Function of GB1 and GB2 subunits in G protein coupling of GABA_B receptors

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Many G protein-coupled receptors (GPCRs) have recently been shown to dimerize, and it was suggested that dimerization may be a prerequisite for G protein coupling. γ -aminobutyric acid type B (GABA_B) receptors (GPCRs for GABA, a major inhibitory neurotransmitter in the brain) are obligate heterodimers of homologous GB1 and GB2 subunits, neither of which is functional on its own. This feature of GABA_B receptors allowed us to examine which of the eight intracellular segments of the heterodimeric receptor were important for G protein activation. Replacing any of the three intracellular loops of GB2 with their GB1 counterparts resulted in nonfunctional receptors. The deletion of the complete GB2 C terminus significantly attenuated the receptor function; however, the proximal 36 residues were sufficient for reconstitution of wild type-like receptor activity. In contrast, the GB1 C terminus could be deleted and GB1 intracellular loops replaced with their GB2 or mGluR1 equivalents without affecting the receptor function. In addition, a large portion of the GB1 i2 loop could be replaced with a random coil peptide without any functional consequences. Thus, GB2 intracellular segments are solely responsible for specific coupling of GABA_B receptors to their physiologic effectors, G_i and G protein-activated K⁺ channels. These findings strongly support a model in which a single GPCR monomer is sufficient for all of the specific G protein contacts.

G protein-coupled receptors (GPCRs) are involved in the detection of numerous physiological signals, including hormones, neurotransmitters, and various sensory stimuli (light, odorants, pheromones, and some tastants). Given this variety of ligands, it is not surprising that GPCRs constitute the largest known superfamily of membrane receptors. All GPCRs share the same basic topology (the extracellular N terminus, seven transmembrane stretches with six intervening extracellular and intracellular loops, and the intracellular C terminus), and are further classified into six families based on the sequence homology. Although GPCRs were traditionally thought to function as monomers, many instances of homodimerization or heterodimerization have now been reported in different GPCR families (for review, see ref. 1). One commonly proposed rationale for GPCR dimerization is that the surface area of a GPCR monomer is barely large enough to contact the α and $\beta\gamma$ subunits of a heteromeric G protein simultaneously (1–3). (The largest distance between receptor-interacting sites of the α and $\beta\gamma$ subunits is 40 Å, whereas the longest dimension of the inactive rhodopsin monomer is 42 Å.) This is a difficult model to test because for GPCRs that are functional in homodimeric form, intracellular sequences of each monomer must contain binding sites for both α and $\beta\gamma$ subunits, even if each of the two GPCR subunits in a dimer interacts with only one G protein subunit.

γ -aminobutyric acid type B (GABA_B) receptors, GPCRs for major brain inhibitory neurotransmitter GABA, are heterodimers of two homologous subunits, GB1 and GB2 (4–7). Both GABA_B receptor subunits belong to the GPCR family C (class III), together with metabotropic glutamate receptors (mGluRs), extracellular Ca²⁺-sensing receptor, and some pheromone and taste receptors. Only heterodimeric GABA_B receptors are functional (4–6), and the surface expression of assem-

bled complexes is regulated through a dimerization-dependent trafficking checkpoint (8, 9). The C-terminal coiled-coil interaction of GB1 and GB2 is important for assembly-dependent trafficking, but additional protein–protein contacts in GABA_B receptor complex have been demonstrated, including, although not limited to, noncovalent N-terminal interactions (8–11).

GABA_B receptors couple to the pertussis toxin-sensitive G proteins, G_i and G_o. The GB1/GB2 heterodimeric receptor complex contains a total of eight different intracellular sequences (three cytoplasmic loops plus the C terminus of GB1, and three cytoplasmic loops plus the C terminus of GB2), all of which potentially could play a role in G protein coupling. To determine which intracellular segments are actually important for GABA_B receptor signaling, we systematically tested the functional role of each of the eight segments. The function of different chimeric, truncated, and mutated GB1 and GB2 proteins was assayed by coexpressing them with GIRK1/GIRK2 (Kir3.1/3.2) channels in *Xenopus* oocytes; GIRK1 and GIRK2 were chosen because they form the neuronal G protein-activated K⁺ channel that underlies the GABA-induced slow inhibitory postsynaptic potentials in central neurons (12). Using this approach, we demonstrate that all four GB2 intracellular segments, but none of the GB1 intracellular segments, are necessary for coupling of GABA_B receptors to their physiologic effectors, GIRK channels. These findings support the idea that a single GPCR monomer is sufficient for specific receptor interactions with the G protein.

Materials and Methods

Molecular Biology. Standard molecular biology protocols were adopted from ref. 13. For oocyte expression, constructs were made either in pGemHE (14) or pGemHEm (pGemHE vector with modifications in the linearization linker). GIRK1, GIRK2, wild-type (wt) GB1, wt GB2, GB1 Δ 102, GB1_{ASRR}, GB1_{AA/ASRR}, and GB2 coiled-coil mutant GB2-CC were used in the form described in ref. 8. Because they are functionally identical, we used hemagglutinin (HA)-tagged and nontagged constructs interchangeably throughout the study. All mutant DNA constructs were constructed by sequential overlap extension PCR. C-terminal truncations of GB2 were numbered from the C terminus: GB2 Δ 195 protein extends two, GB2 Δ 192 protein five, and GB2 Δ 161 protein 36 aa beyond the seventh transmembrane segment. For insertion of mGluR1 sequences in GB1, oligonucleotides were designed based on the mGluR1 sequence RAT-GPCGR (GenBank accession no. M61099). In construction of intracellular loop chimeras, the loop sequences were defined as follows: i1 loops, YNSHVR YIQNSQP (GB1), KNRNQKLIK-MSSP (GB2), and LYRDTPVVKSSSR (mGluR1); i2 loops, HTVF TKKEEKK EWRKTLEPWK (GB1), HAIFKNV KMKK-

Abbreviations: GPCR, G protein-coupled receptor; GABA, γ -aminobutyric acid; mGluR, metabotropic glutamate receptor; GIRK, G protein-activated K⁺ channel; wt, wild type; HA, hemagglutinin.

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KIIKDQK (GB2), and ARILAGSKKKICTRKPRFMSAWA (mGluR1); and i3 loops, ETKSVSTEKINDHR (GB1), ETRN-VSIPALNDSK (GB2), and KTRNVPANFNEAK (mGluR1). All PCR-amplified stretches of DNA were verified by sequencing. The expression of all nonfunctional chimeric proteins was confirmed by Western blotting of oocyte homogenates (not shown).

Electrophysiology. Stage V–VI *Xenopus* oocytes were prepared and maintained as described in Collins *et al.* (15). cRNAs were prepared by using AmpliScribe T7 kits (Epicentre Technologies, Madison, WI), and oocytes were injected with ≈ 1 –2 ng of GIRK1/GIRK2 cRNAs and ≈ 5 –10 ng of each receptor subunit cRNA, 24–48 h before recording. Recordings were done in modified ND96 solution (100 mM NaCl/2 mM KCl/1.8 mM CaCl_2 /1 mM MgCl_2 /6 mM Hepes, pH 7.4) or 40K solution (where 40 mM NaCl was replaced with 40 mM KCl). GABA (Research Biochemicals, Natick, MA) was dissolved in 40K solution and applied by bath superfusion. Currents were measured by using standard two-electrode voltage clamp recording (GeneClamp 500B amplifier, PCLAMP software, Axon Instruments, Foster City, CA). Current-voltage relationships were assayed by square voltage steps from -130 to $+30$ mV, in 10-mV increments, from the holding potential of -30 mV. To correct for leak and endogenous oocyte currents, traces recorded in modified ND96 solution in the beginning or end of each recording were subtracted off-line from traces recorded in 40K solution. For plots and statistical comparisons, currents recorded between 100 and 116.6 ms after the start of the voltage pulse were averaged to reduce the random and 60-Hz noise. For summary plots and statistical comparisons, only currents recorded at -120 mV were analyzed. The relative activation of GIRK current by GABA depended on the size of the basal current (the larger the basal current, the smaller the relative activation); this was true for all active receptors tested and is a general feature of the oocyte expression system (N. Dascal, personal communication). We tried to minimize this effect, in each batch, by analyzing only the oocytes that showed basal currents comparable to the oocytes injected with control (wt) receptor constructs. Nonetheless, the data points collected were not normally distributed and, where appropriate, were statistically analyzed using Kruskal–Wallis one-way ANOVA on ranks.

Surface Labeling. Surface expression of assembled GABA_B receptor complexes was assayed in COS7 cells as described in Margeta-Mitrovic *et al.* (8). In brief, extracellularly tagged wt GB1 subunit (GB1-HA) was expressed alone or in combination with nontagged wt or mutant GB2 subunits. Then 24–36 h after transfection, extracellularly accessible HA epitopes were labeled with an anti-HA primary Ab (3F10, Roche Molecular Biochemicals; 0.2 $\mu\text{g}/\text{ml}$) and a horseradish peroxidase-conjugated secondary Ab (Jackson ImmunoResearch no. 112–036-062, 1:1,000), and bound horseradish peroxidase was then quantitated by using the TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). For each combination of constructs, experiments were carried out using two 35-mm tissue culture dishes.

Results

The C Termini of GB1 and GB2. To assess the functional role of GB1 and GB2 C termini, we constructed C-terminal truncation mutants of both GABA_B receptor subunits; the deletion boundary was set 2 aa beyond the seventh transmembrane segment (constructs GB1 Δ 102 and GB2 Δ 195). Functional properties of the truncated receptor subunits were tested by coexpressing them with GIRK1/GIRK2 K⁺ channels in *Xenopus* oocytes; results of these experiments are summarized in Fig. 1.

Deletion of the GB1 C terminus had no effect; the GB1 Δ 102/GB2 receptor complex was functionally indistinguishable from

Fig. 1. GB1 and GB2 C-terminal truncation mutants were functionally assayed by coexpression with GIRK1/GIRK2 channels in *Xenopus* oocytes. In this assay, binding of GABA to functional GABA_B receptors results in activation of inwardly rectifying K⁺ current; see *Materials and Methods* for experimental details. The I–V plots from representative cells (A–C) and the summary of all of the cells tested (D) are shown. In this and subsequent figures, GB1 subunit is schematically represented in black and GB2 subunit in gray; the notch in GB1 subunit represents the GABA-binding site. In all figures, summarized data (which were not normally distributed) are represented as statistical box charts (the horizontal lines in the box denote 25th, 50th, and 75th percentile values, the error bars denote 5th and 95th percentile values, the asterisks denote 1st and 99th percentile values, and the square symbol in the box denotes the mean). For summary plot and statistical analysis, currents were measured at -120 mV; GABA responses are expressed as a percentage of basal GIRK currents recorded in 40K solution just before GABA application. (A) Coexpression of the GB1 C-terminal truncation mutant GB1 Δ 102 with GB2 resulted in receptors indistinguishable from wt GABA_B receptors in their ability to activate GIRK channels. (B) Deletion of the GB2 C terminus in the GB1 Δ 102/GB2 Δ 195 receptor complex significantly attenuated its ability to mediate GIRK current activation (Kruskal–Wallis one-way ANOVA on ranks, followed by the Dunn’s posttest). (C) GABA_B receptor function was preserved when the truncation of the GB2 C terminus was less severe, leaving intact the proximal 36 residues.

Fig. 2. GB1^{ASRR}, the GB1 subunit with mutation in the endoplasmic reticulum retention/retrieval signal RSRR, is functional in oocytes only when expressed with wt GB2 (A and B). In contrast, the double mutant GB1^{AA/ASRR}, which harbors an additional mutation in the dileucine endocytosis motif, is functional when coexpressed with either wt GB2 (C) or with GB2-CC, the coiled-coil mutant of GB2 (D). Thus, the C-terminal coiled-coil interaction of GB1 and GB2 is important for masking of both trafficking signals in the GB1 C terminus but does not play a role in the GABA_B receptor coupling of GABA_B receptors. The I-V plots from representative cells are shown.

wt GB1/GB2 complex (Fig. 1A and D). In contrast, deletion of the GB2 C terminus significantly impaired the ability of GABA_B receptors to activate GABA_B receptors to activate GABA_B current upon application of 100 μM (Fig. 1B) or 1 mM GABA (not shown). Essentially the same results were obtained with GB2Δ192 (not shown), a slightly longer truncation mutant that was reported as fully functional in ref. 9. In contrast, coexpression of GB1Δ102 with GB2Δ161, a GB2 truncation mutant extending 36 aa beyond the seventh transmembrane segment, resulted in the formation of fully functional GABA_B receptors (Fig. 1C and D).

In these experiments, GB2 truncation mutants GB2Δ195, GB2Δ192, and GB2Δ161 were coexpressed with GB1Δ102 to avoid intracellular retention of receptor complexes by unmasked trafficking signals in the GB1 C terminus. However, to exclude the possibility that the poor function of the GB1Δ102/GB2Δ195 and GB1Δ102/GB2Δ192 complexes was caused by the simultaneous deletion of both GB1 and GB2 C termini, we looked for ways to test the function of GB2 truncation mutants coassembled with full-length GB1. We found previously that GB1^{ASRR} (GB1 subunit with a mutation in the endoplasmic reticulum retention/retrieval motif RSRR) still required coiled-coil interaction with GB2 to give rise to functional GABA_B receptors in oocytes (8). However, the double trafficking mutant GB1^{AA/ASRR}, which lacks the functional dileucine endocytosis motif in addition to the RSRR signal, forms functional receptors when coexpressed either with GB2 or GB2-CC, the coiled-coil mutant of GB2 (Fig. 2). This finding indicates that the normal function of the coiled-coil interaction is to mask both trafficking signals in the C terminus of GB1, and that, at least in oocytes, a latent dileucine motif in GB1 can mediate receptor down-regulation upon disruption of both the RSRR signal and the coiled-coil interaction. When we coexpressed GB1^{AA/ASRR} with GB2Δ195, however, application of GABA resulted only in a small GABA_B current activation, no different from the activation induced by GB1Δ102/GB2Δ195 receptors (not shown). Taken together, these data suggest that only the proximal portion of GB2 C terminus plays an important (but not essential) role in the G protein coupling of GABA_B receptors.

Fig. 3. (A) GB1 intracellular loops can be exchanged with intracellular loops from GB2 [construct GB1(2i1-3)] without any effect on the GABA_B receptor function. (B) Similarly, replacement of GB1 loops with loops from mGluR1 [construct GB1(mG1i1-3)] results in fully functional receptors. mGluR1-derived sequences are represented in black outline. (C) Replacing the middle 11 residues in the GB1 i2 loop with a random coil peptide [construct GB1(i2xg11)] does not affect the receptor function. The sequence of i2 loop in GB1(i2xg11) is indicated, with replacement residues boxed in gray. The I-V plots from representative cells (A–C) and the summary plot (D) are shown.

GB1 Intracellular Loops. To assess the function of GB1 intracellular loops, we replaced them with the equivalent loops from GB2 [construct GB1(2i1-3)] or from mGluR1 [construct GB1(mG1i1-3)]; mGluR1 was chosen because it has the same overall structure as GABA_B and other family C receptors but couples to G_q instead of G_{i/o}. When coexpressed with GB2, GB1(2i1-3), the GB1 subunit with all three intracellular loops from GB2, was as effective as wt GB1 in activating GABA_B currents (Fig. 3A and D).

Fig. 4. All three GB2 intracellular loops are essential for GPCR channel coupling: coexpression of GB1 with individual intracellular loop chimeras GB2(1i1), GB2(1i2), and GB2(1i3) resulted in receptors without any functional activity, despite wt-like surface expression levels (E). The I–V plots from representative cells (A–C) and the summary plot (D) are shown. The surface expression of plasma membrane-expressed receptor complexes was determined in mammalian COS7 cells by using chemiluminescence-based assay (for details, see *Materials and Methods*). For these experiments, the extracellular domain of GB1 subunit was tagged with an HA epitope.

Similar results were observed with GB1(mG1i1–3), the GB1 subunit containing all three intracellular loops from mGluR1 (Fig. 3 B and D).

The results of these experiments suggest that the structure of GB1 intracellular loops is not critical for the GABA_B receptor function. To test this further, we replaced the middle 11 residues of the GB1 i2 loop (sequence KKEEKKEWRKT) with a peptide GGGASSASGGG that is predicted to form a random coil structure [construct GB1(i2xg11)]. When coexpressed with GB2, this mutant receptor also formed heterodimeric complexes functionally indistinguishable from wt GABA_B receptors (Fig. 3 C and D).

GB2 Intracellular Loops. To test the function of GB2 intracellular loops, we replaced them with the equivalent loops from GB1, individually and in combination. When GB1 was coexpressed with the triple loop chimera GB2(1i1–3), GB2 subunit containing all three intracellular loops from GB1, application of GABA failed to activate GIRK current (not shown). However, this receptor complex was not expressed on the cell surface to the same extent as wt GB1/GB2 complex (not shown); thus it was possible that a partial folding or assembly defect might have contributed to the functional failure. We thus assessed the function of GB1 coexpressed with GB2(1i1), GB2(1i2), and GB2(1i3), GB2 mutant proteins in which GB2 intracellular loops were exchanged individually with their GB1 counterparts (Fig. 4). None of these mutant receptor complexes was able to activate GIRK1/GIRK2 channels (Fig. 4 A–D), although they were

expressed on the cell surface to the same degree as wt receptor complexes (Fig. 4E).

Because the i2 loop was reported as critical for G protein coupling in family C GPCRs (16, 17), we also tested the combination GB1(2i2)/GB2(1i2), where all of the intracellular elements of GABA_B receptor complex were preserved, but the i2 loops were exchanged between subunits. Even this relatively conservative mutation resulted in receptors that failed to activate GIRK current upon GABA application (not shown). Thus, all of the GB2 intracellular loops are essential for G protein coupling of GABA_B receptors, and, at least in the case of the i2 loop, context-dependent. In contrast, the GB1 intracellular loops appear not to be critical for GABA_B receptor function.

Discussion

GPCRs were traditionally thought to function as monomers. However, although GPCR dimerization has now been demonstrated in organisms from yeast to human by using genetic, biochemical, structural, physiological, and pharmacological approaches, as well as fluorescence energy transfer in living cells, its functional significance is still not clear (for review, see ref. 1). Because the surface area of a GPCR receptor barely appears sufficiently large to contact both the α and $\beta\gamma$ subunits of a G protein simultaneously, it was proposed that a GPCR dimer is necessary for interactions with trimeric G proteins (e.g., see refs. 1–3). Because heterodimerization is a prerequisite for the plasma membrane localization and function of GABA_B recep-

tors, we were able to start addressing this question by examining the functional role of each of the eight intracellular segments.

We found that all GB2 intracellular segments were important for receptor coupling to GIRK channels (Figs. 1 and 4). In contrast, the C terminus of GB1 could be deleted and GB1 intracellular loops replaced with GB2 loops without any effect on the GIRK channel coupling (Figs. 1 and 3). Similarly, the $G_{i/o}$ /GIRK coupling of $GABA_B$ receptors was not affected when GB1 intracellular loops were replaced with those from mGluR1, a weakly homologous receptor that normally couples to G_q (Fig. 3). [This receptor complex, GB1(mG1i1–3)/GB2, did not couple to G_q , as assayed by the lack of activation of endogenous Ca^{2+} -dependent Cl^- current (not shown).] Most strikingly, a large portion of the GB1 i2 loop could be replaced with a glycine-rich random coil peptide without any effect on the receptor complex function (Fig. 3). Thus, it appears that GB1 C terminus is functionally dispensable and that GB1 intracellular loops are not necessary for specific G protein coupling of $GABA_B$ receptors.

Among the four GB2 intracellular segments, all three loops were essential for GIRK channel coupling, and the proximal part of the C terminus, although not essential, appeared to also play an important role. Similar findings were reported for mGluR1, where the i2 loop is absolutely essential for G protein coupling specificity, but other intracellular elements (i1, i3, and the proximal part of the C terminus) are also important (16, 17). Because both the GB2 and mGluR1 intracellular loops, as well as a random coil peptide, could replace respective GB1 sequences without any effect on $GABA_B$ receptor function, it is possible that all G protein contacts are limited to GB2 (Fig. 5A). In an alternative model consistent with the data, the GB2 subunit underlies all of the specific interactions with $G_{i/o}$, whereas i1 and/or i3 loops of GB1 provide contacts for $G_{\beta\gamma}$ subunit and/or part of the G_α surface that is conserved between different α subunits (Fig. 5B). Further work will be required to differentiate between these possibilities.

In agreement with the results shown here, it was previously reported that the C terminus of GB1 is not essential for $GABA_B$ receptor function (9, 10). The results regarding the C terminus of GB2 are more equivocal: one group reported wt-like agonist affinity and G protein coupling for the complex of GB1 and GB2 C-terminal truncation mutants (9), whereas another group found this receptor complex to be functional but with significantly smaller potency than wt receptors (10). Both of these groups used chimeric G proteins G_{q15} or G_{q05} in mammalian cells to assay the function of $GABA_B$ receptor mutants; the receptor activation was then measured through accumulation of intracellular Ca^{2+} (fluorometric imaging assay). It is possible that the function of some less potent and/or less efficacious receptors can be documented in this type of assay because of the high degree of amplification in the signal transduction pathway. In contrast, in more physiologic GIRK-based assay used here, binding of GABA to functional $GABA_B$ receptors on the plasma membrane results in an increase in GIRK current. This assay is very stringent because amplification exists only between the receptor and G protein, and cooperative binding of several $\beta\gamma$ subunits is required for channel activation (18–20). As a result, the less efficacious receptors are likely to appear inactive when tested this way. It is thus possible that the loss of potency in fluorometric imaging assay translates to a loss of efficacy by using GIRK channels as the effector system; however, even application of 1 mM GABA did not result in additional GIRK activation (not shown). Alternatively, the GB2 C terminus may be important for coupling to native $G_{i/o}$ G proteins and not to $G_{q(i/o)}$ chimeras. The third possibility is that, because of the low degree of amplification in the GIRK signaling pathway, the loss of efficacy we observed may reflect the reduced number of assembled $GABA_B$ receptor complexes on the cell surface. In this scenario,

Fig. 5. $GABA_B$ receptors are constitutive heterodimers. Subunit interactions include the noncovalent contacts between the N-terminal domains and the C-terminal coiled-coil interaction. As illustrated here, contacts between core transmembrane domains are likely; however, they have not been demonstrated experimentally. Conformational changes induced by binding of GABA result in the activation of the $G_{i/o}$ G protein through specific interactions between $G_{\alpha i/o}$ and the intracellular segments of GB2. GB1 intracellular segments may not be involved in the G protein coupling (A) or may provide contacts for the $\beta\gamma$ subunit and/or conserved parts of the α subunit (B). Gray spheres represent GABA.

the C terminus of GB2 would not be important for direct G protein coupling but would play a role in either forward trafficking of the assembled complexes or their stabilization on the cell surface. These possibilities remain to be tested.

Is any part of GB1 besides the N-terminal domain important for $GABA_B$ receptor function? Using accumulation of inositol phosphates as the final read-out, Galvez *et al.* (21) reported that GB1_{1/2}/GB2 receptor complexes, which have heteromeric N-terminal domains but only GB2 heptahelical domains, still coupled to G_{q19} , although with a smaller potency and efficacy than wt receptors. We tested analogous receptor combination by using the GIRK channel assay system (for construct details, see ref. 22); this receptor complex (GB1_{1/2}/GB2) was completely incapable of activating GIRK channels even on application of 1 mM GABA (not shown). Again, this difference is likely a result of the different effector systems used for the experiments. Nonetheless, together with the clear demonstration that GB1 intracellular sequences can be replaced with the equivalent GB2 sequences without any impairment of function, these data suggest that the extracellular loops and/or transmembrane segments of GB1 play an important role in $GABA_B$ receptor activation. This is further supported by the finding that the GB1 splice variant GB1e, which is truncated just before the first transmembrane segment, assembles with GB2 but does not form

a functional GABA_B receptor (11). It is possible that the extracellular loops and/or transmembrane hydrophobic core of GB1, together with parts of GB2, form contact points for the ligand-binding dimeric domain; this remains to be investigated. The intracellular loops of GB1 might provide nonspecific G protein contacts. In addition, intracellular parts of GB1 may be involved in signaling pathways not involving G proteins or in the regulation of subcellular GABA_B receptor localization. For example, it was recently shown that the C terminus of GB1 binds transcription factors CREB2 and ATFx (23), as well as members of the 14–3–3 family of signaling proteins (24).

In summary, the GB2 subunit of the dimeric GABA_B receptor harbors all of the specific G protein-binding sites, whereas the GB1 subunit either does not interact with a G protein, or provides nonspecific contacts for $\beta\gamma$ subunit and/or conserved

parts of the α subunit. These findings provide important constraints for models of receptor/G protein interaction and are thus of importance for the whole field of G protein-coupled receptors.

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