Filippov et al., 2000). Significantly the GABA_{B(1)} and GABA_{B(2)} subunits colocalize at extrasynaptic sites and can be copurified from neuronal membranes (Kaupmann et al., 1998a; Benke et al., 1999). Each GABA_B receptor subunit contains a 30 amino acid leucine zipper motif within its intracellular C-terminal domain (White et al., 1998). Leucine zipper peptides of GABA_{B(1)} (LZ1) and GABA_{B(2)} (LZ2) form a parallel heterodimeric coiled coil but do not undergo significant homodimerization (Kammerer et al., 1999). GABA_{B(1)} is retained in the ER when expressed by itself (Couve et al., 1998). The GABA_{B(2)} subunit facilitates the intracellular trafficking of GABA_{B(1)} from the ER toward the plasma membrane and pro-

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motes the high-affinity agonist conformation of the receptor (Marshall et al., 1999). Despite the identification of heterodimerization as a requirement for function, the mechanism whereby GABA_{B(2)} induces an increase in cell surface expression of GABA_{B(1)} remained enigmatic.

Here we used mutagenesis, functional expression, surface photoaffinity labeling, and immunocytochemistry to explore the molecular interactions that underlie intracellular retention and heteromer formation of GABA_B receptors. We demonstrate that GABA_{B(1)} carries an ERR/R signal located four residues C-terminal of LZ1. This signal restricts plasma membrane incorporation of the GABA_{B(1)} subunit in human embryonic kidney 293 (HEK293) cells and neurons. Exposure of the ERR/R signal is masked by C-terminal interactions between GABA_{B(1)} and GABA_{B(2)}, whereas other family 3 GPCRs are ineffective in shielding. In contrast to the prevailing model, it appears that C-terminal interaction between GABA_{B(1)} and GABA_{B(2)} is not essential for dimerizing the receptor complex per se but rather controls surface trafficking.

MATERIALS AND METHODS

Generation of mutant expression plasmids. All constructs were subcloned into the cytomegalovirus-based eukaryotic expression vector pCI (Promega, Madison, WI). To allow detection of transiently expressed receptors, the N-terminal 16 residues of rat GABA_{B(1a)} (Kaupmann et al., 1997) were replaced by 36 residues encoding the mGlu5 signal peptide, M¹VLLILSVLLLKEDVRGSAQS²², followed by the c-myc epitope, TRE²⁵QKLISEEDL³⁴TR. The GABA_{B(1)} signal peptide was replaced by the mGlu5 signal peptide because the latter is known to accurately release the N-terminal hemagglutinin (HA) epitope tag (Ango et al., 1999). Likewise the N-terminal 41 residues of rat GABA_{B(2)} (Kaupmann et al., 1998a) were replaced by the mGlu5 signal peptide followed by the HA epitope, TRY²⁵PDVDPDYA³³TR. The presence of the c-myc and HA epitope was found to affect neither the pharmacology nor the expression level of recombinant receptors. A cassette strategy was used to engineer all mutations and truncations. Artificial *XhoI* and *EcoRV* sites were introduced by silent site-directed mutagenesis of G²⁶⁹¹C and A²⁶⁹⁴G in GABA_{B(1a)} and C²⁵⁰⁸T in GABA_{B(2)}, respectively. Chimera R1CR2 was made by overlap extension PCR. GABA_{B(1a)} was amplified using a forward primer (bp 1941–1958) and a chimeric reverse primer containing GABA_{B(1a)} (bp 2539–2559) and GABA_{B(2)} (bp 2224–2244) sequences. The GABA_{B(2)} C-terminal tail was amplified by using chimeric primer complementary to the one described above and a reverse primer (bp 2803–2823) with an in-frame 4xHis tag and a stop codon followed by an *XhoI* site. The two PCR products were joined by PCR and subcloned into the *Clal* (bp 2245)/*XhoI* sites of GABA_{B(1a)}. Chimera R2CR1 was made as described above, with primers containing both GABA_{B(2)} (bp 2194–2223) and GABA_{B(1a)} sequences (bp 2560–2588). The GABA_{B(1a)} C-terminal tail was amplified using reverse primer (bp 2863–2880) with an in-frame 4xHis tag and a stop codon followed by an *NotI* site. The 4xHis tags were added to monitor expression of the R2T749_{his}, R1CR2_{his}, and R2CR1_{his} constructs. We alternatively used the HA tag at the N terminus of R2T749. R2T749_{his} and R2T749_{ha} show no overt differences in expression levels, as well as in pharmacological and functional properties. The final PCR product was subcloned into the *RsrII* (bp1661)/*NotI* sites of GABA_{B(2)}. The GABA_{B(1a)} C-terminal truncations R1I860, R1T872, and R1K886 were constructed by ligating complementary oligonucleotides with appropriate overhangs into *BclI*²⁵⁷⁶/*EcoRI* (3' cloning site). Using *XhoI*/*EcoRI* sites we made R1L915, R1L921, and R1P928. By ligating complementary oligonucleotides into *EheI*²⁷⁷¹/*EcoRI* sites we made R1G940 and R1G952. GABA_{B(1a)} alanine scanning from residue R⁹²² to P⁹²⁸ and mutants with S⁹²⁵→D, RR^{922/924}→AA, and RRR^{922/924/925}→AAA changes were constructed by site-directed mutagenesis and subcloned into *XhoI*/*EcoRI*. The GABA_{B(1a)} LZ1 (bp 2650–2751) (Kammerer et al., 1999) mutant R1[PPP] (LIV^{897/901/908}→PPP) was made using the primer 5'-GAA AAC CGA GAA CCC GAG AAG ATC CCC GCT GAG AAA GAG GAG CGC CCC TCT GAA CTG CG-3'. The LZ1 deletion mutant R1ΔLZ was generated from wild-type (WT) GABA_{B(1a)} using complementary 44mer oligonucleotides flanking LZ1 (bp 2626–2649 and 2752–2772). We used overlap extension PCR to construct

R1LZ2 by amplifying LZ2 (bp 2354–2454) (Kammerer et al., 1999) with chimeric primers complementary to WT GABA_{B(1a)} and GABA_{B(2)} (bp 2353–2373, 2431–2454). The amplified LZ2 domain was then inserted into R1ΔLZ. The GABA_{B(2)} C-terminal truncation R2T749 was made by ligating complementary oligonucleotides with appropriate overhangs in *MluI* (bp 2136)/*XbaI* (3' cloning site); truncations R2L791, R2E811, and R2P820 were constructed by taking advantage of *PshAI* (bp 2368)/*XbaI* sites. CR2 (R⁷¹⁴-L⁹⁴¹) and CR2P820 (R⁷¹⁴-P⁸²⁰) were made by removing the N-terminal and transmembrane domain (TMD)1–6 domains from the WT and R2P820 GABA_{B(2)} constructs by *MluI* digestion and religation, thereby preserving the previously inserted HA tag. R2[PPP] with mutation of LIL^{798/802/805}→PPP was made using primer 5'-GAA AAC CAC CGC CCG CGG ATG AAG CCC ACA GAG CCG GAC AAA GAC TTG-3'. The LZ2 deletion in R2ΔLZ was made using complementary 48mer oligonucleotides flanking LZ2 (bp 2311–2334 and 2437–2460). We made chimera R2LZ1 by inserting LZ1 into WT GABA_{B(2)}. PCR using a forward primer duplicating sequences complementary to GABA_{B(2)} (bp 2311–2334) and GABA_{B(1a)} (bp 2650–2672) amplified LZ1; reverse primer corresponded to chimeric GABA_{B(1a)} (bp 2729–2751) and GABA_{B(2)} sequences (bp 2437–2460). All constructs were verified by sequencing.

Cell culture and radioligand binding assays. Mammalian cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Earle's MEM (Life Technologies, Basel, Switzerland) supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells were harvested for membrane preparation 2 d after transfection. Preparation of cell lysates and [¹²⁵I]CGP64213 binding assays was performed as described (Kaupmann et al., 1997). [¹²⁵I]CGP64213 and [¹²⁵I]CGP71872 were radiolabeled (ANAWA, Wangen, Switzerland) to a specific activity of 2000 Ci/mmol. All ligands were synthesized in house.

Immunoblot. Cellular membranes were solubilized in Laemmli sample buffer (125 mM Tris, pH 6.8, 1% SDS, 25 mM DTT, 5% glycerol/bromophenol blue). Proteins (10–20 μg per lane) were separated by SDS-PAGE using 4–15% gradient gels and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore AG, Volketswil, Switzerland) by standard wet electrophoretic transfer. Blocking was overnight in NET-G buffer (150 mM NaCl, 50 mM EDTA, 500 mM Tris HCl, pH 7.4, 0.5% v/v Triton X-100, 2.5% w/v gelatin) at 4°C. Incubation with primary and secondary antibodies was for 45 min at room temperature. Polyclonal rabbit antisera Ab174.1, Ab176a, and C22 were as described (Malitschek et al., 1998; Kaupmann et al., 1998a,b). Polyclonal antisera N22 was derived against the GABA_{B(2)} N-terminal peptide A¹⁶⁴-L²¹⁵ and produced as described (Malitschek et al., 1998). Antibodies were diluted in NET-G buffer as follows: Ab174.1 (1:2000), Ab176a (1:2000), N22 (1:2000), C22 (1:3000), and goat anti-rabbit IgG H+L POD (1:3000; Bio-Rad, Glattpburg, Switzerland). Detection was with enhanced chemiluminescent Western Blot reagents (Amersham, Durbendorf, Switzerland) and exposure to Biomax maximum resolution x-ray films (Kodak, Cambridge, UK).

Surface labeling experiments. Given that [¹²⁵I]CGP71872 cannot permeate the plasma membrane, photoaffinity labeling of intact cells reveals receptor/ligand interactions that occur at the extracellular binding site (Malitschek et al., 1999). HEK293 cells were transfected to six-well plates 6 hr after transfection. After an additional 36 hr incubation, cells were washed twice with cold HEPES buffer, pH 7.6 (Life Technologies). Half of the cells were used for photoaffinity labeling of surface receptors (S), the other half for labeling of receptors in the cell homogenate (H). For labeling of surface receptors, living cells were incubated in the dark for 1 hr at room temperature with 0.8 nM [¹²⁵I]CGP71872. Cells were washed with cold HEPES buffer to remove unbound [¹²⁵I]CGP71872 before UV cross-linking (Kaupmann et al., 1997). Photoaffinity-labeled cells were collected, and the radioactivity was determined in a gamma counter (Packard, Zurich, Switzerland). Photoaffinity labeling of receptors in the membrane fraction was as described (Kaupmann et al., 1997). For SDS-PAGE, the pellets of cells and cell lysates were resuspended in HEPES buffer containing 0.1% SDS. An aliquot was taken for protein determination (Micro BCA Protein Assay Reagent Kit, Pierce, Rockford, IL). Twenty micrograms of total protein of cell and membrane fraction were loaded onto a 4–15% gradient gel. Photoaffinity-labeled proteins were detected using autoradiography. The radioactivity incorporated into the surface (S) and homogenate (H) fraction was normalized to the respective amount of protein in each fraction before the percentage S/H ratio was calculated. Presumably because of the differences in the radiolabeling procedure for surface and homogenate recep-

tors, the percentage S/H sometimes exceeds the theoretical value of 100%.

Immunofluorescence studies. HEK293 cells were grown on eight-well glass slides coated with poly-D-lysine (Becton Dickinson, Basle, Switzerland) and transfected (0.05 μ g of GABA_{B(1a)}/0.2 μ g of GABA_{B(2)} DNA in 5 μ l of H₂O) with FuGene6 (Roche Molecular Biochemicals, Rotkreuz, Switzerland). In control experiments the empty pCI vector substituted for GABA_B plasmids. Dissociated cerebellar granule cells were isolated from 7-d-old mice and plated on poly-L-ornithine-coated 35 mm glass coverslips (Van-Vliet et al., 1989); after 7 d in culture, cells were transfected (Ango et al., 1999) with c-myc and HA-tagged GABA_{B(1a)} and GABA_{B(2)} constructs, respectively. Cells were fixed 48 hr after transfection with 4% paraformaldehyde in PBS for 20 min and blocked with 1% BSA and 2% heat-inactivated goat serum in PBS for 1 hr. Cell surface receptor expression was assayed by labeling cells with either anti-c-myc (clone 9E10, Roche Molecular Biochemicals; 1:200) or anti-HA (clone 12CA5, Roche Molecular Biochemicals; 1:80) monoclonal antibodies for 2 hr. After extensive washing, cells were incubated for 1 hr with secondary Cy3-conjugated affinity-purified goat anti-mouse IgG (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA). Intracellular receptor expression was assayed with permeabilized cells (0.4% saponin added to the blocking/washing buffers and to the primary and secondary antibody solutions). Immunofluorescence microscopy was with a Zeiss Axioplan microscope using a 20 $\times$ Plan-Neofluar objective. Images were collected with a Hamamatsu 3 CCD digital camera.

Measurement of $\Delta[Ca^{2+}]_i$ by fluorometry. For measurement of $\Delta[Ca^{2+}]_i$, all transfections included G α_{q05} to couple GABA_B receptors to PLC (Franeck et al., 1999). HEK293 cells (1.5×10^6) were electroporated (250 V, 300 μ F; BioRad Gene Pulser) with 5 μ g of cDNA constructs in a total volume of 150 μ l buffer (50 mM K₂HPO₄, 20 mM CH₃COOK, 20 mM KOH, pH 7.4). Transfected cells were resuspended in culture medium and split into three wells of 12-well plates that were coated with 100 mg/ml poly-D-lysine. Twenty-four hours after transfection, the cells were incubated at room temperature for 1 hr in a HEPES buffer, pH 7.6 (Life Technologies) containing 10 μ g/ml of the calcium indicator fura-2 AM, 0.5% Pluronic F-127 (Molecular Probes, Eugene, OR), and 1% (v/v) dimethyl sulfoxide. Glass coverslips carrying dye-loaded cells were mounted into a perfused cuvette (2 ml/min) in a fluorescence spectrophotometer (F-4500, Hitachi). Changes in $[Ca^{2+}]_i$ were monitored by measuring the ratio of fura-2 fluorescence (510 nm) excited at 340 and 380 nm, switched at 1.6 Hz. The viability of transfected cells was tested by application of 10 μ M ATP. Control experiments showed no changes in $[Ca^{2+}]_i$ levels after application of 1 mM GABA in HEK293 cells transfected with pcDNA1 vector and in HEK293 cells expressing GABA_{B(1)}+G α_{q05} , GABA_{B(2)}+G α_{q05} , or G α_{q05} . No quantitative comparison between experiments was made, because the signal amplitude depends on the transfection efficiency.

Fluorometric image plate reader. Transfected HEK293 cells were plated into poly-D-lysine coated 96-well plates (Becton Dickinson). After transfection (48–72 hr), cells were loaded for 45 min with 2 μ M fluo-4 AM (Molecular Probes) in HBS (Life Technologies) containing 50 μ M probenecid (Sigma, Buchs, Switzerland). Plates were washed and transferred to a FLIPR (Molecular Devices, Crawley, UK). Concentration–response curves were recorded with three to eight wells per concentration as a change of fluorescence over baseline ($\Delta F/F$). Data were fitted using Igor Pro (Wavemetrics) with a logistic equation using weighted nonlinear regression. Curves and data points were normalized to their asymptotic maximal values or for negative subunit combinations to the respective values of the positive controls measured on the same plate.

Electrophysiology. Concatemers of Kir3.1/3.2 subunits (Kaupmann et al., 1998b) were cotransfected with GABA_B cDNA combinations into HEK293 cells. Whole-cell patch-clamp recordings were performed at room temperature 48–72 hr after transfection in a bath solution consisting of (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, 5 HEPES, pH 7.4. Patch pipettes were pulled from borosilicate glass capillaries, heat-polished to give a tip resistance of 3–5 M Ω , and filled with 140 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM Na₂ATP, 100 μ M cAMP, 100 μ M GTP, and 5 mM HEPES, pH 7.3. Currents were elicited at a holding potential of –90 mV by application of GABA in a high potassium extracellular buffer (25 mM KCl, 115.6 mM NaCl). Data were recorded with an Axopatch 200B using pClamp6.

RESULTS

Previous studies indicated that GABA_B receptor assembly and trafficking are intricately linked processes and that functional

activity of the GABA_{B(1,2)} heteromer correlated with GABA_{B(1)} cell surface expression (Couve et al., 1998; White et al., 1998). Functional expression in heterologous systems therefore provides a fast and sensitive means of identifying structural elements that are necessary for receptor maturation. In this study we constructed chimeric, truncated, and point mutated GABA_{B(1)} and GABA_{B(2)} mutants and coexpressed them in HEK293 cells and cultured neurons. Immunoblot analysis was used to verify that all receptor mutants were expressed. For functional analysis we measured G α_{q05} signaling to phospholipase C (PLC), as manifested by GABA-elicited increases in cytosolic calcium ($[Ca^{2+}]_i$) (Franeck et al., 1999) and G $\beta\gamma$ stimulation of Kir3.1 + 3.2 type K⁺ channels ($I_{Kir3.1 + 3.2}$) (Kaupmann et al., 1998b). Surface expression was studied using immunocytochemistry and quantified using photoaffinity labeling with the membrane-impermeable GABA_{B(1)}-specific ligand [¹²⁵I]CGP71872 (Kaupmann et al., 1997). The surface expression and functional data are summarized in the Table.

Reciprocal exchange of the C terminus allows the assembly of functional heterodimers

A likely location for a putative ERR/R signal in GABA_{B(1)} is the cytoplasmic C-terminal domain. We therefore first investigated whether differences in the carboxyl-tails between GABA_{B(1)} and GABA_{B(2)} affect GABA_{B(1)} cell surface targeting and, as a consequence of this, prevent function. We generated two chimeric subunits, R1CR2 and R2CR1, in which the entire C-terminal domains of GABA_{B(1)} (CR1) and GABA_{B(2)} (CR2) were interchanged (Fig. 1*A,B,D,E*). The results show that exchange of the C-terminal domains does not allow for homomeric (R1CR2, R2CR1; data not shown) or homodimeric receptor (R1+R1CR2, R2CR1+R2) (Fig. 1*B*) function regardless of the C-terminal domains. It appears that transfer of the C termini leads to functional expression whenever the C termini are complementary (R1+R2, R1CR2+R2CR1) or from GABA_{B(2)} alone (R1CR2+R2) (Fig. 1*B*). The functional R1CR2+R2 receptor heterodimerizes through domains other than the leucine zippers, because LZ2 does not dimerize with itself (Kammerer et al., 1999). The above results are consistent with the presence of an ERR/R signal in the C terminus of GABA_{B(1)} that impedes surface trafficking and function when not neutralized by C-terminal GABA_{B(2)} sequences, as is the case with R2CR1+R1 (Fig. 1*B*). Surface expression of the C-terminal exchange mutants was tested using photoaffinity labeling. The results confirm that GABA_{B(1)} is retained intracellularly when containing its own C terminus (R1) (Fig. 1*E*) or expressed with R2CR1, but not when containing the GABA_{B(2)} C terminus (R1CR2) (Fig. 1*E*).

Removal of the GABA_{B(1)} C terminus allows the assembly of functional heterodimers

The above results raised the possibility that receptor dimerization and functional cell surface expression can occur in the absence of C-terminal domains. We therefore removed the C-terminal domains of GABA_{B(1)} (R1K886) and GABA_{B(2)} (R2T749) (Fig. 1*A,C,D,E*). When expressed individually the C-terminally truncated subunits do not yield functional responses (data not shown). However, R1K886 is functional in combination with GABA_{B(2)} (R1K886+R2) and truncated GABA_{B(2)} (R1K886+R2T749) (Fig. 1*C*). The R1K886+R2T749 receptor emphasizes that heteromerization and G-protein signaling do not depend on C-terminal sequences, including the leucine zipper domains. This indicates that TMD and/or N-terminal domains are sufficient to

Table 1. Surface expression and functional coupling of GABA_B receptor mutants

	Subunit combination	Surface photoaffinity labeling	Surface immunostaining	G-protein coupling
Wild type	R1+R2	91	+	+
	R1	6	—	—
	R1_myc	21	—	—
C-terminal exchanges	R1CR2_his	146	+	—
	R1+R1CR2_his	n.d.	+	—
	R1+R2CR1_his	7	n.d.	—
R1 C-terminal deletions	R1I860_myc	141	+	+ ^a / ₊ ^b
	R1T872_myc	116	+	+ ^a / ₊ ^b
	R1K886	160	+	+ ^a / ₊ ^b
	R1K886_myc	129	+	+ ^a / ₊ ^b
	R1L915_myc	131	+	+ ^a / ₊ ^b
	R1L921_myc	73	+	+ ^a / ₊ ^b
	R1P928_myc	5	—	— ^a / ₊ ^b
	R1G940_myc	13	—	— ^a / ₊ ^b
	R1G952_myc	6	—	— ^a / ₊ ^b
Alanine scanning	R1[ASRRHPP]_myc	105/385 ^a	+	+ ^a / ₊ ^b
	R1[RAARRHPP]_myc	6/36 ^a	—	— ^a / ₊ ^b
	R1[RSARRHPP]_myc	68/80 ^a	+	+ ^a / ₊ ^b
	R1[RSRRHPP]_myc	23/56 ^a	+	+ ^a / ₊ ^b
	R1[RSRRHAP]_myc	8/7 ^a	—	— ^a / ₊ ^b
	R1[RSRRHPA]_myc	4/24 ^a	—	— ^a / ₊ ^b
	R1[RSRRHPA]_myc	10/28 ^a	—	— ^a / ₊ ^b
	R1[RDRRHPP]_myc	40/183 ^a	+	+ ^a / ₊ ^b
	R1[ASARRHPP]_myc	126/106 ^a	+	+ ^a / ₊ ^b
	R1[ASAAHPP]_myc	152/181 ^a	+	+ ^a / ₊ ^b
R2 C-terminal deletions	R1+R2T749_his	23	—	—
	R1+R2L791_ha	17	—	—
	R1+R2E811_ha	34	—	+
	R1+R2P820_ha	254	+	+
Leucine zipper deletions and exchanges	R1ΔLZ1	284	+	—
	R1ΔLZ1+R2	225	+	+
	R1ΔLZ1+R2T749_his	150	n.d.	+
	R1ΔLZ1+R2[PPP]	140	n.d.	+
	R1LZ2	30	—	—
	R1LZ2+R2	177	+	+
	R1LZ2+R2T749_his	40	+	+
	R2ΔLZ2+R1	24	—	—
	R2ΔLZ2+R1ΔLZ1	103	n.d.	+
	R2LZ1+R1	49	—	+
	R2LZ1+R1LZ2	153	+	+
Proline mutations within leucine zippers	R1[PPP]	7	—	—
	R1[PPP]+R2	135	+	+
	R1+R2[PPP]	108	n.d.	+
	R1[PPP]+R2[PPP]	70	+	+
	R1I860_myc+R2[PPP]	247	+	+
	R1[PPP]+R2T749_his	n.d.	—	—

Surface photoaffinity labeling was determined using the membrane-impermeable antagonist [¹²⁵I]CGP71872 and quantified as the percentage of radioactivity incorporated at the cell surface receptors versus the radioactivity incorporated in the cell homogenate (percentage S/H). Surface immunofluorescence was determined with anti-myc and anti-HA antibodies. For functional measurements we used G_{α_q5} coupling to phospholipase C and G_{βγ} coupling to Kir3 channels. No differences were observed between G_{α_q5} and G_{βγ} coupling. +, Signal; —, no signal; n.d., not determined.

^aExperiments with co-expressed R2T749_his.

^bExperiments with co-expressed WT GABA_{B(2)}.

form an interface that supports assembly of a functional heteromeric receptor. This is in line with homodimerization of mGlu5 and Ca²⁺-sensing receptors that occurs in the N-terminal extracellular domains (Romano et al., 1996; Ward et al., 1998; Bai et al., 1999; Ray et al., 1999). The R1+R2T749 (Fig. 1C) and

R1K886+R2CR1 (Fig. 1D) subunit combinations are not functional. These results are consistent with the presence of an ERR/R signal in the C terminus of GABA_{B(1)} that impedes surface trafficking and function. In agreement with this proposal, the R1CR2+R2T749 combination, where the putative ERR/R

Figure 1. Functional expression and cell surface targeting of C-terminal exchange and deletion mutants in HEK293 cells. *A*, Immunoblot analysis of WT GABA_{B(1a)} (R1a), WT GABA_{B(2)} (R2), and mutant proteins. Antibodies directed to N- (176, N22) and C-terminal (174, C22) epitopes of GABA_{B(1)} (176, 174) and GABA_{B(2)} (N22, C22) were used. *B*, *C*, *D*, Functional analysis of C-terminal exchange and deletion mutants in fluorometer. Functional coupling to PLC shows representative Ca^{2+} transients as measured by changes in fura-2 fluorescence intensity ratios ($R_{340/380}$, $\Delta[Ca^{2+}]_i$). Bars below traces indicate application of drugs. GABA (100 μ M)-evoked changes in $[Ca^{2+}]_i$ are reversibly inhibited by coapplication of the GABA_B receptor antagonist CGP54626A (1 μ M). Functional coupling to Kir3.1 + 3.2 channels shows current responses ($I_{Kir3.1 + 3.2}$) to GABA (100 μ M for 10 sec, white arrow), CGP54626A (1 μ M for 60 sec, gray arrow), and GABA+CGP54626A (for 10 sec, black arrow) in transfected HEK293 cells. All results are representative of at least three independent experiments. Schematic representation of GABA_{B(1)} (white) and GABA_{B(2)} (gray) receptors: boxes represent the TMDs; LZ1 and LZ2 are shown in black. Where present, C-terminal histidine tags (his) are indicated. *E*, Surface targeting of WT and mutant GABA_{B(1)} proteins expressed individually and in combination with WT and mutant GABA_{B(2)}. Intact cells (S) and cell homogenates (H) were photoaffinity-labeled with the membrane-impermeable, GABA_{B(1)}-specific antagonist [¹²⁵I]CGP71872 and subjected to SDS-PAGE. Labeled proteins were detected by autoradiography. The radioactivity incorporated into the surface (S) and homogenate (H) fraction was normalized to the respective amount of protein in each fraction before the percentage S/H ratio was calculated.

signal of GABA_{B(1)} has been replaced by the GABA_{B(2)} sequence, yields functional receptors (Fig. 1D). For all subunit combinations tested, functional analysis does not reveal any differences between G $\beta\gamma$ ($I_{Kir3.1 + 3.2}$) and G α_{q05} signaling ($\Delta[Ca^{2+}]_i$) (Fig. 1B, C, D). Photoaffinity labeling (Fig. 1E) studies

confirm that WT GABA_{B(1)} targets to the cell surface in combination with WT GABA_{B(2)} (R1+R2) but not in combination with R2T749 (R1+R2T749). R1K886, devoid of any putative ERR/R signal, is efficiently delivered to the cell surface when expressed alone (Fig. 1E).

The RSRR motif prevents surface trafficking of GABA_{B(1)}

To further localize the signal preventing GABA_{B(1a)} surface expression, we sequentially truncated a total of 113 residues of its 960 amino acids C terminus (Fig. 2*A,B*). For functional studies, we coexpressed the resulting mutants with WT GABA_{B(2)} (positive control) or R2T749 to avoid shielding of the putative ERR/R signal (Fig. 2*C*). Our results with R2T749 coexpression show that functional activity (Fig. 2*C*) and surface trafficking (Figs. 2*F*, 6*A*) are impaired when <32 amino acids are truncated from the GABA_{B(1)} C terminus (R1P928). Sequential truncation beyond P⁹²⁸ up to TMD7 (mutants R1L921 through R1I860) abrogates intracellular retention (Figs. 2*F*, 6*A*), and functional expression is restored (Fig. 2*C*). We conclude that the information necessary for retention is located in a seven amino acid segment between L⁹²¹ and P⁹²⁸, in the vicinity of the LZ1 (Fig. 2*A*). Known ERR/R motifs within this segment are RSR and RR because they match the consensus motives first identified in K_{ATP} channels (Zerangue et al., 1999) and human invariant chain Iip33 polypeptides (Schutze et al., 1994).

The regions involved in coupling the mGlu receptors to G-proteins are thought to be the second/third intracellular loops and C-terminal domains (Pin et al., 1994; Francesconi and Duvoisin, 1998). Pharmacological analysis of C-terminal mGlu1 splice variants has shown that although the rank order of agonist potencies is identical, the potencies differ between variants (Flor et al., 1996). We therefore explored whether deletion of C-terminal domains in both subunits changes the pharmacological profile of the R1I860+R2T749 receptor (Fig. 2*D,E*). [¹²⁵I]CGP64213 displacement curves with agonists [GABA, L-baclofen, 3-aminopropylphosphonic acid (APPA)] and antagonists (CGP54626A, CGP35348) are similar to those obtained with WT receptors. Similarly, concentration–response curves obtained in FLIPR analysis do not show significant differences compared with WT receptors (Fig. 2*E*). We conclude that C-terminal domains do not influence G-protein coupling, because this would likely result in altered agonist pharmacology.

To pinpoint the critical residues responsible for ERR/R, we used alanine-scanning mutagenesis to change each residue from R⁹²² to P⁹²⁸ (Fig. 3*A*). When coexpressed with R2T749 to prevent shielding of the ERR/R signal, only mutation of R⁹²² (R1[ASRRHPP]), R⁹²⁴ (R1[RSARHPP]), and R⁹²⁵ (R1[RSR-AHPP]) yielded functional receptors (Fig. 3*B*). The resulting motif, RXRR, matches both the RXR consensus defined for K_{ATP} channels and the RR retention signal of Iip33 polypeptides. Although the S⁹²³→A mutation (R1[RARRHPP]) does not allow the assembly of functional receptors, signaling is obtained with the S⁹²³→D mutation (R1[RDRRHPP]) (Fig. 3*B*). The latter replacement is therefore not permissible in the ERR/R signal, and the position between the two arginine residues cannot accommodate large side chains, similar as for the RXR motif in K_{ATP} channels (Zerangue et al., 1999) and the cystic fibrosis transmembrane conductance regulator (Chang et al., 1999). Subunits with multiple mutations in the RSRR motif, as in R1[ASARHPP] and R1[ASAAHPP], all yield functional heteromeric receptors when coexpressed with the C-terminally truncated R2T749 subunit (Fig. 3*B*). Stressing the importance of the RSRR motif, we can rescue the nonfunctional combination R1+R2CR1 by inactivating the two ERR/R signals (R2CR1[ASARHPP]+R1[ASARHPP]) (Fig. 3*C*). A mutated ERR/R signal allows for coupling not only to PLC (Fig. 3*B*) but also to Kir3.1 + 3.2 (Fig. 3*D*), revealing no differences in the competence

to signal through Gα_{qo5} and Gβγ. Similarly, dose–response curves in FLIPR analysis do not reveal significant pharmacological differences between WT GABA_{B(1,2)} and R1[ASAAHPP]+R2T749, with the exception of the potency for APPA that is somewhat altered by the mutations (pEC₅₀ value 6.0 ± 0.1 vs 6.7 ± 0.05 for WT) (Fig. 3*E*). The R1+R2T749 subunit combination with an active ERR/R signal is not functional (Fig. 3*E*). The above data suggest that mutation of the RSRR motif enables GABA_{B(1)} to escape from the ER. We directly analyzed the surface localization of GABA_{B(1)} mutants in photoaffinity labeling and immunofluorescence experiments (Figs. 3*F*, 6*A*). When expressed by themselves, mutants R1[ASRRHPP], R1[RSARHPP], R1[ASARHPP], and R1[ASAAHPP] are most efficiently transported to the cell surface. The arginine residues thus cooperate in mediating full ER retention. R1[RSRAHPP] and R1[RDRHPP] show relatively inefficient escape (low surface expression levels), indicating that the mutated ERR/R motif is not fully functional and allows some leakage. In R2T749 coexpression experiments, efficient trafficking of alanine mutants to the surface (Fig. 3*F*) always correlates with functional responses (Fig. 3*B*). In general, coexpression of R2T749 with alanine mutants increased surface trafficking relative to the mutants expressed individually (Fig. 3*F*). Subunit coexpression may stabilize the heteromeric receptor complex during export from the secretory apparatus or at the cell surface.

In summary, deletion and alanine-scanning analysis indicate that the RSRR motif is sufficient to prevent escape of the GABA_{B(1)} protein from the early secretory apparatus and that no other ERR/R signal is operative. Importantly, surface targeting of RSRR mutants does not confer homomeric function (data not shown). In the heteromeric receptor complex, GABA_{B(2)} therefore not only mediates surface trafficking but also participates in intracellular signaling. The dominant role of the RSRR signal in GABA_{B(1)} on cell surface expression is intriguing. Remarkably, GABA_{B(2)}, which efficiently targets to the cell surface by itself, contains several putative ERR/R signals in its C terminus as well (RHR, RLR) (Fig. 4*A*). Possibly these signals are inactive because the sequence context prevents their exposure in the ER. Alternatively, ER resident proteins other than GABA_{B(1)} mask them.

C-terminal GABA_{B(2)} sequences are sufficient to mask the ERR/R signal of GABA_{B(1)}

The above data indicate that C-terminal sequences of GABA_{B(2)} antagonize the GABA_{B(1)} ERR/R signal. We therefore made serial truncations in the C terminus of GABA_{B(2)} to map the shielding sequences (Fig. 4*A,B,C,F*). The data show that the C-terminal 130 residues, including seven residues of LZ2, can be removed without loss of function (R1+R2E811) (Fig. 4*C*). Because the ERR/R signal is in close proximity of the C terminus of LZ1, GABA_{B(2)} sequences directly complementary to the ERR/R signal (T⁸²³ through I⁸²⁶) (Fig. 4*A*) are unnecessary for its shielding. Additional truncation of LZ2 (R2L791) (Fig. 4*C*) results in loss of function, suggesting that the coiled-coil interaction between GABA_{B(1)} and GABA_{B(2)} is destabilized to the point where no shielding is possible anymore. The LZ2 residues between L⁷⁹¹ and E⁸¹¹ therefore are important for engaging LZ1 into a coiled-coil interaction that propagates a C-terminal conformation that neutralizes the ERR/R signal.

Expression of the otherwise nonfunctional R1+R2T749 combination together with the C terminus of GABA_{B(2)} (CR2) (Fig. 4*B*) results in complementation and recovery of intracellular signaling (Fig. 4*D*). The concentration–response curves in

Figure 2. Progressive C-terminal GABA_{B(1)} deletion mutants indicate that residues between P⁹²⁸ and L⁹²¹ impair surface expression. **A**, C-terminal domain of GABA_{B(1)} with LZ1 (E⁸⁸⁴–S⁹¹⁷, shaded). The C-terminal residues of the deletion mutants are shown in white letters. The RSRR sequence encoding a putative ERR/R signal in close proximity to LZ1 is boxed. **B**, Immunoblot analysis of GABA_{B(1a)} deletion mutants with antibody 176. All mutants were tagged with the myc epitope. **C**, Functional expression of GABA_{B(1a)} deletion mutants together with C-terminally truncated GABA_{B(2)} (R2T749_his) to prevent shielding of the putative ERR/R. As a control, all GABA_{B(1)} mutants yield functional responses together with WT GABA_{B(2)} (R2). **D**, Binding pharmacology of a C-terminally truncated receptor (R1I860+R2T749, closed symbols) and WT GABA_{B(1a,2)} (R1+R2, open symbols) receptors. Inhibition of [¹²⁵I]CGP64213 binding by agonists (GABA, L-baclofen, APPA) and antagonists (CGP54626A, CGP35348) using membranes from transfected HEK293 cells is shown. The affinities for agonists and antagonists do not differ significantly between mutant and WT receptors. The pIC₅₀ values for R1+R2T749_his and R1+R2 are as follows: GABA, 5.0 ± 0.02 and 5.2 ± 0.02; L-baclofen, 4.7 ± 0.03 and 5.1 ± 0.03; APPA, 5.9 ± 0.01 and 6.2 ± 0.03; CGP54626A, 8.7 ± 0.01 and 8.7 ± 0.01; CGP35348, 4.8 ± 0.01 and 4.9 ± 0.01, respectively. Results from typical experiments performed in triplicate are shown. Immunoblotting and photoaffinity labeling verified GABA_B protein expression (data not shown). **E**, FLIPR analysis. Concentration–response curves for agonists at WT receptors (R1+R2, open symbols) and R1I860+R2T749 (filled symbols). Data points show average and SEM of relative fluorescence changes normalized to their maximum (I/I_{max}). Lines are Hill equations fitted to the data points. pEC₅₀ values for GABA, L-baclofen, and APPA are 5.8 ± 0.1 and 5.7 ± 0.1, 5.9 ± 0.1 and 5.7 ± 0.05, 6.7 ± 0.05 and 6.5 ± 0.05 for WT and R1I860+R2T749 receptors, respectively (mean ± SEM; n = 4–8). Hill coefficients for GABA, L-baclofen, and APPA are 1.1 ± 0.2 and 1.3 ± 0.2, 1.1 ± 0.2 and 1.3 ± 0.2, 1.4 ± 0.3 and 1.2 ± 0.2 for WT and R1I860+R2T749 receptors, respectively (mean ± SEM; n = 4–8). **F**, Surface targeting of individually expressed C-terminal GABA_{B(1)} deletion mutants.

Figure 4. Influence of the GABA_{B(2)} C-terminal domain on GABA_{B(1)} surface expression and function. *A*, The C-terminal domains of GABA_{B(1)} and GABA_{B(2)} with LZ1 (E⁸⁸⁴-S⁹¹⁷, shaded) and LZ2 (T⁷⁸⁵-D⁸¹⁸, shaded) are shown. The C-terminal residues of GABA_{B(2)} deletion mutants are depicted in white letters. The RSRR motif and RXR consensus sequences in GABA_{B(2)} are boxed. *B*, Immunoblot analysis of GABA_{B(2)} mutants with N-terminal (N22) and C-terminal (C22) antibodies. The antibodies do not recognize mutant CR2P820 (R⁷¹⁴ through P⁸²⁰), which are missing N- and C-terminal epitopes. Expression of CR2P820 was confirmed in immunofluorescence studies with anti-HA antibodies (data not shown). *C*, Functional expression of C-terminally truncated GABA_{B(2)} receptors together with WT GABA_{B(1)}. As a control, all GABA_{B(2)} mutants yield functional responses when expressed together with R1860_myc, which is devoid of the domain containing the RSRR signal. *D*, The C-terminal domain of GABA_{B(2)} (CR2, R⁷¹⁴ through L⁹⁴¹), expressed as a membrane-bound protein, rescues the otherwise functionally inert R1+R2T749 subunit combination *in trans*. CR2 is not able to mask the RSRR signal when two GABA_{B(1)} C termini are present (R2CR1+R1). *E*, FLIPR analysis. Agonist concentration–response curves for the triple transfection of R1+R2T749+CR2. pEC₅₀ values for GABA, L-baclofen, and APPA are 5.5 ± 0.1, 5.4 ± 0.1, and 6.5 ± 0.1, respectively (mean ± SEM; n = 4). Hill coefficients for GABA, L-baclofen, and APPA are 0.9 ± 0.1, 1.0 ± 0.2, and 1.1 ± 0.2, respectively (mean ± SEM; n = 4). *F*, Surface targeting of GABA_{B(1)} in combination with C-terminally truncated GABA_{B(2)}, R2CR1, and the C-terminal GABA_{B(2)} fragments CR2 and CR2P820.

(Fig. 2*C,E*), it follows that in the dimer the TMDs of GABA_{B(2)} are involved in addressing the effector system. A truncated CR2 fragment with a deletion of the 121 residues C terminal of the zipper domain, CR2P820, very efficiently exports WT GABA_{B(1)} to the cell surface (Figs. 4*F*, 6*A*). Therefore the shielding information provided by the GABA_{B(2)} C terminus resides between TMD7 and P⁸²⁰.

We previously observed functional responses in a minor fraction of transfected HEK293 cells expressing GABA_{B(1)} or GABA_{B(2)} alone (Kaupmann et al., 1998a,b). We speculated that homomeric GABA_{B(1)} responses were the consequence of forcing receptors to their effectors at the cell surface, because of the artificially high expression levels observed in heterologous cells.

The lack of functional responses obtained with monomeric GABA_{B(1)} expressed at the cell surface (e.g., R1CR2, R1K886, and R1+CR2P820) makes this explanation less likely. Rare responses obtained with GABA_{B(1)} and GABA_{B(2)} may depend on additional factors that are limiting or absent in most HEK293 cells.

C-terminal coiled-coil interaction is involved in, but not absolutely necessary for, masking the RSRR signal

One structural feature that is characteristic of GABA_B receptor subunits, and not shared by other family 3 GPCRs, is the presence of leucine zippers in their C termini. The previous experiments demonstrate that a C-terminal fragment of GABA_{B(2)}, CR2P820, which includes LZ2, can traffic WT GABA_{B(1)} to the cell surface (Fig. 4F). The close proximity of the RSRR motif to the LZ1 (Fig. 5A) suggests that the ERR/R signal is inactivated upon coiled-coil dimerization. To test the role of the leucine zippers on targeting and function, we deleted LZ1 (R1ΔLZ1) and LZ2 (R2ΔLZ2) from the WT subunits (Fig. 5A,B). Surprisingly, LZ1 deletion does not impair functional assembly in R1ΔLZ1+R2 (Fig. 5B). However, deletion of LZ1 in R1ΔLZ1 may alter the structural context in which the ERR/R signal is placed and render it inactive, as has been observed for the RXR motif in Kir6.2 (Zerangue et al., 1999). This is supported by the fact that the R1ΔLZ1+R2T749 receptor, where no GABA_{B(2)} shielding is provided, is functional (Fig. 5B). Photoaffinity labeling (Fig. 5F) and surface immunofluorescence (Fig. 6A) experiments demonstrate that R1ΔLZ1 is indeed efficiently released to the cell surface. Conformational changes imposed by the deletion are therefore responsible for R1ΔLZ1 surface delivery, rather than a shielding of the ERR/R signal through coiled-coil independent interactions. Deletion of the zipper in GABA_{B(2)} is sufficient to prevent functional assembly in combination with WT GABA_{B(1)} (R2ΔLZ2+R1) (Fig. 5B), suggesting that preventing the coiled-coil interaction impairs masking of the ERR/R signal. R2ΔLZ2 does constitute functional heterodimers with GABA_{B(1)} subunits that have an impaired (R2ΔLZ2+R1ΔLZ1) (Fig. 5B) or missing (R2ΔLZ2+R1I860) (Fig. 5B) ERR/R signal, demonstrating that the introduced mutation does not generally affect functionality.

We further tested the roles of the zipper domains by exchanging them between subunits. The resulting mutants R1LZ2 and R2LZ1 produce functional receptors in all combinations tested (Fig. 5C). Small responses obtained with R1LZ2+R2T749 demonstrate that replacement of LZ1 by LZ2 imposes conformational constraints that impair proper functioning of the RSRR motif. The R1LZ2 receptor expressed alone is not detectable at the surface by immunostaining, suggesting that a small amount of mutant protein escapes the ER (Fig. 6A). However, this leakage of R1LZ2 from the ER may explain the coupling obtained in combination with WT GABA_{B(2)} (R1LZ2+R2) (Fig. 5C), in the absence of coiled-coil interaction between the two LZ2 domains (Kammerer et al., 1999). The R2LZ1+R1 combination with LZ1 in both subunits yields barely functional receptors (Fig. 5C), again in the absence of coiled-coil interaction between the two LZ1 domains (Kammerer et al., 1999). Because this receptor combination consistently yields tiny responses, we conclude that significant but not absolutely tight shielding is taking place. R1LZ2+R2LZ1 assembles a highly functional receptor, demonstrating that swapped zippers enable efficient surface targeting (Fig. 5B).

Because the leucine zipper deletion and exchange experiments were not absolutely conclusive because of possible confor-

mational alterations forced by the changes, we decided to destabilize the coiled-coil interaction between GABA_{B(1)} and GABA_{B(2)} by introducing proline mutations. Leucine zippers form amphipathic α -helices that coil around each other in a left-handed supertwist. The sequences of coiled coils are characterized by a heptad repeat of seven residues denoted *a*–*g* (Fig. 5A). The stability of a coiled coil is determined by interactions between residues in the hydrophobic interface (positions *a* and *d*) (Lupas, 1996; Malashkevich et al., 1996). We therefore introduced proline residues, which are expected to break helices, at three of these critical positions in LZ1 (R1[PPP]) and LZ2 (R2[PPP]) (Fig. 5A). In contrast to R1ΔLZ1 and R1LZ2, the R1[PPP] subunit is very tightly retained in the ER, as shown by the lack of responses on coexpression with R2T749, where no shielding of the ERR/R is provided (R1[PPP]+R2T749) (Fig. 5D). This is confirmed in both surface photoaffinity (Fig. 5F) and immunofluorescence (Fig. 6A) labeling experiments. Clearly, surface trafficking is not inherent to R1[PPP], and its ERR/R signal is still fully active. As expected, receptor function is recovered by replacing R2T749 with WT GABA_{B(2)} (R1[PPP]+R2) (Fig. 5D). R2[PPP] is able to form functional receptors with WT GABA_{B(1)}, demonstrating that the ability to mask the ERR/R signal is retained after mutation of LZ2 (Fig. 5D). As a control, R2[PPP] also forms functional assemblies with R1I860, where no ERR/R signal is operative (Fig. 5D). The R1[PPP]+R2[PPP] combination, where α -helices are impaired in both leucine zippers, is not expected to undergo significant coiled-coil interaction (Fig. 5D,E). Nevertheless, R1[PPP]+R2[PPP] exhibits functional responses, emphasizing that in the heteromer the coiled-coil interaction is not absolutely necessary for shielding of the RSRR motif. The agonist pharmacology of R1[PPP]+R2[PPP] is reminiscent of that of WT receptors (Fig. 5E).

Altogether, the experiments with mutated leucine zipper domains led us to conclude that (1) LZ1 is necessary for exposure of the RSRR motif and (2) the coiled-coil interaction between the two subunits is involved in, but not absolutely necessary for, shielding the ERR/R signal.

The RSRR signal is recognized in neurons

We next wanted to study whether the RSRR signal was active in primary neurons. Because GABA_{B(1)} and GABA_{B(2)} are expressed in virtually all neurons (Marshall et al., 1999), we were unable to perform experiments in the absence of endogenous GABA_B protein. Although this supposedly complicates analysis in neurons, overexpression of the heterologous proteins by the cytomegalus virus promoter should lead to a saturation of the targeting machinery. This presumably overrides any possible influence on targeting by endogenous GABA_B subunits, and therefore the mutant proteins are expected to exhibit their inherent targeting phenotype. In good agreement with this assumption, the targeting of WT and mutant GABA_B receptors in HEK293 cells (Fig. 6A) are mirrored in transfected cerebellar neurons (Fig. 6B). Although GABA_{B(1)} is retained intracellularly when expressed alone (R1) (Fig. 6B), it is directed to the cell surface when its ERR/R signal is inactivated by mutagenesis (R1[4S4RHPP]) (Fig. 6B) or when expressed together with WT GABA_{B(2)} (R1+R2) (Fig. 6B).

Ca²⁺-sensing and mGlu receptors do not support surface expression of GABA_{B(1)}

Targeting information is associated with the carboxyl tails of many neurotransmitter receptors and ion channels. It is therefore

Figure 5. Functional analysis and surface expression of leucine zipper point, exchange, and deletion mutants. *A*, The sequences of the predicted GABA_{B(1)} (R1) and GABA_{B(2)} (R2) coiled-coil domains (shaded) are characterized by a heptad repeat of seven residues denoted *a–g*, with a 3,4-hydrophobic repeat of mostly apolar residues at positions *a* and *d*. Proline mutations were introduced at the L⁸⁹⁷I⁹⁰¹V⁹⁰⁸ and L⁷⁹⁸I⁸⁰²L⁸⁰⁵ core residues of GABA_{B(1)} and GABA_{B(2)}, respectively. The ERR/R signal, spaced by four residues from LZ1, is boxed. *B*, Functional analysis of LZ1 (R1 Δ LZ1) and LZ2 (R2 Δ LZ2) deletion mutants. *C*, Functional analysis of LZ1 (R1LZ2) and LZ2 (R2LZ1) exchange mutants. *D*, Functional analysis of LZ1 R1[PPP] and LZ2 R2[PPP] point mutants. *E*, FLIPR analysis. Agonist concentration–response curves for R1[PPP]+R2[PPP]. pEC₅₀ values for GABA, L-baclofen, and APPA are 5.4 ± 0.1 , 5.2 ± 0.1 , and 6.8 ± 0.05 , respectively (mean $\pm$ SEM; $n = 4$). Hill coefficients for GABA, L-baclofen, and APPA are 0.8 ± 0.3 , 1.2 ± 0.2 , and 1.1 ± 0.3 , respectively (mean $\pm$ SEM; $n = 4$). *F*, Surface targeting of LZ1 GABA_{B(1)} mutants expressed individually and in combination with WT and mutated GABA_{B(2)}. Expression of all mutants was verified on immunoblots (data not shown).

tempting to speculate that proteins other than GABA_{B(2)} recognize and shield the GABA_{B(1)} RSRR signal. Because coiled-coil interaction does not appear to be an absolute requirement to mask the ERR/R signal, these proteins may not necessarily contain leucine zippers. The RXR consensus is present in cyto-

plasmic domains of various transmembrane proteins. For example, it is found in the C-terminal domains of most mGlu receptors, with the exception of mGlu2 and mGlu3. It was claimed that mGlu4 receptors, which form homodimers and are structurally related to GABA_B receptors, could act as a chaperone for

Figure 6. Surface expression of myc- and HA-tagged GABA_B receptor mutants. *A*, Surface expression in transfected HEK293. The C-terminal deletion mutant R1L921 is expressed at the cell surface, whereas R1P928 is retained intracellularly. Substitution of alanine for R⁹²² (R1[ASRRHPP]), R⁹²⁴ (R1[RSARHPP]), and to a lesser extent R⁹²⁵ (R1[RSRAHPP]) results in surface expression of GABA_{B(1)}, whereas substitution of S⁹²³ (R1[RARRHPP]) does not affect intracellular retention. A C-terminal fragment of GABA_{B(2)} encoding residues R⁷¹⁴ through P⁸²⁰ (CR2P820) efficiently traffics WT GABA_{B(1)} to the plasma membrane. R2L791 with a more extensive C-terminal deletion is unable to deliver WT GABA_{B(1)} to the surface. Removal of the LZ1 is sufficient to direct R1ΔLZ1 to the cell surface, indicating that LZ1 is required for correct exposure of the ERR/R signal. R1LZ2 does not reveal surface immunoreactivity but is detectable at the plasma membrane on coexpression with either R2LZ1 or WT GABA_{B(2)}. R1[PPP] does not show significant surface immunoreactivity when expressed alone but is targeted to the plasma membrane on coexpression with either WT GABA_{B(2)} or R2[PPP]. Coexpression with R2T749 is insufficient to traffic R1[PPP] to the cell surface, because no shielding of the fully functional ERR/R in R1[PPP] is provided. For detection of intracellular myc and HA epitopes, (permeabilized) transfected cells were permeabilized with saponin. *B*,

Figure 7. Ca²⁺-sensing (CaSR) and mGlu receptors do not traffic WT GABA_{B(1)} to the cell surface. Coexpressed proteins were always from identical species (rat or human). Surface photoaffinity-labeling experiments are as outlined in Figure 1*E*.

GABA_{B(1)} (Sullivan et al., 2000). In that study, coexpression with mGlu4 led to the surface expression of GABA_{B(1)} as determined by immunoblot analysis and flow cytometry. However, mGlu4+GABA_{B(1)} heteromers were not formed, and no functional coupling of the monomer was observed.

We systematically analyzed in photoaffinity labeling experiments whether Ca²⁺-sensing and mGlu receptors mask the ERR/R signal in GABA_{B(1)} and facilitate surface expression (Fig. 7). None of the receptors that were analyzed, including mGlu4, were able to convincingly deliver GABA_{B(1)} to the cell surface. Consistent with this finding, none of the expressed proteins yielded functional GABA_B receptors in combination with GABA_{B(1)} and GABA_{B(2)} (data not shown). It is of note that mGlu5 and the Ca²⁺-sensing receptors are as ineffective as the other mGlu receptors in trafficking GABA_{B(1)}, although they are well known to homodimerize (Romano et al., 1996; Ward et al., 1998; Bai et al., 1999; Ray et al., 1999). These data demonstrate that the shielding of the ERR/R signal contained in the cytoplasmic domain of GABA_{B(1)} has structural requirements provided by the GABA_{B(2)} backbone but not by the related mGlu or Ca²⁺-sensing receptor proteins.

DISCUSSION

To date, the physiological significance of GPCR dimerization remains largely unknown. GABA_B receptors are currently the only example of where function depends on dimerization. GPCRs other than GABA_B, such as the serotonin (Xie et al., 1999), opioid (Jordan and Devi, 1999), dopamine D₁/adenosine A₁ receptors (Ginés et al., 2000) and angiotensin II AT₁/bradykinin B₂ (Abdalla et al., 2000) have recently been shown to assemble from distinct subunits. Heteromerization can change pharmacological properties and potentiate signal transduction. This raises the possibility that heteromerization is a relevant and more common principle among GPCRs. Conceptually, this largely increases the

Surface expression in transfected primary cerebellar granule cells. Neurons transfected with myc-tagged WT GABA_{B(1)} (R1) alone do not show cell surface immunoreactivity. Strong c-myc surface staining is detected with cells cotransfected with WT GABA_{B(1)} and GABA_{B(2)} (R1+R2) and with mutant R1[ASARHPP]. For surface labeling, transfected cerebellar neurons were incubated with anti-c-myc antibody in culture medium for 1 hr, washed, and fixed with paraformaldehyde. Neurons were incubated for 1.5 hr with anti-mouse IgG Texas Red conjugate (1:1000), washed extensively, mounted on coverslips, and viewed with a 63× magnification objective. For labeling of permeabilized cells, paraformaldehyde-fixed neurons were permeabilized with 0.05% Triton X-100 before incubation with primary and secondary antibodies. Three independent experiments were performed.

already vast repertoire of GPCRs and may significantly change our view of how these receptors operate. Understanding the mechanisms that govern assembly of functional heteromeric GPCRs is therefore a fundamental problem and may reveal dimerization partners that otherwise are difficult to discover.

For any protein that oligomerizes in the ER, the proper tertiary and quaternary structures must be achieved before the release. Sorting mechanisms are in place to discriminate between the unassembled subunits and completed oligomers. These mechanisms include, for example, the exposure and masking of short discrete ERR/R motifs. The best-characterized signals are the luminal KDEL sequences of soluble and type II transmembrane proteins (Munro and Pelham, 1987) and the cytoplasmic KK sequences of type I membrane proteins (Teasdale and Jackson, 1996). Other signals include RR (Schutze et al., 1994) or RXR (Zerangue et al., 1999) in the cytoplasmic domain of the retained protein.

It was shown that GABA_{B(2)} can target to the cell surface by itself (Marshall et al., 1999). In contrast, GABA_{B(1)} is retained in the ER in the absence of coexpressed GABA_{B(2)}, and heterodimerization results in a profound enhancement of surface expression (Couve et al., 1998; Marshall et al., 1999). The mechanism of plasma membrane targeting was not understood. We now show that a four amino acid motif in GABA_{B(1)}, RSRR, tightly inhibits the surface expression of monomers. The RSRR motif combines two known ERR/R signals: RXR (Zerangue et al., 1999) and RR (Schutze et al., 1994). One would assume that proteins possessing this enhanced motif would be retained more efficiently in the ER than those featuring the minimal motif.

GABA_{B(2)} was found in yeast two-hybrid screens using the GABA_{B(1)} C terminus as a bait, suggesting that the leucine zippers were responsible for heteromerization of the GABA_{B(1,2)} complex (White et al., 1998; Kammerer et al., 1999; Kuner et al., 1999). We show that a GABA_{B(2)} C-terminal fragment, CR2P820, is sufficient to reverse the ER retention imposed by the RSRR motif (Fig. 4D). The close proximity of the RSRR motif to the C-terminal LZ1 suggests that the coiled-coil interaction is responsible for masking the ERR/R signal. In support of such an involvement, shielding of the ERR/R signal is lost once serial deletions of the C terminus of GABA_{B(2)} progress into the leucine zipper domain (Fig. 4C) or when the LZ2 is deleted in R2ΔLZ2 (Fig. 5B). Surprisingly, however, mutation of the leucine zipper in R1[PPP] still allows the formation of functional surface receptors together with WT GABA_{B(2)} and R2[PPP] (Fig. 5D,E). These latter results suggest that some shielding of the ERR/R signal still occurs when leucine zipper interactions are partly or completely destabilized. It does not imply, however, that in WT receptors the coiled-coil interaction is not involved in masking the ERR/R signal. Our conclusion from these seemingly conflicting results is that the leucine zipper interaction is involved in, but not absolutely necessary for, masking the ERR/R signal. Accordingly, functional interactions between GABA_{B(1)} and GABA_{B(2)} can still be obtained when leucine zipper interactions are weakened or prevented by mutation or deletion. From a therapeutic point of view, it appears that drugs that disrupt the coiled-coil interaction will not necessarily reduce the number of GABA_B receptors expressed at the cell surface. Possibly the coiled-coil domains also serve functions other than GABA_B surface trafficking and support interaction with additional proteins, such as recently shown for ATF-4 (Nehring et al., 2000).

Human GABA_{B(1)} polymorphisms were described, but none of them involve the LZ1 domain or the RSRR motif (Sander et al., 1999). Known GABA_{B(1)} splice variants conserve the ERR/R

signal and the LZ1 domain (Isomoto et al., 1998) (GeneBank accession number AJ012187). Moreover, two C-terminal splice variants of GABA_{B(2)} (our unpublished data) do not alter the LZ2 domain. This emphasizes the importance of the coiled-coil domains for receptor maturation. We did not observe any trafficking of GABA_{B(1)} to the surface when coexpressed with several family 3 GPCRs. However, it has been shown recently that ionotropic and metabotropic receptors are able to undergo a physical association (Liu et al., 2000). Proteins with unrelated structure therefore may traffic GABA_{B(1)} to the cell surface. Although GABA_{B(1)} monomers do not efficiently couple through Gα and Gβγ, it is possible that once at the cell surface GABA_{B(1)} signals through G-protein independent pathways, as shown for the mGlu receptors (Heuss et al., 1999). The interdependence between heteromerization, exit of GABA_{B(1)} from the ER, and functional activity makes it difficult to separate the role of individual receptor domains. However, no obvious functional properties of heterologous GABA_B receptors are lost when both C termini are deleted. It follows that the primary determinants for heteromeric receptor assembly and function do reside in the N-terminal domain and TMD.

The ER trafficking signal identified here was first characterized in K_{ATP} channels (Zerangue et al., 1999). This signal was proposed to serve as a quality control mechanism that ensures only the surface expression of properly assembled octameric channels. Because for WT subunits only GABA_{B(1,2)} complexes are shown to be capable of robust signal transduction, a similar mechanisms may exist that prevents monomeric GABA_{B(1)} from expressing on the cell surface. Mutations leading to truncation of the C terminus of SUR1 are one cause of a severe, recessive form of persistent hyperinsulinemic hypoglycemia of infancy, most likely because aberrant K_{ATP} channels are formed (Zerangue et al., 1999). Because GABA_{B(1)} monomers are functionally inert when expressed on the plasma membrane, they would not have obvious harmful effects. An important aspect of GPCR regulation is the control of the number of receptors expressed on the cell surface. The ERR/R signal identified here is poised to play a dynamic role in controlling the level of surface expression of heterodimeric receptors. Slow trafficking rates may create a large intracellular pool of GABA_{B(1)} protein that is ready to go to the cell surface in the event that GABA_{B(2)} is provided. For the GABA_B receptors, ERR/R may therefore represent a means to rapidly regulate surface expression levels, as proposed similarly for Homer1b and mGlu5 (Roche et al., 1999).

After submission of this manuscript a similar study reporting the identification of the RSRR motif in GABA_{B(1)} was published (Margeta-Mitrovic et al., 2000). The authors of that study reached conclusions that are mostly similar to ours. However, our data do not support the conclusion that “interaction of GABA_{B(1)} and GABA_{B(2)} through the C-terminal coiled-coil α helices seems to be required not only for the shielding of the retention/retrieval signal RSRR in GABA_{B(1)} but also for the functional activity of the fully assembled complex.” Margeta-Mitrovic et al. (2000) base their statement on an experiment with point mutations in the coiled-coil and RSRR regions of GABA_{B(1)}. Although they observe surface expression of that GABA_{B(1)} mutant, they do not see function on coexpression with GABA_{B(2)}. In contrast, we observe in all of our experiments with either deleted (Figs. 1C,D, 2C,E, 3B,D,E, 4C,D) or mutated (Fig. 5B,C,D,E) coiled-coil domains, functional heteromeric receptors whenever the two subunits are coexpressed at the cell surface. This shows convincingly that the N-terminal domain and TMD of the two subunits

are sufficient to assemble a functional heteromeric complex provided GABA_{B(1)} exits the ER.

Note

The Nomenclature Committee of the International Union of Pharmacology recommendation for nomenclature of GABA_B receptors will be GABA_{B(1x,2x)}, where 1 and 2 refer to the subunits and x refers to the splice variants.

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