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A Trafficking Checkpoint Controls $GABA_B$ Receptor Heterodimerization

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Summary

Surface expression of $GABA_B$ receptors requires heterodimerization of GB1 and GB2 subunits, but little is known about mechanisms that ensure efficient heterodimer assembly. We found that expression of the GB1 subunit on the cell surface is prevented through a C-terminal retention motif RXR(R); this sequence is reminiscent of the ER retention/retrieval motif RKR identified in subunits of the ATP-sensitive K^+ channel. Interaction of GB1 and GB2 through their C-terminal coiled-coil α helices masks the retention signal in GB1, allowing the plasma membrane expression of the assembled complexes. Because individual $GABA_B$ receptor subunits and improperly assembled receptor complexes are not functional even if expressed on the cell surface, we conclude that a trafficking checkpoint ensures efficient assembly of functional $GABA_B$ receptors.

Introduction

G protein–coupled receptors (GPCRs) constitute the largest known superfamily of membrane receptors, and are involved in the regulation of numerous physiological functions, including hormonal signaling and slow synaptic neurotransmission. While all GPCRs have the same basic membrane topology (extracellular N-terminal domain, seven transmembrane domains, and intracellular C terminus), they generally share little sequence homology and are classified in six large families (A–F; Kolakowski, 1994; see also The G-protein-coupled Receptor Database at www.gcrdb.uthscsa.edu).

In the mammalian brain, the major inhibitory neurotransmitter is γ-aminobutyric acid (GABA). The G protein–coupled receptor for GABA, the $GABA_B$ receptor, is known to mediate presynaptic inhibition of neurotransmitter release as well as the slow inhibitory postsynaptic potential. Two $GABA_B$ receptor genes, each encoding several splice variants, have been cloned to date ($GABA_BR1$, referred to as GB1 in the text [Kaupmann et al., 1997], and $GABA_BR2$, referred to as GB2 in the text [Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Kuner et al., 1999]). Both $GABA_B$ receptors have a large extracellular N-terminal domain with homology to bacterial periplasmic amino acid binding proteins and belong to GPCR family C together with metabotropic glutamate receptors, extracellular Ca^{2+}-sensing receptors, and vomeronasal pheromone receptors.

Hetero- or homomultimerization of GPCRs has been suggested before, but the functional significance of such protein complexes has been unclear (reviewed by Hébert and Bouvier, 1998, and Bockaert and Pin, 1999). Interestingly, dimerization seems to be a particularly important feature of family C GPCRs: homodimerization has been observed with metabotropic glutamate and extracellular Ca^{2+}-sensing receptors (Romano et al., 1996; Bai et al., 1998), and heterodimerization has been reported for $GABA_B$ receptors (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Kuner et al., 1999). It has been demonstrated that coassembly of GB1 and GB2 is required for formation of functional $GABA_B$ receptors that can activate G protein–activated inwardly rectifying K^+ channels (GIRKs), and that each subunit expressed alone is unable to mediate GIRK activation (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Kuner et al., 1999). $GABA_B$ receptor heterodimerization is at least partly mediated through the interaction of coiled-coil α helices in the C termini of both subunits (Kammerer et al., 1999; Kuner et al., 1999); however, the possibility of additional protein–protein interactions has not been excluded.

Folding and assembly of multimeric protein complexes is typically a tightly controlled process that ensures the expression of an appropriate number of fully assembled complexes on the cell surface. The assembly of multimeric protein complexes is often tightly coupled to the trafficking of their individual components, preventing monomers and incompletely assembled complexes from reaching the cell surface. One example of this quality control process is the assembly-dependent trafficking of ATP-sensitive K^+ (K_{ATP}) channels which couple the metabolic state of the cell to its membrane excitability (Zerangue et al., 1999). These channels are octameric complexes that consist of four pore-forming inwardly rectifying α subunits (Kir6.1/2) and four sulphonylurea binding β subunits (SUR1/2A/2B) that belong to the ATP binding cassette family of proteins. Both K_{ATP} subunits contain a short motif, RKR, that functions as an endoplasmic reticulum (ER) retention/retrieval signal and prevents the cell surface expression of individual subunits and partially assembled complexes. Interestingly, the RKR motif is located within the C terminus of Kir6.2 and in a cytoplasmic loop of SUR1, demonstrating relative positional independence in contrast to the previously identified ER retention/retrieval motif KKXX whose activity depends on its position at the very end of the C terminus (Teasdale and Jackson, 1996).

We have investigated assembly-dependent trafficking of heterodimeric $GABA_B$ receptors, and the relevance of this quality control process for the control of $GABA_B$ receptor functional activity. It was shown previously that GB2 trafficks to the cell surface independently of GB1 (White et al., 1998; Martin et al., 1999) and that it does not bind $GABA_B$ receptor ligands with appreciable affinity (Jones et al., 1998; Kaupmann et al., 1998a). In contrast,

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GB1 binds all $GABA_B$-specific ligands with the same rank order of potencies as native $GABA_B$ receptors. Compared with native receptors, GB1 exhibits similar affinity for antagonists but 100-fold lower affinity for agonists (Kaupmann et al., 1997). However, heterologously expressed GB1 is retained in the ER compartment of mammalian fibroblasts, epithelial cells, and primary neurons in culture and is not expressed on the plasma membrane (Couve et al., 1998). We now report that ER retention of GB1 is due to the RSRR motif in its C terminus, a sequence related to the RKR ER retention/retrieval motif in K_{ATP} channel subunits. This retention signal is shielded by the interaction of GB1 and GB2 through their coiled-coil domains, thereby allowing the assembled heterodimeric complex to traffick to the cell surface. Interestingly, cell surface–expressed GB1 subunit, as well as surface-expressed heterodimeric receptor complex with only partly associated subunits, failed to respond to GABA by activating GIRK currents. Thus, only fully assembled heterodimeric $GABA_B$ receptor complexes are functionally active, and a trafficking checkpoint ensures their efficient assembly.

Results

Identification of Trafficking Signals in the C Terminus of GB1

To reliably measure only the receptor protein expressed on the cell surface, we introduced hemagglutinin (HA) epitopes in N-terminal extracellular domains of GB1 (splice variant 1a) and GB2. After expressing these constructs in COS7 cells, we quantitated the surface protein using an assay developed by Zerangue et al. (1999). In this assay, extracellularly exposed epitopes are labeled with a specific primary antibody followed by a horseradish peroxidase– (HRP-) conjugated secondary antibody, and antibody bound to the cell surface is quantitated by measurement of the chemiluminescence. Results of this assay for wild-type GB1 and GB2 are shown in Figure 1. In agreement with previously published results (Couve et al., 1998; White et al., 1998), expression of wild-type HA-tagged GB1 by itself did not result in surface labeling above background levels, while coexpression of nontagged GB2 (in a 1:1 ratio) resulted in a robust stimulation of surface labeling (Figure 1A). Increased surface expression of GB1 did not result from a change in the total GB1 protein levels, as shown in Western blots of cleared cell lysates (Figure 1A). We also assayed the surface expression of HA-tagged GB2. Again in agreement with published data (White et al., 1998; Martin et al., 1999), HA-GB2 trafficked to the cell surface when expressed by itself (Figure 1B). However, its surface expression, as well as the total protein level as detected by Western blotting, was decreased by coexpression of nontagged GB1. The decrease in protein levels of HA-GB2 did not result from nonspecific effects of coexpression, as the total protein level of GB1 was not affected by cotransfection. Reduced surface expression of HA-GB2 may simply reflect the decrease in the total HA-GB2 protein. Alternatively, the observed reduction of the HA-GB2 surface expression could have resulted from masking of the HA epitope in the GB1/HA-GB2 assembled complex. The latter possibility is more likely, as we were not able to visualize subcellular distribution of GB2 upon coexpression of GB1 using immunofluorescence (data not shown).

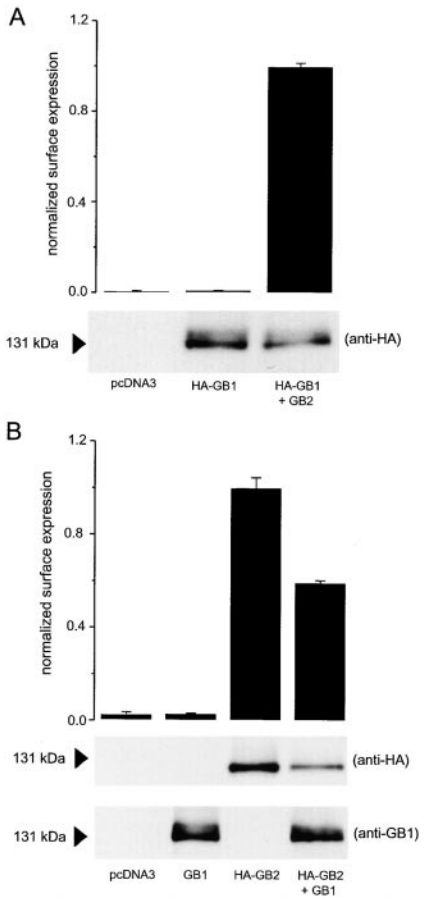

Figure 1. GB1 Is Present on the Cell Surface Only When Coexpressed with GB2

Extracellular HA epitopes were introduced in N-terminal domains of GB1 and GB2 to allow measurement of exclusively cell surface–expressed protein. Twenty-four hours after transfection in COS7 cells, HA-tagged GB1 or GB2 present on the cell surface was detected with an anti-HA primary and an HRP-conjugated secondary antibody; the bound antibody was quantitated by analytical luminometry. Each surface labeling assay shown in this and subsequent figures is a representative example from several experiments with similar results. In this and subsequent figures data plotted represent mean ± S.E.M. for three 35 mm dishes that were assayed in an individual experiment, and are normalized to measurements obtained with coexpression of HA-GB1 and GB2. Western blots of total HA-tagged protein or of nontagged GB1 in cleared cell lysates are shown beneath the plots.

(A) HA-GB1 subunit of $GABA_B$ receptor expressed by itself is not detectable on the cell surface, but coexpression of nontagged GB2 subunit results in significant cell surface labeling. Total HA-GB1 protein level was not significantly affected by coexpression of GB2.

(B) HA-GB2 subunit is present on the cell surface when expressed alone. Coexpression of nontagged GB1 results in a decrease in HA-GB2 cell surface labeling and in total HA-GB2 protein. Decrease in HA-GB2 protein was specific, as GB1 protein levels were not affected by coexpression.

To identify the signal in GB1 that prevents its plasma membrane expression, we analyzed surface expression of several C-terminal deletion constructs; results of these experiments are summarized in Figure 2Cb. Complete deletion of the C terminus (except for the first two amino acids beyond the seventh transmembrane

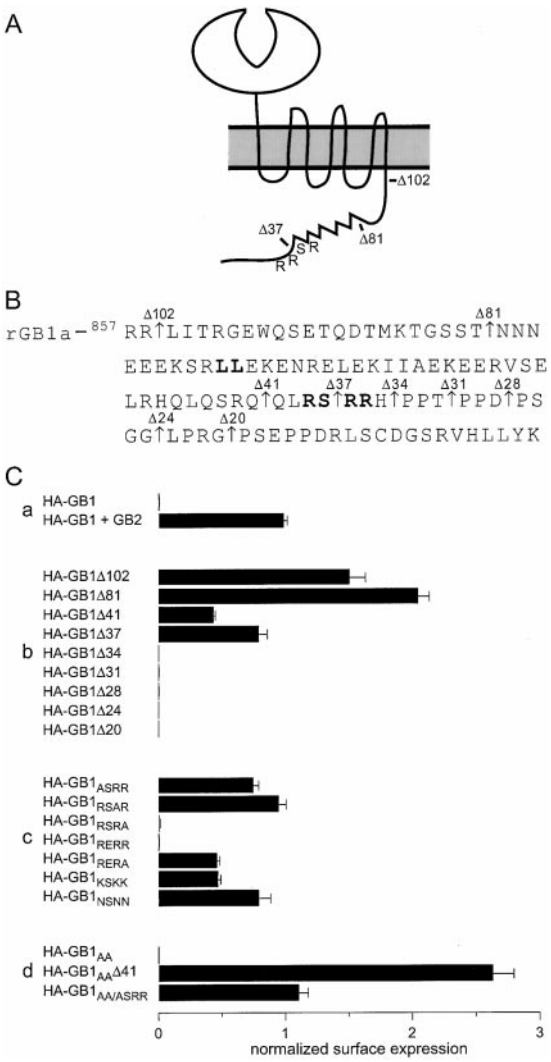

Figure 2. Identification of Molecular Determinants of GB1 Cell Surface Expression

(A) Schematic diagram of $GABA_B$ receptor GB1 subunit.

(B) Sequence of rat GB1a C terminus. The C-terminal end of individual C-terminal deletion constructs is indicated by arrows. The retention motif RSRR identified through these experiments, as well as a di-leucine internalization motif that plays a role in GB1 trafficking, are shown in bold.

(C) Cell surface expression data for individual HA-GB1 mutants.

(a) Cell-surface expression of wild-type HA-GB1 and of HA-GB1 coexpressed with GB2.

(b) Sequential C-terminal deletions implicated the RSRR cluster (residues 922–925 in rat GB1a) in mediating the intracellular retention of HA-GB1.

(c) Mutagenesis of residues within RSRR cluster indicated that sequence determinants of the HA-GB1 intracellular retention motif are RXR(R).

(d) Replacing two leucines (L889 and L890 in rat GB1a) of the di-leucine motif with alanines did not result in surface expression of HA-GB1 but did increase surface expression of HA-GB1Δ41 and $HA\text{-}GB1_{ASRR}$.

domain; construct HA-GB1Δ102) resulted in surface expression above the levels observed when wild-type HA-GB1 was coexpressed with GB2. Large surface expression was also observed with constructs HA-GB1Δ81 and HA-GB1Δ41 but not with construct HA-GB1Δ20, which did not induce surface labeling above background levels. As these results suggested that the region flanked by deletions Δ41 and Δ20 may contain a retention signal, we constructed an additional series of deletions covering those 20 amino acids (constructs HA-GB1Δ37, Δ34, Δ31, Δ28, and Δ24). Of these deletions, only construct HA-GB1Δ37 was expressed on the cell surface. These results suggested that an arginine-rich sequence RSRR (left intact in Δ34 but disrupted in Δ37) could be responsible for the intracellular retention of GB1; this sequence is reminiscent of the ER retention/retrieval motif RKR identified in subunits of the K_{ATP} channel (Zerangue et al., 1999).

We analyzed specific molecular determinants of the RSRR sequence using different point mutants; results from these experiments are summarized in Figure 2Cc. Constructs $HA\text{-}GB1_{ASRR}$ and $HA\text{-}GB1_{RSAR}$, but not the construct $HA\text{-}GB1_{RSRA}$, were expressed on the cell surface, indicating that arginine is required in the first and the third but not in the fourth position of the arginine cluster. In addition, as in the RKR motif of K_{ATP}, these arginines could not be replaced with lysines or asparagines (both $HA\text{-}GB1_{KSKK}$ and $HA\text{-}GB1_{NSNN}$ constructs were expressed on the cell surface). Interestingly, substitution of serine with glutamic acid in the RSRR motif (construct $HA\text{-}GB1_{RERR}$) did not result in the surface expression of GB1, but construct $HA\text{-}GB1_{RERA}$ did show appreciable surface expression. Thus, although the last arginine in the RSRR sequence is not required for the ER retention/retrieval of GB1, it does contribute to the strength of the retention signal. Based on these results, we define the sequence of the retention motif in GB1 as RXR(R).

While expression of deletion constructs HA-GB1Δ102, Δ81, Δ41, and Δ37 in COS7 cells resulted in significant surface labeling in each case, we observed appreciable differences in the extent of their plasma membrane expression. In particular, HA-GB1Δ41 was expressed at a significantly lower level than HA-GB1Δ81, suggesting an additional plasma membrane expression determinant in the region flanked by these deletions. This region contains a potential di-leucine motif (sequence EKSRLL), a signal generally known to play a role in the endosomal and *trans*-Golgi network targeting of different proteins (Trowbridge et al., 1993; Petris et al., 1998; Kirchhausen, 1999). To determine the effect of this sequence on the trafficking of GB1, we replaced the key leucine residues (L889 and L890 in rat GB1a) with alanines in the wild-type construct as well as in HA-GB1Δ41 and $HA\text{-}GB1_{ASRR}$ mutants, and assayed their surface expression (Figure 2Cd). Expression of $HA\text{-}GB1_{AA}$ in COS7 cells did not result in surface labeling above background, indicating that the di-leucine motif by itself was not responsible for the intracellular retention of GB1. However, surface expression of $HA\text{-}GB1_{AA}$Δ41 was strongly (6-fold) enhanced relative to the expression of HA-GB1Δ41, and was comparable to the expression of HA-GB1Δ81. In addition, cell surface expression of $HA\text{-}GB1_{AA/ASRR}$ was enhanced relative to the expression of $HA\text{-}GB1_{ASRR}$, although the difference was smaller than with the two deletion constructs.

We also determined the distribution of wild-type and mutant HA-GB1 proteins by immunofluorescent staining (Figure 3). As expected, visualization of surface-expressed HA epitopes by labeling of cells with an anti-HA antibody prior to permeabilization (Figure 3A) gave

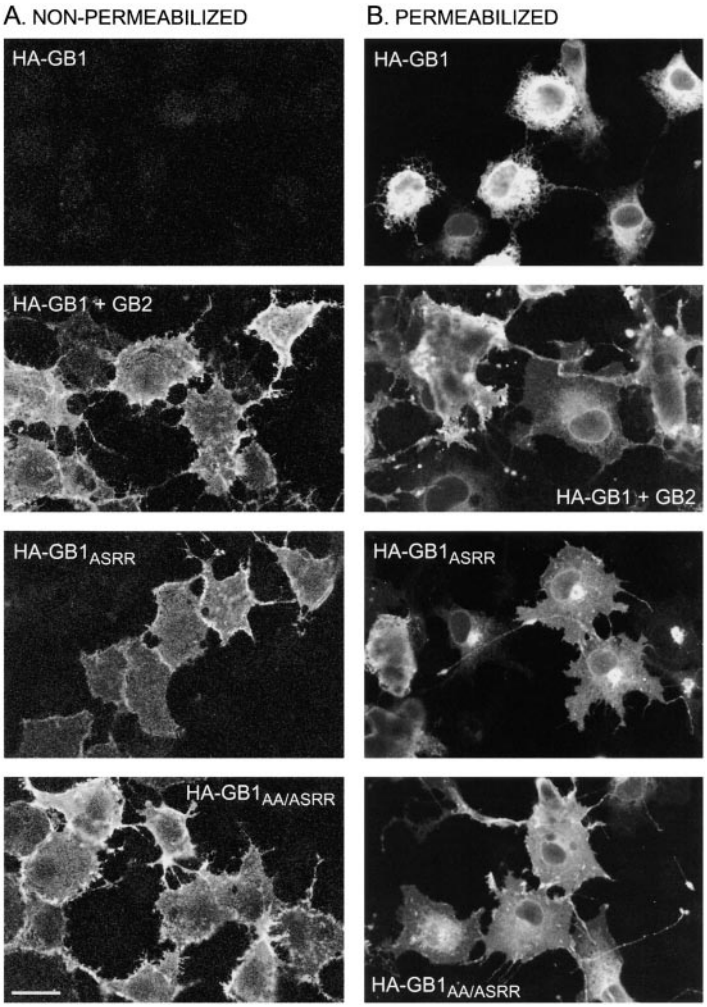

Figure 3. Disruption of the RSRR Motif Allows ER Exit of GB1 in the Absence of GB2, While Additional Mutagenesis of the Di-Leucine Motif Prevents Accumulation of GB1 in a *trans*-Golgi Network-like Compartment

(A) To visualize only cell surface–expressed HA-tagged protein, 24 hr after transfection COS7 cells were labeled with the anti-HA antibody prior to permeabilization. As in the quantitative surface expression assay, wild-type HA-GB1 was not present on the cell surface unless coexpressed with GB2. In contrast, mutants HA-GB1$_{ASRR}$ and HA-GB1$_{AA/ASRR}$ were expressed on the cell surface.

(B) To visualize total cell HA-tagged protein, permeabilized COS7 cells were labeled with the anti-HA antibody 72 hr after transfection. HA-GB1 accumulated in a reticular perinuclear ER-like compartment; coexpression of GB2 resulted in greatly reduced labeling of that compartment and in cell surface expression of HA-GB1. Similar expression pattern was seen with HA-GB1$_{ASRR}$, except that this mutant also accumulated in a juxtanuclear compartment consistent with its localization to the *trans*-Golgi network. Labeling of this compartment was abolished by additional mutation of the di-leucine motif (HA-GB1$_{AA/ASRR}$). A similar pattern of HA-GB1 distribution was seen when immunostaining was done 24 hr after transfection (data not shown), except that at that time point a fraction of HA-GB1 was accumulated in the ER in all cases. Scale bar, 25 μm.

the same results as quantitative surface labeling (Figures 1 and 2). Subcellular localization of wild-type and mutant HA-GB1 proteins was visualized by immunofluorescent staining of permeabilized COS7 cells (Figure 3B). HA-GB1 expressed alone strongly labeled a reticular perinuclear compartment, consistent with its retention in the ER. Coexpression of GB2 abolished the ER accumulation of HA-GB1 and resulted in a strong surface staining; an analogous staining pattern was observed with HA-GB1$_{ASRR}$. Interestingly, while HA-GB1$_{ASRR}$ was strongly expressed on the cell surface, it also accumulated in a juxtanuclear compartment, consistent with its targeting to the *trans*-Golgi network (Petris et al., 1998). Labeling of that structure was not observed with HA-GB1$_{AA/ASRR}$, a construct with mutations in both the di-leucine motif and the retention signal RSRR; this result is consistent with the di-leucine motif–mediated internalization of surface-expressed HA-GB1 and its subsequent localization to the *trans*-Golgi network.

GB1/GB2 Protein–Protein Interactions and Their Role in the Cell Surface Trafficking of the Receptor Complex

The identified ER retention/retrieval signal RSRR in GB1 is positioned at the very end of the coiled-coil domain (boundaries of the domain were determined using Pair-Coil algorithm with cutoff defined as $p = 0.5$; Berger et al., 1995). This coiled-coil domain was shown to participate in the formation of the GABA$_B$ receptor heterodimeric complex (Kammerer et al., 1999; Kuner et al., 1999). Thus, we wanted to determine whether the interaction of the coiled-coil α helices of GB1 and GB2 was involved in the shielding of the retention signal in GB1, allowing the cell surface expression of the assembled complexes. For this purpose we introduced four mutations in the a and d positions in the middle of the predicted coiled-coil domains of GB1 (mutations L897E, I901A, K904A, and V908A; construct HA-GB1-CC) and GB2 (mutations N793A, L796A, I800A, and L803A; construct GB2-CC). Because amino acids in the a and d positions in coiled-coil α helices generally form the hydrophobic interaction interface, the mutations in HA-GB1-CC and GB2-CC are expected to prevent the coiled-coil interaction between GB1 and GB2.

Like HA-GB1, HA-GB1-CC alone did not exhibit surface labeling above control levels. However, while coexpression of GB2 with HA-GB1 stimulated surface expression of HA-GB1, coexpression of GB2 with HA-GB1-CC did not result in an increase in the surface labeling (Figure 4B). Similarly, coexpression of GB2-CC with HA-GB1 failed to stimulate HA-GB1 surface expression. These results indicate that interaction of coiled-coil α

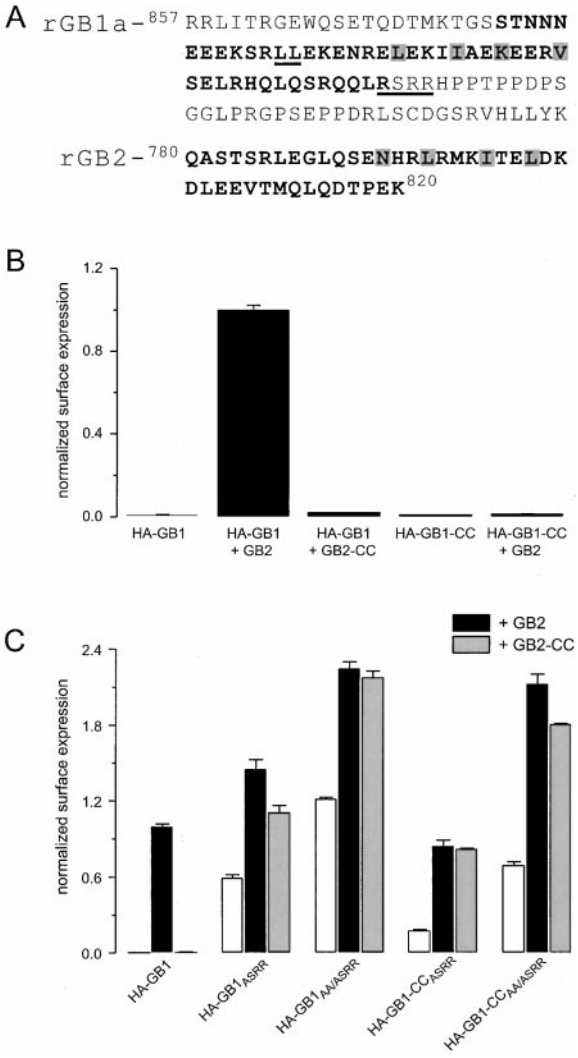

Figure 4. Protein–Protein Interactions in the GB1/GB2 Heterodimer

(A) Sequence of the C terminus of rat GB1a and of the coiled-coil domain of rat GB2. Coiled-coil portions of the receptor sequences (determined using the PairCoil algorithm, $p > 0.5$) are shown in bold. Residues mutated to disrupt the hydrophobic interaction interfaces in either GB1 or GB2 (-CC constructs) are shaded in gray. (They were mutated as follows: L897E, I901A, K904A, and V908A in rat GB1a; N793A, L796A, I800A, and L803A in rat GB2.) The RSRR motif and two leucines of the di-leucine motif are underlined.

(B) Surface expression of HA-GB1 constructs in the presence or absence of GB2 or GB2-CC was assayed in COS7 cells. Disruption of the interaction interface in the coiled-coil α helix of GB1 (construct HA-GB1-CC) or GB2 (construct GB2-CC) abolished the ability of GB2 to promote expression of HA-GB1 on the cell surface.

(C) Surface expression of different HA-GB1 constructs in COS7 cells was assayed when they were expressed alone (white bars), coexpressed with GB2 (black bars), or coexpressed with GB2-CC (gray bars). Only wild-type GB2 could enhance surface expression of wild type HA-GB1. In contrast, surface expression of all HA-GB1 retention signal mutants (including constructs with disrupted coiled-coil interfaces) was increased by coexpression of either GB2 or GB2-CC.

helices of GB1 and GB2 is required for the shielding of the retention motif in GB1, allowing the ER exit of the assembled complexes.

To investigate whether GB1/GB2 interactions were abolished when their coiled-coil interfaces were disrupted, we examined how coexpression of GB2 or GB2-CC affected surface expression of different GB1 mutants (Figure 4C). Coexpression of GB2 potentiated surface expression not only of HA-GB1, HA-$GB1_{ASRR}$, and HA-$GB1_{AA/ASRR}$ but also of HA-GB1-CC_{ASRR} and HA-GB1-$CC_{AA/ASRR}$, two mutants with disrupted coiled-coil interfaces. Similarly, GB2-CC enhanced surface expression of all HA-GB1 retention signal mutants but not of wild-type HA-GB1. (Total HA-GB1 protein levels were similar in all cases and thus could not account for the observed changes in surface protein levels [data not shown].) Taken together, the data suggest that GB1 and GB2 can interact through domains other than their coiled-coil α helices. However, while these interactions can enhance surface expression of GB1 with the mutated retention signal, only the interaction of the coiled-coil domains allows shielding of the intact retention signal and the surface expression of the assembled wild-type receptor complexes.

Interestingly, HA-GB1-CC_{ASRR} was expressed on the surface to a lesser extent than HA-$GB1_{ASRR}$, but this difference was abolished by mutating the di-leucine motif EKSRLL involved in GB1 internalization from the cell surface (construct HA-GB1-$CC_{AA/ASRR}$). Thus, the di-leucine motif–mediated internalization appears to play a larger role in GB1 trafficking in the context of the coiled-coil mutations, perhaps due to partial unfolding of the GB1 C terminus under those conditions.

Retention of a Reporter Protein by the GB1 C Terminus Can Be Abolished by Coiled-Coil-Mediated Interaction with GB2

To determine whether the C terminus of GB1 can confer ER retention/retrieval to another protein, we fused the last 104 amino acids of GB1 to the C terminus of the CD4 glycoprotein and measured the surface expression of this reporter construct (CD4-GB1T; Figure 5A). The GB1 sequence was less effective in retaining CD4 fusion proteins than the last 36 amino acids from the C terminus of Kir6.2 (CD4-36T) or the previously characterized ER retention/retrieval sequence KKXX (CD4-KKXX), resulting in surface labeling above background levels. However, the surface expression of CD4-GB1T was greatly decreased in comparison with surface expression of CD4-AAXX. In addition, mutating the retention sequence RSRR in the C terminus of the reporter protein to ASRR or NSNN resulted in a substantial increase in the surface expression of the relevant fusion constructs. In contrast, the last 197 amino acids (the whole C terminus) of GB2 did not cause the intracellular retention of the fusion protein CD4-GB2T; this is consistent with the observation that GB2 can traffick to the cell surface independently of GB1. Taken together, these data suggest that the retention signal RSRR in the C terminus of GB1 can confer intracellular retention when transferred to another protein, as previously shown for the tail of Kir6.2 containing the ER retention motif RKR (Zerangue et al., 1999).

We have also investigated whether GB2 can promote surface expression of CD4-GB1T through the shielding of the retention signal RSRR in the C terminus of the fusion protein (Figure 5B). Indeed, coexpression of CD4-GB1T and GB2 in a 1:1 ratio resulted in a 2.5-fold increase of CD4-GB1T surface expression. This result was

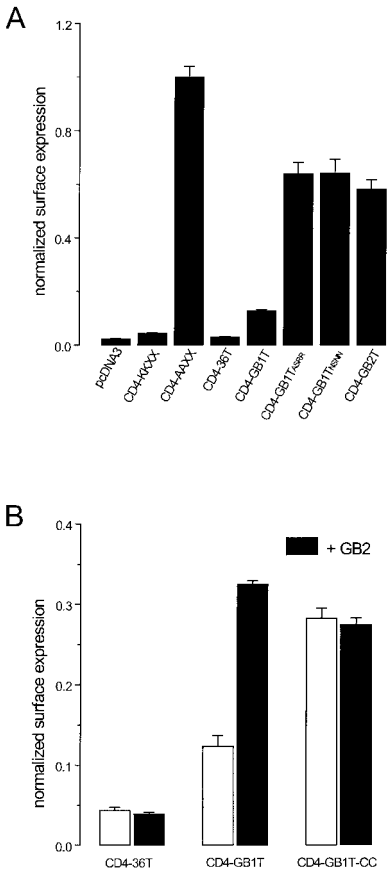

Figure 5. RSRR Retention Motif of GB1, as Well as Its Shielding, Functions in the Context of the CD4 Reporter Protein

Wild-type or mutated C termini of GB1 or GB2 were fused to the C terminus of human CD4 glycoprotein, and cell surface expression of the fusion proteins was assayed with an anti-CD4 primary antibody; all results were normalized to the value measured for CD4-AAXX.

(A) Fusion of the GB1 C terminus to the CD4 protein (CD4-GB1T) markedly diminished its surface expression, while similar fusion of the GB2 C terminus (CD4-GB2T) was without a strong effect. Mutations in the RSRR sequence in the fused GB1 tail (CD4-GB1T_{ASRR}, CD4-GB1T_{NSNN}) attenuated the intracellular retention conferred by the wild-type tail and allowed surface expression of the fusion protein.

(B) Cell surface expression of CD4-GB1T was stimulated by coexpression of GB2; this stimulation was specific (it did not affect surface expression of CD4-36T, the last 36 amino acids of Kir6.2 fused to the CD4 protein) and was abolished by mutations affecting the coiled-coil interaction interface in the tail of GB1 (surface expression of CD4-GB1T-CC was not affected by coexpression of GB2).

specific, because coexpression of GB2 did not promote surface expression of CD4-36T. To test whether shielding of the retention signal was dependent on the interaction of coiled-coil domains in this context, we constructed the CD4-GB1T fusion protein with mutations in this domain (CD4-GB1T-CC; same mutations as described for GB1-CC; Figure 4A). While by itself this construct was expressed on the cell surface somewhat more than CD4-GB1T, GB2 was not able to promote its surface expression further. Thus, we conclude that the interaction of coiled-coil domains in the C termini of GB1 and GB2 is necessary and sufficient for shielding of the retention motif RSRR in the tail of GB1, and that this effect persists even in the absence of the extracellular and transmembrane domains of GB1.

Only Completely Assembled GB1/GB2 Heterodimers Are Functional

Previous studies have shown that GB1 expressed by itself cannot activate ion channel effectors such as GIRKs in *Xenopus* oocytes, and that only very rare activation of GIRKs by GB1 is observed in mammalian cell lines (Jones et al., 1998; Kaupmann et al., 1998a, 1998b; White et al., 1998; Kuner et al., 1999). As GB1 expressed alone is retained in the ER, it is not known whether it can activate GIRKs if expressed on the cell surface by itself. To test this, we injected GIRK1 and GIRK2 cRNAs together with different GB1 cRNAs in *Xenopus* oocytes, and recorded currents induced in response to 100 μM GABA 24 hr after injection (Figure 6). As demonstrated before, GB1 or GB2 expressed alone were not able to stimulate GIRK currents (data not shown). However, coexpression of GB1 and GB2 resulted in a robust GABA-induced GIRK current (Figure 6A). When HA-$GB1_{ASRR}$ (which trafficks to the cell surface in the absence of GB2) was coexpressed with GIRKs, we did not observe any induced GIRK currents in response to 100 μM GABA (Figure 6B) or 1 mM GABA (data not shown). The same result was observed with $GB1_{NSNN}$, HA-GB1Δ81, and HA-GB1-CC_{ASRR} (data not shown). Moreover, the same results were observed 48 hr after oocyte injection (data not shown). Thus, like GB2, GB1 expressed on the cell surface by itself cannot activate GIRK channels, indicating that only GB1/GB2 heterodimeric complexes are functionally active. Because our surface expression data suggested that GB1 and GB2 interacted through domains additional to coiled-coil α helices, we investigated whether those interactions were sufficient for the functional activity of the receptor complex by coexpressing HA-GB1-CC_{ASRR} and GB2 together with GIRK1 and GIRK2. Even though expressed on the oocyte surface at levels comparable to wild-type HA-GB1/GB2 complexes (data not shown), the putative HA-GB1-CC_{ASRR}/GB2 receptor complexes were not able to stimulate GIRK current (Figure 6D). In contrast, coexpression of GB2 with HA-$GB1_{ASRR}$ resulted in a physiologically active receptor complex (Figure 6C). Thus, the coiled-coil domain interactions are necessary (although not necessarily sufficient) for the physiological activity of $GABA_B$ receptors.

Discussion

Trafficking Signals in the C Terminus of GB1

Individual subunits of many multimeric protein complexes are retained in the ER, and for some membrane proteins this serves as a quality control mechanism preventing cell surface expression of partially assembled or misfolded complexes (Zerangue et al., 1999). Recently, a novel ER retention/retrieval signal RKR has been identified in α and β subunits of the K_{ATP} channel (Zerangue et al., 1999). This signal can function in N or C termini as well as in cytoplasmic loops and is thus potentially involved in trafficking and quality control of many membrane proteins.

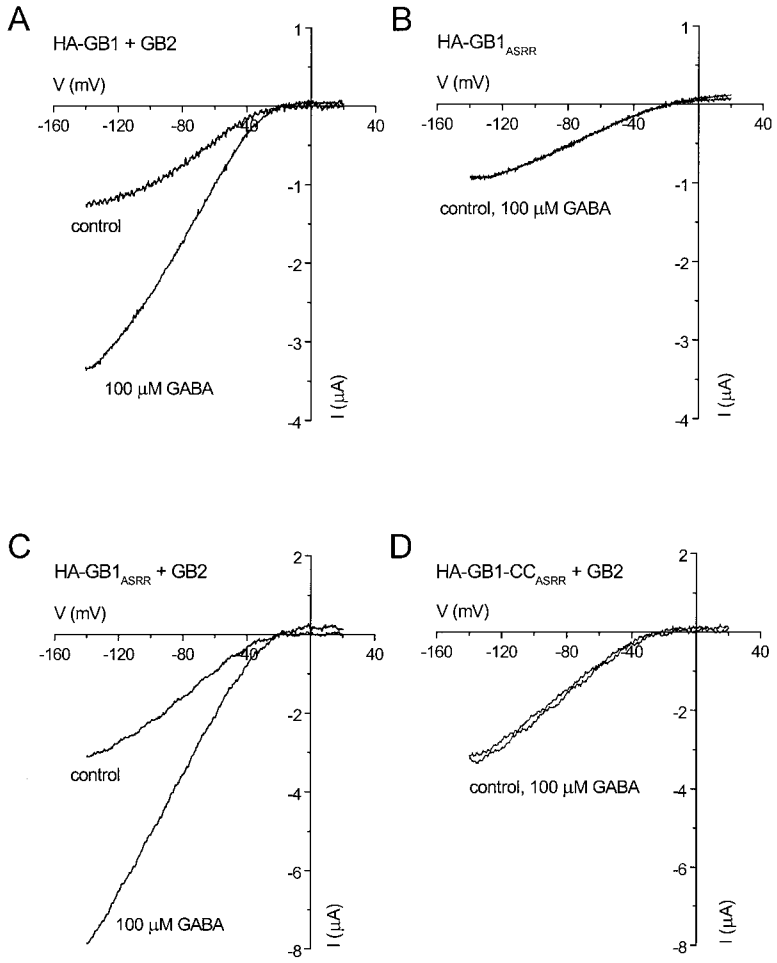

Figure 6. Only Fully Assembled $GABA_B$ Receptor Complexes Can Activate GIRK Channels in *Xenopus* Oocytes

Xenopus oocytes were injected with GIRK1 and GIRK2 cRNAs (~1 ng/oocyte) and $GABA_B$ receptor subunit cRNAs (~5 ng/oocyte) 24 hr before standard two-electrode voltage clamp recording. Currents were recorded in 40K solution with or without 100 μM GABA; the holding potential was −30 mV. Ramp traces (from −140 to 20 mV; ramp speed 1 mV/ms) were recorded every 3 s; all traces were corrected for leak current recorded with the same protocol in ND96 solution.

(A) Coexpression of HA-GB1 and GB2 resulted in activation of GIRK current in the presence of GABA.

(B) HA-$GB1_{ASRR}$ failed to activate GIRK channels in response to GABA.

(C) HA-$GB1_{ASRR}$ coexpressed with GB2 activated GIRK channels in response to GABA.

(D) Coexpression of HA-GB1-CC_{ASRR} and GB2 did not result in GABA-induced GIRK current.

In this work we show that a similar motif, RSRR or more generally RXR(R), is involved in the ER retention of GB1, one subunit of the heterodimeric $GABA_B$ G protein–coupled receptor. The properties of the GB1 retention signal RSRR are very similar to the properties of the RKR signal in K_{ATP} subunits: (1) arginines in the first and the third position are essential for its function; (2) it functions in the middle of the C terminus—i.e., it is relatively position independent; and (3) its function is independent of the general protein context. The first two properties clearly distinguish both the K_{ATP} and the GB1 retention signals from the classical ER retention/retrieval motif KKXX, which consists of a di-lysine motif two residues from the very end of the C terminus (reviewed by Teasdale and Jackson, 1996), as well as from the previously described di-arginine ER retention/retrieval motif whose function depends on its position at the very beginning of the N terminus (Schutze et al., 1994). However, while the second position in the RKR motif of Kir6.2 is quite tolerant to substitutions, some amino acids (i.e., glutamate) in that position are not compatible with a functional retention signal. This was not the case with the RSRR motif of GB1; while we have not done extensive mutagenesis in the second position, substitution of serine with glutamate was tolerated unless we simultaneously replaced the last arginine with alanine. Thus, the additional arginine residue in the fourth position, while not essential by itself, strengthens the overall retention signal. This suggests that there may exist a family of RXR-based ER retention/retrieval signals with varying degrees of retention strength and penetrance, allowing very precise regulation of protein trafficking patterns. Our identification of RXR(R) as an ER retention/retrieval signal in a G protein–coupled receptor, i.e., a protein completely distinct from either ATP binding cassette proteins or inwardly rectifying K^+ channels, underscores the generality of RXR-based motifs, and suggests that they may be important in the regulation of trafficking and assembly of many different membrane proteins.

We have also investigated the role of a di-leucine internalization signal EKSRLL (reviewed by Trowbridge et al., 1993; Kirchhausen, 1999) in GB1 trafficking. While mutating this motif to EKSRAA in the wild-type background (construct HA-$GB1_{AA}$) did not cause GB1 plasma membrane expression, the same mutation potentiated surface expression of different GB1 retention signal mutants. Interestingly, combination of an upstream di-leucine internalization motif and an RXR-type ER retention motif is also present in Kir6.2 (Zerangue et al., 1999), raising the possibility that the two motifs may be parts of a single protein trafficking control module. The di-leucine motif may represent an additional checkpoint that prevents inappropriate surface expression of unas-

sembled or partly associated subunits, as any escaped subunit would be internalized and transported either to the *trans*-Golgi network, from where it could be retrieved back to the ER, or to the lysosome, where it could be degraded.

Protein–Protein Interactions in the $GABA_B$ Receptor Heterodimer

It has been established that GB1 and GB2 interact through coiled-coil α helices located in the C termini of both proteins (Kammerer et al., 1999; Kuner et al., 1999); however, the possibility that these receptor subunits interact through other domains has not been excluded. Thus, we investigated which protein–protein interactions were important for the shielding of the retention signal in GB1 and the ER exit of assembled $GABA_B$ receptor complexes. In addition, we examined whether the binding of coiled-coil α helices represented the sole interaction between the receptor subunits.

The retention signal RSRR is located at the very end of the coiled-coil domain in GB1. Thus, it seemed possible that the interaction of GB1 and GB2 coiled-coil α helices shielded the retention signal in GB1, allowing the ER exit of assembled complexes. This indeed turned out to be the case: when we disrupted the hydrophobic interaction interface in either $GABA_B$ receptor subunit through mutagenesis of four residues in the a and d positions, the stimulation of GB1 plasma membrane expression by coexpression of GB2 was abolished. This was also true in the context of the CD4 reporter protein, indicating that the interaction of coiled-coil domains is both necessary and sufficient for the masking of retention signal and the release of the assembled complexes from the ER.

Coexpression of GB2 increased the surface expression of all HA-GB1 retention signal mutants tested, possibly due to the increased stability of heteromeric receptor complexes on the cell surface, or to a forward trafficking signal in GB2; the mechanism for this potentiation was not further investigated. This effect of GB2 was observed even upon its coexpression with two coiled-coil mutants of GB1 (HA-GB1-CC_{ASRR} and HA-GB1-$CC_{AA/ASRR}$). Similarly, plasma membrane expression of HA-GB1 retention signal mutants, but not of wild-type HA-GB1, was potentiated upon coexpression of GB2-CC. Thus, GB1 and GB2 retained the ability to interact after their coiled-coil interaction interfaces were disrupted, suggesting the existence of additional protein–protein interactions. Indeed, the additional interactions are likely, considering that (1) many other GPCRs seem to homo- or heterooligomerize through transmembrane domains (Hébert and Bouvier, 1998; Bockaert and Pin, 1999), and (2) the most closely related family C GPCRs form homodimers through covalent interactions of their extracellular N-terminal domains (Romano et al., 1996; Bai et al., 1998). These covalent interactions are mediated through the cysteine-rich domain that immediately precedes the first transmembrane domain and is absent in GB1 and GB2. However, additional noncovalent interactions between the large N-terminal domains are likely, and as these domains share the same basic fold in all family C GPCRs (Galvez et al., 1999), it is possible that some of the interactions are conserved. The fact that assembly with GB2 increases the agonist affinity of GB1 (Kaupmann et al., 1998a; White et al., 1998) further supports this possibility, as such an effect is more likely to arise from interactions of N-terminal ligand binding domains than from the C-terminal interactions. While the data presented here suggest that—in addition to the interaction of coiled-coil α helices—other protein–protein interactions are important for heterodimerization of $GABA_B$ receptors, their precise nature and function remain to be identified.

A Trafficking Checkpoint Ensures Efficient Assembly of Functional $GABA_B$ Receptors

Only coexpression of GB1 and GB2 allows robust coupling of recombinant $GABA_B$ receptors to downstream effectors in different heterologous expression systems. However, experiments testing functional ability of individual $GABA_B$ receptor subunits gave ambiguous results. For example, coupling to adenylyl cyclase was reported for both GB1 (Kaupmann et al., 1997) and GB2 (Kuner et al., 1999; Martin et al., 1999; for counterexample, see White et al., 1998). In contrast, neither GB1 nor GB2 expressed by themselves could mediate GABA-induced stimulation of GTPγS binding, while robust stimulation was observed with brain $GABA_B$ receptors and GB1/GB2 heterodimers (White et al., 1998). Coupling of either GB1 or GB2 to ion channel effectors such as GIRKs or voltage-gated Ca^{2+} channels could not be demonstrated in *Xenopus* oocytes but was observed with very low frequency in mammalian heterologous expression systems (Jones et al., 1998; Kaupmann et al., 1998a, 1998b; White et al., 1998; Kuner et al., 1999). Although GB2 is clearly expressed on the cell surface without GB1, it is ineffective in activating GIRK channels (White et al., 1998; Martin et al., 1999). However, it was possible that some of the inconsistencies observed with GB1 were due to its intracellular retention and inefficient plasma membrane expression. It was therefore important to determine whether GB1 could function independently if expressed alone on the plasma membrane, and to elucidate the roles of the individual subunits in the assembled heterodimer. To address these questions, we coexpressed cell surface–expressing GB1 mutants with GIRK1 and GIRK2 in *Xenopus* oocytes and assessed their ability to stimulate GIRK currents in response to GABA. Interestingly, none of the tested mutants was able to activate GIRKs. In addition, GIRK channels could not be activated by GABA in cells coexpressing GB1-CC_{ASRR} and GB2, although surface expression experiments suggested the formation of complexes with partly associated subunits under those conditions. Our results therefore suggest that neither GB1 nor heterodimers with partly associated subunits can functionally couple to ion channel effectors. This is in agreement with the finding that chimeric proteins containing the GB1 N terminus fused to the membrane-spanning and C-terminal domains of metabotropic glutamate receptor 1 are not functionally active, although they bind GABA- and $GABA_B$-specific ligands with wild-type affinity (Malitschek et al., 1999). In fact, it appears that only fully assembled heterodimers represent functional $GABA_B$ receptors. Consistent with this view, Benke et al. (1999) could not detect GB1a, GB1b, or GB2 homodimers in

the brain and indeed demonstrated that all GB2 protein was found in heterodimeric complexes with either GB1a or GB1b in native membranes. In the light of these findings, the weak and inconsistently observed functional activity of either GB1 or GB2 expressed alone in mammalian cells is probably due to the low-level endogenous expression of $GABA_B$ receptor subunits in these cells. Indeed, there is low-level expression of different GB1 splice variants in many peripheral tissues (Isomoto et al., 1998), and the same could be true for GB2 (or some yet unidentified $GABA_B$ receptor subunit that couples to GB1).

Together with previously reported observations, our results suggest that GB2 acts not only as a chaperone or translocator for GB1 but also as an equal partner in the fully assembled heterodimer: it modulates the agonist binding affinity of GB1 (Kaupmann et al., 1998a; White et al., 1998) and seems to play a crucial role in coupling of the receptor complex to G proteins and downstream effectors. Indeed, interaction of GB1 and GB2 through the C-terminal coiled-coil α helices seems to be required not only for the shielding of the retention/retrieval signal RSRR in GB1 but also for the functional activity of the fully assembled complex. However, it remains to be determined whether both receptor subunits or only one of them interact directly with downstream effectors. In addition, as the GB1 distribution in the brain does not completely overlap with distribution of GB2 (Kaupmann et al., 1998a; Kuner et al., 1999), and neither subunit can function alone, it is very likely that there are additional $GABA_B$ receptor subunits not yet identified.

In the assembly of octameric K_{ATP} channel complexes, ER retention of the K_{ATP} subunits mediated by RKR motif prevents the cell surface expression of partially assembled complexes that are functional but improperly regulated and hence deleterious to the cell. Here, we demonstrate that a trafficking checkpoint also regulates assembly and expression of $GABA_B$ receptor heterodimers. In contrast to K_{ATP} channels, however, individual $GABA_B$ receptor subunits—as well as complexes with partly associated subunits—are not functional. It is possible that uncontrolled expression of GB1 on the cell surface could have unfavorable consequences for the cell, for example by titrating out putative receptor binding proteins. In addition to preventing GB1 monomers from reaching the cell surface, the trafficking checkpoint ensures efficient ER assembly and plasma membrane trafficking of heterodimers, the sole known functional $GABA_B$ receptor species to date.

Experimental Procedures

Molecular Biology

Standard molecular biology protocols were adopted from Ausubel et al. (1997). Mammalian expression constructs were all in pcDNA3 (Invitrogen). For oocyte expression, constructs were either in pGemHE (Liman et al., 1992) or in pGemHEm (pGemHE vector with modifications in the linearization linker). To ensure more efficient cRNA expression, GIRK2 cDNA was also subcloned in pGemHEm vector. Wild-type GB1a and GB2 cDNAs were constructed by amplifying overlapping stretches of DNA from rat brain cDNA (Clontech) using polymerase chain reaction (PCR); specific oligonucleotides were designed based on the sequences published in the database. For GB2, the first 243 base pairs (up to the NheI site) were assembled using overlapping oligonucleotides; some silent mutations were introduced to decrease the repetitiveness of the sequence. The HA epitope (YPYDVPDYA) was introduced by sequential overlap extension PCR; the epitope was introduced at the N terminus, after residue 50 in GB1a and after residue 56 in GB2 (counted from the initiator methionine). All truncated and mutated constructs of GB1a and GB2, as well as CD4 fusion proteins, were constructed by PCR. For CD4 constructs, wild-type or mutated C termini of either GB1 or GB2 were fused to human CD4 cDNA through an artificial NotI site engineered after the last codon. All PCR-amplified stretches of DNA were verified by sequencing.

Surface Labeling

Surface labeling assay was adopted for mammalian cells based on the single oocyte surface labeling assay developed by Zerangue et al. (1999). COS7 cells plated in 35 mm tissue culture dishes were transfected with Fugene (Roche), and surface expression of all constructs was assayed 24 hr after transfection. Cells were fixed with 4% formaldehyde in PBS (30 min), blocked in PBS with 1% goat serum (30 min), and then labeled with primary antibody for 1 hr and with an appropriate HRP-coupled secondary antibody for 20 min. Chemiluminescence of the whole 35 mm dish was quantitated in TD-20/20 luminometer (Turner Designs) after 15 s incubation in Power Signal Elisa solution (Pierce). Extensive washing between steps and before chemiluminescence measurement was necessary for good signal-to-background ratio; all steps were performed at room temperature. For the detection of HA-tagged proteins, we used anti-HA rat monoclonal antibody (3F10, Roche, 0.2 μg/ml) and goat anti-rat HRP-conjugated $F(ab')^2$ fragments (Jackson, 1:1000 dilution); surface expression of CD4 fusion proteins was detected by anti-CD4 mouse monoclonal antibody (mAb 1779, Chemicon, 1:1000 dilution) and goat anti-mouse HRP-conjugated IgG (Jackson, 1:1000 dilution). For each construct or combination of constructs, surface expression was assayed in three 35 mm dishes, and each experiment was repeated several times. Protein surface expression in individual oocytes was determined as described by Zerangue at al. (1999).

Western Blotting

COS7 cells were transfected with Fugene. 24 hr after transfection, cells were collected and lysed for 60 min at 4°C in lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Nonidet P-40 [pH 7.6]; supplemented with protease inhibitors). Cleared cell lysates were solubilized in standard SDS sample buffer, resolved with 8% SDS–PAGE gels and electroblotted to nitrocellulose filters. Tris-buffered saline with 0.1% Nonidet P-40 (TBS-N) and 5% nonfat dried milk was used for blocking and for the dilution of primary and secondary antisera. For the detection of HA-tagged proteins, we used rat monoclonal anti-HA antibody (3F10, Roche, 0.2 μg/ml) and goat anti-rat HRP-conjugated IgG (Jackson, 1:1000 dilution). Detection of nontagged GB1 was performed with guinea pig antiserum GP311 as described in Margeta-Mitrovic et al. (1999). After thorough washing of filters in TBS-N buffer, bound antibodies were detected with the enhanced chemiluminescence system (Amersham).

Immunofluorescence

COS7 cells grown on glass chamber slides were transfected with Fugene, and immunostained 24 hr (nonpermeabilized cells) or 72 hr (permeabilized cells) after transfection. Nonpermeabilized cells were blocked with 5% goat serum in PBS (30 min at 4°C), labeled with primary antibody (2 hr at 4°C), fixed with 4% formaldehyde in PBS (30 min), blocked in 5% goat serum in PBS with 0.1% Triton X-100 (30 min), and labeled with secondary antibody (1 hr); after cells were fixed, all steps were done at room temperature. Permeabilized cells were fixed with 4% formaldehyde in PBS (30 min), blocked in 5% goat serum in PBS with 0.1% Triton X-100 (30 min), and labeled with primary antibody for 2 hr and secondary antibody for 1 hr; all steps were done at room temperature. In both experiments we used anti-HA monoclonal rat primary antibody (3F10, Roche, 1 μg/ml) and Cy3-conjugated donkey anti-rat secondary antibody (Jackson, 1:500). Immunofluorescence was visualized with a BioRad confocal microscope.

Electrophysiology

Stage V–VI *Xenopus* oocytes were prepared and maintained as described in Collins et al. (1997). cRNAs were prepared using AmpliScribe T7 kits (Epicenter Technologies), and oocytes were injected

with ~1 ng GIRK1/GIRK2 cRNAs and ~1 or 5 ng of each receptor subunit cRNA 24 to 48 hr before recording or surface protein measurement. Recordings were done in modified ND96 solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 6 mM HEPES [pH 7.4]) or in 40K solution (where 40 mM NaCl was replaced with 40 mM KCl). GABA (RBI) was dissolved in 40K solution and applied by bath superfusion. Currents were measured using standard two-electrode voltage clamp recording (GeneClamp 500B amplifier, PCLAMP software [Axon Instruments]).

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