Direct Detection of the Interaction between Recombinant Soluble Extracellular Regions in the Heterodimeric Metabotropic γ-Aminobutyric Acid Receptor

Rei Nomura, Yoshikazu Suzuki, Akira Kakizuka, and Hisato Jingu

The γ-aminobutyric acid (GABA) receptor is a heterodimeric receptor consisting of two complementary subunits, GABA_a1 receptor (GBR1) and GABA_a2 receptor (GBR2). GBR1 is responsible for GABA binding, whereas GBR2 is considered to perform a critical role in signal transduction toward downstream targets. Therefore, precise communication between GBR1 and GBR2 is thought to be essential for the proper signal transduction process. However, biochemical data describing the interaction of the two subunits, especially for the extracellular regions, are not sufficient. Thus we began by developing a protein expression system of the soluble extracellular regions. One of the soluble recombinant GBR1 proteins exhibited a ligand binding ability, which is similar to that of the full-length GBR1, and thus the ligand-binding domain was determined. Direct interaction between GBR1 and GBR2 extracellular soluble fragments was confirmed by co-expression followed by affinity column chromatography and a sucrose density gradient sedimentation. In addition, we also found homo-oligomeric states of these soluble extracellular regions. The interaction between the two soluble extracellular regions caused the enhancement of the agonist affinity for GBR1 as previously reported in a cell-based assay. These results not only open the way to future structural studies but also highlight the role of the interaction between the extracellular regions, which controls agonist affinity to the heterodimeric receptor.

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter of the central nervous system that activates two types of receptor, the ionotropic GABA_A/C receptors, to produce fast synaptic inhibition, and the metabotropic GABA_B receptor, to elicit a slow and prolonged inhibitory response. The GABA_B receptor, a G-protein-coupled receptor (GPCR), modulates neurotransmitter release from pre-synaptic neurons or hyperpolarization of post-synaptic membranes (1, 2). The GABA_B receptor belongs to the class C GPCR, together with metabotropic glutamate receptors (mGluR1–8), a Ca²⁺-sensing receptor and some pheromone and taste receptors. Each class C GPCR member consists of three functional regions: a large N-terminal extracellular region (ECR) that binds ligands, a seven-spanning transmembrane region, and a cytoplasmic region. Another feature of these receptors is that they function as dimers. mGluRs and Ca²⁺-sensing receptor form functional homodimers, and the crystal structures of the homodimeric ligand-binding domains (LBDs) of mGluR1, mGluR3, and mGluR7 have been determined (3, 4). On the other hand, the GABA_B receptor forms a heterodimer consisting of two subunits, GABA_B1 receptor (GBR1) and GABA_B2 receptor (GBR2).

The two GBR subunits share ~35% amino acid sequence similarity. The first GBR subunits, isolated by expression cloning using a specific synthetic radiolabeled ligand (5, 6), are two isoforms, named GBR1a and GBR1b. They differ in the N terminus, where GBR1a possesses a repeat of consensus sequences for the complement protein (also called Sushi domain), and it is missing in the GBR1b. Because no significant differences have been revealed about the two main GBR1 isoforms in a number of pharmacological studies, we focused on GBR1a to simplify the biochemical study (GBR1a is termed GBR1 hereafter). When a recombinant GBR1 is expressed by itself in COS cells, it remains intracellular, and the transfected cells lack ligand binding ability (7). In further studies, the GBR2 subunit was discovered by several groups (8–11), and its co-localization with GBR1 in the brain was reported (8, 10, 11). Subsequently, when GBR1cDNA was co-transfected with GBR2cDNA simultaneously, both subunits were expressed on the cell surface, and the cells clearly showed ligand binding activity. Interestingly, GBR2 increased the affinities of agonists to GBR1, but not that of an antagonist (9, 10). In addition, yeast two-hybrid experiments indicated that both subunits interact at their coiled-coil intracellular regions. Truncation and amino acid mutation experiments revealed that this association is involved in shielding an ER retention signal close to the coiled-coil region within the GBR1 intracellular region and enables the combined GBR
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subunits to transport to the cell surface (12, 13). However, this C-terminal interaction between both subunits is not essential for dimerizing the receptor complex per se, because removal of the GBR1 C terminus allows the assembly of a functional heterodimer (13, 14). Fluorescence resonance energy transfer experiments also showed that both subunits interact at the ECR on the cell surface (15). Therefore, these previous data indicated that GBR1 and GBR2 interact in the extracellular region and/or transmembrane region; however, the interaction manner at the molecular level has not been completely deciphered.

The amino acids responsible for ligand binding in the GBR1 subunit have been postulated by homology modeling to the crystal structures of the mGluR1-LBD and amino acid mutagenesis experiments (16). The amino acids that possibly participate in the ligand binding are well conserved among several species. However, the corresponding residues in the GBR2 subunit vary among species, and amino acid substitutions of these residues did not prevent GABA activation of the receptor (16). Therefore, GBR2 itself is considered not to bind ligands, whereas G-proteins interact with specific intracellular sites in GBR2 (17–20). The extracellular signals received by GBR1 are transmitted to the cytoplasmic region of GBR2 through an allosteric interaction between the two. The extracellular domains of both GBR1 and GBR2, we performed PCR with the full-length human GBR1 and GBR2 cDNAs, which were kindly provided by Prof. Bernhard Bettler (University of Basel), as templates. In the case of GBR1-ECR, a forward primer was designed with a BamHI site just after the 3'–end of the coding sequence of an endogenous GBR1 signal peptide. The reverse primer was designed with a stop codon, followed by an XbaI site. The PCR product was cloned using the BamHI and XbaI sites of pBlue-Script, which contains a hemagglutinin signal sequence followed by a FLAG epitope (DYKDDDDK) tag just upstream of the BamHI site. The cDNA fragment was excised using the NotI and XbaI sites, which were in multiple cloning sites of pBlue-Script, and the fragment was inserted into the pFastBac DUAL vector, resulting in the transfer vector pGBR1-ECR1. For the longer GBR1-ECR2 and -ECR3 constructs, PCRs were performed with a forward primer with an MfeI site and reverse primers designed at distinct positions with a stop codon followed by a NheI site. The two PCR products were replaced with the MfeI-XbaI fragment in pGBR1-ECR1, resulting in pGBR1-ECR2 and -ECR3. For GBR2-ECR, the forward primer was designed with an Eco47III site just after the coding region of the endogenous signal sequence, and the reverse primer was designed with a sequence encoding His$_{16}$, followed by an XbaI site, at the putative 3'-end of the GBR2-ECRcDNA. The PCR product was exchanged with the mGluR1 cDNA previously cloned into the pFastBac DUAL vector using the Eco47III and XbaI sites. Consequently, an artificial GBR2cDNA, containing a DNA sequence encoding the mGluR1 signal sequence instead of the original signal sequence, was prepared.

Production of Baculoviruses for Protein Expression—Baculoviruses for protein expression were obtained by the Bac-to-Bac baculovirus expression system (Invitrogen). Spodoptera frugiperda (SF-9) cells were propagated in a monolayer at 27 °C in TNM-FH (Grace’s powder medium, 0.4% yeastolate, 0.3% lactalbumin hydrolysate, 0.1% pluronic F-68, 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B). The vector DNA was transformed into DH10Bac Escherichia coli cells (Invitrogen). The recombinant bacmid DNA purified from the DH10Bac cells was then transfected into SF-9 insect cells, using the Cellfectin reagent (Invitrogen). After incubation for 72 h at 27 °C, the viruses were amplified by re-infecting the SF-9 cells to enhance the viral titer. Finally, the viral titer was checked by a plaque formation assay, using an immobilized monolayer culture of SF-9 cells.

Protein Expression and Purification—HighFive cells were cultured in a monolayer at 27 °C in Express Five serum-free medium (Invitrogen) supplemented with 18 mM L-glutamine. The GBR1-ECRs and GBR2-ECR proteins were expressed by inoculating the baculoviruses into the HighFive cells. The cell culture medium was collected 4 days after the inoculation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Baclofen, CGP54626, and [$^3$H]CGP54626 (53.31 Ci/mmol) were purchased from Tocris (UK). GABA was purchased from Wako (Osaka, Japan). Oligonucleotide primers were obtained from Prologo (Japan).

**Construction of Expression Vectors for GBR1-ECRs and GBR2-ECR**—To make expression plasmids for the ECRs of GBR1 and GBR2, we performed PCR with the full-length human GBR1 and GBR2 cDNAs, which were kindly provided by Prof. Bernhard Bettler (University of Basel), as templates. In the case of GBR1-ECR, a forward primer was designed with a BamHI site just after the 3'-end of the coding sequence of an endogenous GBR1 signal peptide. The reverse primer was designed with a stop codon, followed by an XbaI site. The PCR product was cloned using the BamHI and XbaI sites of pBlue-Script, which contains a hemagglutinin signal sequence followed by a FLAG epitope (DYKDDDDK) tag just upstream of the BamHI site. The cDNA fragment was excised using the NotI and XbaI sites, which were in multiple cloning sites of pBlue-Script, and the fragment was inserted into the pFastBac DUAL vector, resulting in the transfer vector pGBR1-ECR1. For the longer GBR1-ECR2 and -ECR3 constructs, PCRs were performed with a forward primer with an MfeI site and reverse primers designed at distinct positions with a stop codon followed by a NheI site. The two PCR products were replaced with the MfeI-XbaI fragment in pGBR1-ECR1, resulting in pGBR1-ECR2 and -ECR3. For GBR2-ECR, the forward primer was designed with an Eco47III site just after the coding region of the endogenous signal sequence, and the reverse primer was designed with a sequence encoding His$_{16}$, followed by an XbaI site, at the putative 3'-end of the GBR2-ECRcDNA. The PCR product was exchanged with the mGluR1 cDNA previously cloned into the pFastBac DUAL vector using the Eco47III and XbaI sites. Consequently, an artificial GBR2cDNA, containing a DNA sequence encoding the mGluR1 signal sequence instead of the original signal sequence, was prepared.
After the addition of protease inhibitors (10 μg/ml leupeptin, 2 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride), the cells were pelleted by centrifugation at 6000 × g for 20 min at 4 °C. Purification of the protein was facilitated by the FLAG and His tags. In the case of the GBR1-ECRs, the supernatant was applied directly to anti-FLAG M2-agarose (Sigma) packed in a disposable column (Bio-Rad). After the column was washed with low salt buffer (10 mM Tris-HCl (pH 7.5) and 20 mM NaCl), the protein bound to the column was eluted by high salt buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 4.7 mM KCl, and 1.8 mM CaCl₂) containing the FLAG peptide at a concentration of 150 μg/ml. The supernatant containing the GBR2-ECR protein was directly applied to nickel-Sepharose (Amersham Biosciences) packed in a disposable column (Bio-Rad). The column was washed with phosphate buffer (0.5 M NaCl and 20 mM sodium phosphate), and the protein bound to the column was eluted with the phosphate buffer containing 150 mM imidazole.

**Western Blotting**—The culture medium was fractionated by SDS-PAGE, and the proteins were electroblotted onto a nitrocellulose membrane. The membrane was blocked for 1 h in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) with 3% bovine serum albumin and then was incubated for 1 h with anti-FLAG and anti-His antibodies at room temperature. The membrane was washed and then incubated for 1 h with anti-mouse IgG conjugated with alkaline phosphatase at room temperature. Color development was done in ALP buffer (40 mM Tris-HCl (pH 9.0), 150 mM NaCl, 1 mM MgCl₂) containing 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium Color Development Substrate (Promega).

**Ligand Binding Assay**—The ligand binding assay was performed by the polyethylene glycol (PEG) precipitation method, as previously described (22). ³H-Labeled CGP54626, GABA, and the protein solution were mixed in 150 μl of the high salt buffer at 4 °C for 1 h. Then, 6-kDa PEG was added to the sample to a final concentration of 15%, along with 3 mg/ml γ-globulin. After vortexing and centrifugation, the precipitated material was washed twice with 1 ml of 40 mM HEPES (pH 7.4) and 2.5 mM CaCl₂ containing 8% 6-kDa PEG, and then was dissolved in 1 ml of water. After the addition of 14 ml of ClearsolII (Nacalai Tesque), the radioactivity was measured using a scintillation counter.

**Density Gradient Centrifugation**—The GBR1-ECR3 protein, partially purified by the anti-FLAG M2 column, the GBR2-ECR protein, partially purified by the nickel column, and a mixture of both proteins were sedimented through 15–35% sucrose gradients formed with the high salt buffer. The gradients were made using a Gradient Master (BioComp, Inc., Minneapolis, MN). Samples were applied to the top of the sucrose gradient and were centrifuged in an SW41 (Beckman) rotor for 22 h at 40,000 rpm. After the centrifugation, the samples were fractionated into 150-μl aliquots by a piston gradient fractionator (BioComp, Inc.). The fractionated samples were subjected to SDS-PAGE and Western blotting with anti-FLAG and anti-His antibodies. An anti-mouse secondary antibody conjugated with horseradish peroxidase was then applied. Proteins were visualized and quantified using an ECL detection kit (Amersham Biosciences).

**RESULTS**

**Expression and Purification of the Extracellular Regions of the GABA₂ Receptor**—Fig. 1 shows diagrams of the recombinant proteins of the GABA₂ receptor ECRs used in the baculovirus infection experiments. To determine the C-terminal ends of the soluble forms of the ECRs by analogy, we first aligned the amino acid sequences of the GABA₂ receptor ECR and the mGluR1-LBD, and then constructed transfer vectors for the expression of the three GBR1-ECRs (GBR1-ECR1–3) and the GBR2-ECR. Because we expected that efficient secretion was a key step to obtain these soluble proteins, we replaced the original signal peptides by exogenous signal peptides, which had been working well in our laboratory. As shown in Fig. 1, the signal peptide from influenza virus hemagglutinin was added to the N termini of the GBR1-ECRs, whereas that of mGluR1 was exploited for GBR2-ECR. Consequently, both proteins were secreted well and accumulated in the culture medium. We then purified these soluble proteins by affinity chromatography. The expression and purification of these extracellular fragments were confirmed by SDS-PAGE followed by silver staining and Western blotting as shown in Fig. 2. The three GBR1 fragments (GBR1-ECR1–3) migrated to positions, slightly lower than 75 kDa. Their sizes correspond to the molecular mass of 64 kDa calculated from the amino acid compositions. The GBR2-ECR was detected at a position around 50 kDa. This size also corresponds to the molecular mass of GBR2-ECR. Thus, all three GBR1-ECRs and GBR2-ECR were confirmed to have been secreted into the culture medium. The purified GBR1-ECR3 and GBR2-ECR proteins were concentrated and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue R250 staining, to assess the purity. Although a few extra bands were observed (Fig. 2, C and D), especially at the high molecular mass regions in the GBR1-ECR3 lane, each protein was pure enough to perform the following biochemical experiments.

**Ligand Binding Assay of the GBR1-ECRs**—We investigated the ligand-binding abilities of GBR1-ECR1–3, using ³H-labeled CGP54626 ([³H]CGP54626) by the PEG precipitation method,
as described under “Experimental Procedures” (Fig. 3A). The final concentration of $[^3H]CGP54626$ was 20 nM. Although $[^3H]CGP54626$ did not bind to GBR1-ECR1 and -ECR2, its binding to GBR1-ECR3 was clear. Furthermore, in the presence of non-labeled GABA, the binding was displaced. This result was intriguing, because the difference between GBR1-ECR2 and GBR1-ECR3 was the five additional amino acid residues at the C terminus of GBR1-ECR3. As a result, these five amino acid residues were shown to be important for the proper protein folding required for ligand binding ability. However, the GBR2-ECR itself did not bind the ligand, consistent with previous reports (16).

We next investigated saturation binding. The amount of specific ligand binding to GBR1-ECR3 increased and plateaued at a concentration of $\sim 40$ nM $[^3H]CGP54626$ (Fig. 3B, squares), while significant binding to the GBR1-ECR2 was not observed (Fig. 3B, triangles). The dissociation constant ($K_d$) of this ligand for GBR1-ECR3 was $8.7 \pm 2.3$ nM. $B_{\text{max}}$ was $1.12 \pm 0.154$ nmol/mg of protein. This was 7% of the theoretical value, which was calculated on the assumption that all of the purified proteins had normal binding activity. Therefore only 7% of the obtained proteins were active. The other 93% were inactive probably because of incorrect protein folding (see “Discussion”). Although the incompleteness of protein purification can affect results of quantitative analyses, which need precise active protein concentration, it does not affect results of qualitative analyses such as a competition binding assay or detection of interaction with other proteins. Therefore we proceeded with the following qualitative analyses using this protein.

The inhibition of $[^3H]CGP54626$ binding by two agonists and an antagonist was examined. The dose-response curves are shown in Fig. 3C. The rank order of inhibition was CGP54626 $\gg$ GABA $\gg$ baclofen (Table 1).

**Interaction between GBR1-ECR3 and GBR2-ECR**—Because both the GBR1-ECR3 and GBR2-ECR proteins were successfully expressed in soluble forms, we investigated whether GBR1-ECR3 and GBR2-ECR interact with each other in vitro. The viruses encoding the two proteins were co-infected into the insect cells simultaneously. The supernatant of the culture medium was concentrated and loaded on the anti-FLAG M2 column. After washing with low salt buffer, the bound material was eluted with a FLAG peptide and subjected to SDS-PAGE and Western blotting. The membranes were incubated with an anti-FLAG antibody and an anti-His antibody. The samples eluted from the column should contain GBR1-ECR3, because it was trapped by the FLAG peptide tag attached at the N terminus, but not the free GBR2 fragment, unless GBR2-ECR was associated with GBR1-ECR3. GBR1-ECR3 was detected with the anti-FLAG antibody, as shown in Fig. 4A, left, whereas a protein band of $\sim 50$ kDa was detected with the anti-His antibody (Fig. 4A, right). This band is possibly GBR2-ECR, because the molecular mass of GBR2-ECR is 50 kDa and it contains the amino acid sequence corresponding to GBR1-ECR1 (A), GBR1-ECR2 (B), and GBR1-ECR3 (C). GBR2-ECR was expressed and purified with a nickel-Sepharose column. The arrows indicate bands corresponding to GBR2-ECR (D). Samples of 1.0 $\mu$g (GBR1-ECR3) and 0.4 $\mu$g (GBR2-ECR) were fractionated and silver stained. Concentrated samples of 4.5 $\mu$g (GBR1-ECR3) and 7.3 $\mu$g (GBR2-ECR) were stained by Coomassie Brilliant Blue (CBB).
His tag at its C terminus. To confirm that this band was not due to nonspecific trapping by the anti-FLAG column, GBR2-ECR alone was expressed independently, and the culture medium was loaded onto the anti-FLAG column and eluted with the FLAG peptide. No protein band was detected in the elution by the anti-His antibody (Fig. 4B). Consequently, this indicates that the GBR2-ECR was bound to the anti-FLAG column through the interaction with the GBR1-ECR3, suggesting that the soluble GBR1 and GBR2 extracellular fragments interact with each other under these solution conditions.

To confirm the direct interaction between GBR1-ECR3 and GBR2-ECR, we next performed a density gradient centrifugation analysis. We made a 15–35% sucrose gradient and applied the GBR1 and GBR2 extracellular fragments to each protein on the top of the gradients. After ultracentrifugation, we made an SDS-PAGE (Sup, supernatant; FT, flow through; Elute, eluted fraction) of each protein on the top of the gradients. The results for GBR1-ECR3 and GBR1-ECR2 are shown in squares and triangles, respectively. Values are the means ± S.E. of at least three experiments performed in triplicate.

### TABLE 1

| Ligand     | GABA | Baclofen | CGP54626 |
|------------|------|----------|----------|
| GBR1-ECR3 | 23.54 ± 0.3 | 44.44 ± 0.4 | 19.47 ± 0.8 |
| GBR1-ECR3 + GBR2-ECR | 6.71 ± 1.7 | 12.38 ± 0.2 | 27.45 ± 6.5 |
| GBR1-ECR3 + mGluR1-LBD | 19.43 ± 0.3 | ND* | ND |

*ND, not determined.

FIGURE 4. Interaction between GBR1-ECR3 and GBR2-ECR. A, GBR1-ECR3 and GBR2-ECR were co-expressed in insect cells. The culture medium was purified with an anti-FLAG-column and was eluted with a FLAG peptide (150 μg/ml). Eluted samples were resolved by SDS-PAGE and immunoblotting with anti-FLAG and anti-His antibodies. The arrows indicate bands corresponding to GBR1-ECR3 and GBR2-ECR. B, GBR2-ECR was expressed, and the culture medium was purified with the FLAG-column and eluted with the FLAG peptide (150 μg/ml). Eluted samples were resolved by SDS-PAGE and immunoblotted with the anti-His antibody. Sup, supernatant; FT, flow through; Elute, eluted fraction.
protein amount on the basis of the ligand binding experiment of affinity change (described below, in the next paragraph). When the two subunits were mixed, a complex of GBR1-ECR3 and GBR2-ECR appeared in the delayed fractions 18–21, as detected by the anti-FLAG antibody (Fig. 5B). These emerged bands, which were reproducibly observed, were not detected when the GBR1-ECR3 was mixed and centrifuged with the control bovine serum albumin (Fig. 5C). When the mixed samples of GBR1-ECR3 and GBR2-ECR were probed with the anti-His antibody, the GBR2 protein was also detected in fractions 9–27 (Fig. 5E). The protein amounts in the dimer position (fractions 18–21) were ~10% of a total GBR1-ECR3 when the protein bands were quantified by densitometry.

**GBR2-ECR Enhances the Agonist Affinity to GBR1-ECR3**—The enhancement of agonist affinities to GBR1 in the presence of GBR2 may be caused by an allosteric interaction between both subunits (9, 10). We next examined whether our purified extracellular fragments showed this affinity change. The inhibition of [3H]CGP54626 binding with agonists, GABA and baclofen, to GBR1-ECR3 alone and to GBR1-ECR3 plus GBR2-ECR (0.5 μg of GBR1-ECR3 and 5 μg of GBR2-ECR) was estimated (Fig. 5, A and B) (Table 1). As shown in Fig. 6, A (GABA) and B (baclofen), the inhibition curves of both agonists for GBR1-ECR3 plus GBR2-ECR were shifted to the left, as compared with those for GBR1-ECR3 alone. The IC<sub>50</sub> values of GABA and baclofen were 23.54 ± 0.3 and 44.44 ± 0.4 μM for GBR1-ECR3 alone, and 6.71 ± 1.7 and 12.38 ± 0.2 μM for GBR1-ECR3 plus GBR2-ECR, respectively. The IC<sub>50</sub> values of both agonists were decreased ~3.5-fold in the presence of GBR2-ECR. For a control experiment, we measured the GABA affinity to GBR1-ECR3 in the presence of mGluR1-LBD (5 μg), instead of GBR2-ECR. The IC<sub>50</sub> value of GABA did not decrease in the presence of mGluR1-LBD (Fig. 6C), indicating that the effect of GBR2-ECR on ligand affinity was specific to GBR2-ECR. This affinity change was clearly observed with the GBR1-ECR3 and GBR2-ECR protein ratios of 1:5 to 1:10. Next, we also estimated the inhibition of [3H]CGP54626 binding with an antagonist, CGP54626 (Fig. 6D). The IC<sub>50</sub> values of CGP54626 were 19.47 ± 0.8 nm for GBR1-ECR3 and 27.45 ± 6.5 nm for GBR1-ECR3 plus GBR2-ECR. Namely, the IC<sub>50</sub> value of the antagonist did not decrease by the addition of GBR2-ECR. These data indicated that the ligand affinity enhancement of the GBR2 extracellular domain occurred specifically when the GBR1 bound agonists.

**DISCUSSION**

**Determination of the C-terminal End of GBR1 Extracellular Region That Possesses Ligand Binding Activity**—In this study, we determined the essential region of the GBR1 for ligand binding. Only the longest GBR1-ECR3 possessed ligand binding ability among the three GBR1-ECRs that were varied in C-terminal length. GBR1-ECR3 has an additional stretch of five amino acids, PPADQ, compared with the GBR1-ECR2. This extension seemed to assist the correct folding of the active GBR1 ligand-binding domain, although something is still necessary for a perfect folding as judged from the maximum ligand binding (see next section). This short linker region may also perform other unknown functions, such as protecting the new protein surface emerging upon ligand-binding domain isolation as a soluble protein. This domain boundary is consistent with the results reported by Deriu et al. (23), who detected it by a truncation experiment using membrane-bound forms of GBR1.

**Binding Activity of the Soluble GBR1 Fragment**—We examined saturation binding activity of the soluble GBR1-ECR3 and GBR2-ECR proteins by the ligand binding assay using RI-labeled antagonist ligand of GABA<sub>B</sub> receptor, [3H]CGP54626. We reported that the K<sub>d</sub> value for GBR1-ECR3 in the presence
of GBR2-ECR was 13.12 ± 2.7 nM (supplemental Fig. S1). Green et al. (24) has reported that $K_v$ value for full-length GBR1 coexpressed with GBR2 in CHO K1 cells is 7.9 nM (determined EDTA-containing binding buffer), which is almost similar to that of our soluble protein. In addition, White et al. (9) has reported that the IC$_{50}$ value of GABA for the full-length GBR1 expressed in HEK293 cells, as determined in a competition binding assay using $[^{3}H]$CGP54626, is 28.8 μM, which is also similar to the value of our soluble protein (23.54 ± 0.3 μM). These correspondences indicated that, even if all of the soluble fragments are not active, the active species in the soluble GBR1-ECR maintained normal ligand binding activity.

However, we found a critical impairment in the maximum ligand binding activity of the soluble GBR1-ECR3 fragment. We estimated that the $B_{max}$ value of $[^{3}H]$CGP54626 for GBR1-ECR3 alone was 1.12 nmol/mg of protein. This value represents ~7% of the theoretical maximum value, which is calculated on the assumption that the binding activity of the purified protein is 100%. Two possibilities can be supposed to explain such low maximum binding: one is that all of the soluble GBR1-ECR3 molecules identically fold, but the folding manner is incorrect. The second possibility is that only 7% of the obtained soluble protein is correctly folded and the other 93% are inactive. The latter possibility seems to be more plausible because $K_v$ value of $[^{3}H]$CGP54626 in the saturation binding and IC$_{50}$ value of GABA in the competition binding assay are similar to those of full-length receptors expressed in mammalian cells. Furthermore, in the density gradient centrifugation experiment to investigate the interaction between GBR1-ECR3 and GBR2-ECR, the amount of protein bands appeared in the dimer position were only ~10% of total GBR1-ECR3. This percentage is almost similar to the percentage (7%) of active soluble fragment estimated from the $B_{max}$ value of the saturation binding. This fact also supports the idea that ~7% of the purified proteins are in active correct folding. A further purification step such as an affinity chromatography using ligand-conjugated resin would be necessary to obtain the full active soluble form of the GBR1 extracellular region, which is essential for precise future biophysical experiments.

Homo- and Hetero-interaction of GBR1 and GBR2 at the Extracellular Region—Interaction between the extracellular regions of GABA$_B$ receptors has been previously demonstrated by Liu et al. (15). Their extracellular regions are tethered to the membrane with the first transmembrane α-helical segment or with an artificial glycosylphosphatidylinositol anchor instead of the original seven-spanning transmembrane region (hereinafter they are designated as ΔGB1b and ΔGB2 for the constructs possessing the single α-helical transmembrane segment). Hence one cannot completely exclude influences from the cellular membrane or the artificial segments using these constructs. In contrast, our soluble GBRs were completely separated from the transmembrane region, and therefore we could directly examine characters of the extracellular regions. We confirmed the interaction between the soluble GBR1 and GBR2 by the density gradient centrifugation and the purification using the affinity column chromatography after co-expression of GBR1 and GBR2 protein. The correct folded 7% of the soluble GBR1-ECR3 properly interacted with GBR2-ECR.

We examined the IC$_{50}$ value of GBR1-ECR3 for GABA, which was 6.71 ± 1.7 μM in the presence of the GBR2-ECR. The result indicated that the interaction of the two subunits caused enhancement of the agonist affinity by 3.5-fold, compared with that of GBR1-ECR3 alone. Liu et al. has also reported the IC$_{50}$ values for the membrane-tethered artificial GBR1-ECRs using $^{125}$I-labeled CGP64213, an antagonist different from that we used. The IC$_{50}$ values of GABA for ΔGB1b alone and for ΔGB1b in the presence of ΔGB2 were 1.0 ± 0.2 μM and 0.4 ± 0.2 μM, respectively. This 2.5-fold enhancement of the agonist affinity was similar to our data. The agreement of the enhancement effect for GBR2-ECR between the previous cell-based assay and our in vitro biochemical assay using recombinant soluble proteins implies that almost all of the isolated GBR2-ECR molecules would be correctly folded. However, the enhancement effects observed in the completely isolated or the membrane-
tethered ECRs are less than that observed in the full-length receptor (10-fold enhancement) (9). This discrepancy may represent a weak interaction between the extracellular regions of GBR1 and GBR2 and may indicate the importance of the intracellular coiled-coil region for the full receptor activity. In the density gradient centrifugation we also identified homo-oligomerization states of extracellular regions of GBR1 and GBR2 (25). Intriguingly, the oligomerization manner of GBR1-ECR3 was apparently different from that of GBR2-ECR. In the case of GBR1-ECR3, the oligomer was found to be formed by intermolecular disulfide bonds, because the oligomerized bands were disappeared in a reducing condition. On the other hand, the GBR2-ECR oligomers were formed not by disulfide bonds but by non-covalent interaction, which was probably a hydrophobic interaction since it was observed in the high salt buffer (118 mM NaCl). These homo-oligomerization states of GBR1 and GBR2 extracellular regions have also been detected by fluorescence resonance energy transfer experiments on the cell surface (15). Also for the full-length construct of GBR1 and GBR2, formation of the homodimers in living cells has been previously reported (25). Although the role of the homo-oligomerization is not evident at present, it may be involved in the regulation of the GABAB receptor has not been completely deciphered. It is known that a destabilization. One such mutation abrogated the ligand binding domain at the open form, so the enhancement of the antagonists. On the other hand, the antagonist leads to stabilize GBR1 ligand-binding domain. The approach of the GBR2 subunit toward the GBR1 subunit may rearrange the conformation of both subunits. The closing angle of the ligand-binding cleft may alter and lead to the higher affinity of the agonists. On the other hand, the antagonist leads to stabilize GBR1 ligand-binding domain at the open form, so the enhancement of the agonist affinity of GBR1 cannot occur in the presence of GBR2-ECR. In the mGluR1 crystal structure, the agonist binds to the cleft of the open-formed protomer and acts as a wedge to keep the angle from closing (34, 35). The dimer interface of the homomeric mGluR1 mainly consists of two α-helices from each protomer. Amino acid mutagenesis of the hydrophobic amino acids that form the interface decreased the protein expression level of the mutant mGluR1 (36), possibly due to destabilization. One such mutation abrogated the ligand binding cooperativity between homomeric protomers (30).

Currently, the actual construction of the dimer interface in the GABA_B receptor has not been completely deciphered. Thus, structural elucidation of the construction manners of the putative extracellular dimer interface(s) in the heteromeric and homomeric dimers as well as the ligand-binding site in the GABAB receptor should provide clues to its allosteric control. Improvement of the folding status of our partially purified extracellular fragments or specific extraction of the active form fragments from the mixture of the active and inactive species may make further biophysical studies possible.

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