Subcellular distribution of GABA_B receptor homo- and hetero-dimers

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GBRs (GABA_B receptors; where GABA stands for γ -aminobutyric acid) are G-protein-coupled receptors that mediate slow synaptic inhibition in the brain and spinal cord. *In vitro* assays have previously demonstrated that these receptors are heterodimers assembled from two homologous subunits, GBR1 and GBR2, neither of which is capable of producing functional GBR on their own. We have used co-immunoprecipitation in combination with bioluminescence and fluorescence resonance energy transfer approaches in living cells to assess directly the interaction between GBR subunits and determine their subcellular localization. The results show that, in addition to forming heterodimers, GBR1 and GBR2 can associate as stable homodimers. Confocal microscopy

indicates that, while GBR1/GBR1 homodimers are retained in the endoplasmic reticulum and endoplasmic reticulum–Golgi intermediate compartment, both GBR2/GBR2 homodimers and GBR1/GBR2 heterodimers are present at the plasma membrane. Although these observations shed new light on the assembly of GBR complexes, they raise questions about the potential functional roles of GBR1 and GBR2 homodimers.

Key words: bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET), GABA_B receptor, heterodimer, homodimer, subcellular localization.

INTRODUCTION

GABA (γ -aminobutyric acid) is the principal inhibiting neurotransmitter of the central nervous system. It mediates fast synaptic inhibition through GABA_A and GABA_C ionotropic receptors as well as slow and prolonged synaptic inhibition through the metabotropic GBR (GABA_B receptor) [1–3]. The GBR mediates both presynaptic inhibition of neurotransmitter release and postsynaptic inhibition of neuronal excitability (reviewed in [4,5]).

To date, molecular cloning has identified two main GABA_B subtypes termed GBR1 and GBR2, which arise from distinct genes. The human GBR1 gene encodes two forms of the receptor, termed GBR1a and GBR1b that differ in their extracellular N-terminal domains, whereas the human GBR2 gene encodes a single form of the receptor [6]. Over the last few years, several lines of evidence have indicated that GBR1 and GBR2 need to heterodimerize to form a functional GBR. These include: (i) the fact that the two receptor subtypes need to be co-expressed to reproduce the pharmacology of the native GBR, (ii) the observation that the two subtypes can be co-immunoprecipitated with one another and (iii) the finding that GBR1 cannot reach the plasma membrane when expressed alone but is trafficked normally to the cell surface when co-expressed with GBR2 [6]. Along with the identification of an ER (endoplasmic reticulum) retention signal in the carboxy tail of GBR1, these observations led to a model whereby heterodimerization between GBR1 and GBR2 conceal the ER retention signal, allowing the export and cell-surface expression of both receptor subtypes [7–9]. The notion that the heterodimer then constitutes the functional form of the receptor is reinforced by the specific roles attributed to each subtype. Galvez et al. [10] have demonstrated that the extracellular N-terminal region of GBR1 is responsible for ligand binding, whereas the corresponding region in

GBR2, which cannot bind GABA on its own, is required for high-affinity agonist binding to GBR1. In contrast, GBR2 and not GBR1 is needed for the activation of the G-protein, suggesting a transactivation mechanism linking the binding of GABA by GBR1 to the activation of GBR2 and its coupling with G-protein [11,12]. The general overlap in the expression pattern of GBR2 and GBR1 in the nervous system has provided additional support to the notion that the heterodimer represents the functional receptor entity.

Despite the wide acceptance of this model, no study has confirmed the existence of GBR1/GBR2 heterodimers in living cells. Also, the possibility that each of the subtypes could form homodimers remains controversial. Indeed, whereas yeast two-hybrid screens and glutathione S-transferase pull-down assays failed to reveal homotropic interaction for either GBR1 or GBR2, the detection of high-molecular-mass species after SDS/PAGE and Western-blot analysis led some authors to propose that both GBR1 and GBR2 could form homodimers (reviewed in [13]).

In the present study, we used a combination of co-immunoprecipitation, BRET (bioluminescence resonance energy transfer), FRET (fluorescence resonance energy transfer) and confocal microscopy approaches to assess the existence and subcellular distribution of GBR1 and GBR2 homo- and hetero-dimers.

EXPERIMENTAL

Materials

All tissue culture media supplies were from Wisent (St-Bruno, QC, Canada). Mouse anti-Myc (9E10) and anti-HA (12CA5) antibodies were produced by our core facility as ascite fluids. Monoclonal rat 3F10 anti-HA antibodies were from Roche Molecular Biochemicals (Laval, QC, Canada), whereas monoclonal mouse

Abbreviations used: γ -AP1, γ -adaptin 1; BRET, bioluminescence resonance energy transfer; CCR5, CC chemokine receptor 5; CFP, cyan fluorescence protein; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; FRET, fluorescence resonance energy transfer; GABA, γ -aminobutyric acid; GBR, GABA_B receptor; GFP10, green fluorescent protein 10; GPCR, G-protein-coupled receptor; HEK-293 cells, human embryonic kidney 293 cells; hTfR, human transferrin receptor; Rluc, *Renilla* luciferase; TGN, trans-Golgi network; YFP, yellow fluorescent protein; EYFP, enhanced YFP.

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anti-phospho ERK (extracellular-signal-regulated kinase) and polyclonal rabbit anti-ERK were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Texas Red, Oregon Green and Alexa 633 fluor dye-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR, U.S.A.). DeepBlueC Coelenterazine was from PerkinElmer Life Sciences (Boston, MA, U.S.A.) and Coelenterazine h was from Molecular Probes. Horseradish peroxidase-conjugated secondary antibodies and Protein G–Sepharose were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). The renaissance chemiluminescence kit was from PerkinElmer Life Sciences. All other reagents were of analytical grade and obtained from various commercial suppliers.

Expression plasmids

Myc–GBR1b and HA–GBR2

A pcDNA3.1 vector (Invitrogen) encoding the full-length cDNA for the human Myc-tagged GBR1b and HA-tagged GBR2 were generated as previously published [14].

GBR1b–Rluc (where Rluc stands for *Renilla luciferase*), GBR1b–GFP10 (where GFP10 stands for green fluorescent protein 10), GBR2–Rluc, GBR2–GFP10

Receptor cDNAs for Myc–GBR1b and HA–GBR2 were amplified by PCR to generate a stop-codon-free fragment that was inserted into the pcDNA3.1 vector. Rluc and GFP10 were then subcloned in-frame with the C-terminus of both GBR1b and GBR2. The resulting plasmids encoded receptors fused at their C-terminus to Rluc and GFP10 molecules.

CCR5–GFP10 (where CCR5 stands for CC chemokine receptor)

The pcDNA3.1–CCR5–GFP10 plasmid was constructed as previously reported in Blanpain et al. [15].

GFP10–hTfR (where hTfR stands for human transferrin receptor)

To generate the pDNA3.1–GFP10–hTfR vector, the GFP10 coding sequence lacking its stop codon was subcloned into the BamHI–NotI restriction sites of the pDNA3.1/Zeo(+) (Invitrogen). The hTfR coding sequence, including its stop codon, was then inserted in-frame into the 3'-end of GFP10.

GBR1a–CFP (where CFP stands for cyan fluorescence protein), GBR1a–YFP (where YFP stands for yellow fluorescent protein) and GBR2–CFP

Receptor cDNAs for Myc–GBR1a and HA–GBR2 were amplified by PCR to generate stop codon-free fragments. The fragments were then subcloned in frame into the 5'-end of the CFP and EYFP (enhanced YFP) into the pAmCyan1-N1 and pZsYellow1-N1 respectively (ClonTech Laboratories UK Limited, Basingstoke, U.K.).

GBR2–YFP

Receptor cDNA for HA–GBR2 was amplified by PCR to generate a stop codon-free fragment. The fragment was then subcloned in-frame to the 5'-end of the YFP into the pcDNA3–EYFP. All the constructs were verified by direct DNA sequencing.

Cell culture and transfections

HEK-293 cells (human embryonic kidney 293 cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS (foetal bovine serum), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For transfection

experiments, cells were seeded at a density of 3×10^6 cells/100 mm dish or 2×10^5 cells in each well of a six-well plate and cultured for 24 h. Transient transfections were then performed using Eugene-6™ (Roche Molecular Biochemicals), as described in the manufacturer's instructions, or by the calcium phosphate precipitation methods [16]. Dulbecco's modified Eagle's medium was replaced 24 h after transfection and cells were cultured for an additional 24 h.

Confocal immunofluorescence microscopy

Cells were grown on poly-D-lysine-treated glass coverslips deposited at the bottom of each well of six-well plates. Labelling of cell-surface receptors was performed 48 h after transfection, using an appropriate anti-sera (1:100) for 1 h at 4°C. Cells were then fixed with 3% (w/v) paraformaldehyde in PBS for 15 min. Subsequently, samples were rinsed and labelled with the appropriate fluorophore-conjugated secondary antibodies (1:500). For intracellular localization, cells were first fixed with 3% paraformaldehyde for 15 min and permeabilized using 0.15% (v/v) Triton X-100 in 0.2% (w/v) PBS/BSA for 10 min at room temperature (21°C), before proceeding with labelling as described above. Non-specific binding was blocked with 0.2% PBS/BSA. The samples were analysed by confocal laser-scanning microscopy using a $\times 100$ oil immersion lens and a Leica DM IBRE confocal microscope. Laser excitation and emission filters for the different labelled dyes were as follows: Oregon Green (green) $\lambda_{ex} = 488$ nm (excitation), $\lambda_{em} = 530/50$ nm (emission; where '50' represents the bandpass of the filter, e.g. 530/50 nm means 530 ± 25 nm); Texas Red (red) $\lambda_{ex} = 568$ nm, $\lambda_{em} = 610/35$ nm; and Alexa633 (blue) $\lambda_{ex} = 633$, $\lambda_{em} = 680/40$. The extent of co-localization was evaluated using the Leica Confocal software.

Immunoprecipitation of total and cell-surface protein expression

Total extracts

Cells grown in 100 mm dishes were washed twice with PBS. Cells were then lysed and proteins solubilized for 20 min. at 4°C in a lysis/solubilization buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P40, 0.5% (w/v) sodium deoxycholate, 50 mM iodoacetamide, 1 mM Pefabloc, 0.1 mg/ml bacitracin, 0.1 mg/ml benzamidine, 2.5 µg/ml leupeptin and centrifuged at 12000 g for 15 min at 4°C to remove unlysed cells. Immunoprecipitation was then initiated by adding mouse monoclonal anti-Myc antibody (clone 9E10) or rat monoclonal anti-HA antibody (clone 3F10) to the supernatant. After an overnight incubation at 4°C, Protein G–Sepharose was added for an additional 3 h. Protein G–Sepharose-coupled antibody–antigen complexes were collected by centrifugation at 12000 g for 2 min and extensively washed. The final pellet was resuspended in a sample buffer containing 60 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 4.5 M urea with or without 100 mM dithiothreitol and prepared for Western-blot analysis.

Cell surface

Cells grown in 100 mm dishes were washed three times with PBS and incubated with blocking buffer [PBS containing 0.2% (w/v) BSA] for 1 h on ice. Whole cells were then incubated with anti-Myc (9E10) or anti-HA (12CA5) antibodies (1:150 dilution) in blocking buffer on ice for 1 h, and washed twice with blocking buffer and twice with PBS. After removing the free antibodies, cells were lysed, Protein G was added and the rest of the procedure performed as above.

Western-blot analysis

Protein samples were resolved by SDS/PAGE (10 % polyacrylamide), transferred on to nitrocellulose and then subjected to immunoblotting using rabbit polyclonal anti-Myc or rat monoclonal anti-HA antibodies at the dilution 1/10 000. The Renaissance™ chemiluminescence kit was used for development of film after Western-blot analysis.

ERK activation

Cells were plated 24 h after transfection in six-well plates at a density of 450 000 cells/well. After 24 h, the cells were serum-starved for 17 h and subsequently the medium was replaced with fresh serum-free medium for another 2 h. After the stimulation of cells with either 10 % FBS or 10 mM baclofen for 5 min at 37 °C, cells were rinsed twice in cold PBS and 100 µl of sample buffer was directly added into each well, and protein was resolved by SDS/PAGE and transferred on to nitrocellulose. Phospho ERK and total ERK were detected by Western-blot analysis using anti-phospho ERK (1/1000) and anti-total ERK (1/20 000).

BRET²

BRET assay

Cells were washed twice with PBS 48 h post-transfection, detached by 5 mM PBS/EDTA and resuspended in PBS containing 0.1 % (w/v) glucose. Cells were then distributed in 96-well microplates (white Optiplate from PerkinElmer Life Sciences) at a density of 150 000 cells/well (~30 µg of proteins). DeepBlueC™ was added at a final concentration of 5 µM and readings were collected using a modified Top-count apparatus (TopCount; Packard Bioscience, Montréal, Québec, Canada) that allows the sequential integration of the signals detected over the 370–450 and 500–530 nm range. The BRET signal is determined by calculating the ratio of the light emitted by the receptor-GFP10 (500–530 nm) over the light emitted by the receptor-Rluc (370–450 nm). The values were corrected by subtracting the background signal detected when the receptor-Rluc constructs were expressed alone.

To monitor the expression level of receptor fused to GFP10 and Rluc, total fluorescence and luminescence of the transfected cells were determined. For fluorescence, cells were placed (150 000 cells/well) in a 96-well white Costar plate with clear bottom, and the fluorescence detected using a Packard Fluoro-Count with an excitation filter at 400 nm and an emission filter at 510 nm. The same cells were then incubated for 10 min with Coelenterazine h (Molecular Probes) at a final concentration of 5 µM, and the total luminescence of cells measured using a LumiCount (Packard Bioscience). For both measurements, the mean of duplicate wells was calculated.

BRET titration curves

BRET titration curves were performed as in [17]. Briefly, cells were co-transfected with a constant amount of receptor-Rluc constructs and increasing concentrations of the receptor-GFP10 plasmids. The BRET values are then plotted as a function of the total fluorescence/luminescence ratios (GFP10/Rluc) as determined above.

FRET

The day before transfection, HEK-293 cells were seeded on sterile glass coverslips in six-well plates. Coverslips were mounted on to the holder 48 h after transfection and positioned on a Leica TCS-NT confocal microscope. Cells were observed using a 63×1.40

water immersion objective using the following filters: YFP λ_{ex} = 490 nm and λ_{em} = 550 nm (long-pass filter), CFP λ_{ex} = 450 nm and λ_{em} = 490/20 nm and FRET λ_{ex} = 450 nm and λ_{em} = 550 nm (long-pass filter).

Protein determination assay

Protein contents were determined using the Bradford (Bio-Rad Laboratories, Mississauga, ON, Canada) protein assay system using BSA as a standard.

RESULTS

Expression of the recombinant receptor in mammalian cells

The subcellular distribution of the GBR1b and GBR2 receptor subtypes was assessed by differential cell-surface immunoprecipitation. For this purpose, GBR1b and GBR2 receptors harbouring different epitope tags were transiently expressed in HEK-293 cells and their expression assessed by Western-blot analysis after total lysate or cell-surface immunoprecipitation. When expressed alone, Myc-GBR1b could be readily detected in total cell extract but not at the cell surface (Figure 1A, lanes 3 and 4), indicating that, as previously reported [13], the receptor is retained inside the cell. In contrast, immunoreactive species of HA-GBR2 expressed alone are detected both at the cell surface and in the total cell extract (Figure 1A, lanes 9 and 10), confirming that this receptor subtype can traffic on its own to the plasma membrane [13]. The notion that GBR2 can favour the transport of GBR1 to the plasma membrane [5,7–9] was also confirmed by the observation that both Myc-GBR1b and HA-GBR2 immunoreactivities could be detected at the cell surface after co-expression of the two receptor subtypes (Figure 1A, lanes 6 and 12).

When considering the receptor immunoreactive species observed, bands with molecular mass corresponding to those expected for monomeric, dimeric and even larger oligomeric forms were detected (Figure 1A, lanes 3, 9 and 10), indicating the formation of homo- and heterodimers.

Interactions between GBR1b and GBR2

Although the presence of high-molecular-mass forms is consistent with the existence of homo- and hetero-dimers, it could also result from the interaction of individual GBRs with polypeptides other than GBR1 or GBR2. To assess directly the possible formation of homo- and hetero-dimers, co-immunoprecipitation experiments were performed in cells co-expressing the Myc- and HA-tagged GBR1b. As shown in Figure 1(B, lane 3), HA-GBR1b immunoreactivity could be detected by immunoprecipitation of the total cell extract with an anti-Myc antibody, indicating inter-molecular interactions between Myc-GBR1b and HA-GBR1b. Such a co-immunoprecipitation revealed the presence of species corresponding to monomeric and dimeric forms as well as higher-order complexes, indicating that, as was previously reported for several GPCRs (G-protein-coupled receptors), the co-immunoprecipitated homodimers are partially resistant to SDS denaturation [14,18,19] and/or that GBR1b remains stably associated with other proteins. No immunoreactivity could be detected when the immunoprecipitation step was performed in whole cells (Figure 1B, lane 4), confirming that the GBR1b could not be trafficked to the plasma membrane despite the apparent formation of a homodimer. This contrasts with the ability of the HA-GBR2 to be co-immunoprecipitated with the Myc-GBR1b both in total extract and in whole cell (Figure 1B, lanes 5 and 6), and is consistent with the postulate that the heterodimerization is needed for cell-surface trafficking of GBR1. Identical results were obtained when

Figure 1 Immunoprecipitation of GBR1b and GBR2

(A) Immunoprecipitation studies: HEK-293 cells expressing Myc-GBR1b (lanes 3 and 4), HA-GBR2 (lanes 9 and 10) or both receptors (lanes 5, 6, 11 and 12) were lysed before (T, total expression) or after (S, cell-surface expression) the addition of anti-Myc (lanes 1–6) or anti-HA (lanes 7–12) antisera. The receptors were then immunoprecipitated (IP) by the addition of Protein G-Sepharose and the immune complexes resolved by SDS/PAGE. The Myc- or HA-tagged receptors were identified by immunoblotting (WB) with either anti-Myc (lanes 1–6) or anti-HA (lanes 7–12) antisera. (B) Co-immunoprecipitation studies. HEK-293 cells expressing Myc-GBR1b and HA-GBR1b (lanes 3 and 4), or Myc-GBR1b and HA-GBR2 receptors (lanes 5, 6, 9 and 10) were lysed and the receptors immunoprecipitated as described above using anti-Myc (lanes 1–6) or anti-HA (lanes 7–10) antisera. The receptors were revealed by immunoblotting with either anti-HA (lanes 1–6) or anti-Myc (lanes 7–10) antisera. The results shown are representative of at least three independent experiments.

the immunoprecipitation step was performed using anti-HA antibody, whereas for immunodetection the anti-Myc antibody was used (Figure 1B, lanes 9 and 10).

To overcome the possibility that dimerization observed in immunoprecipitation experiments could result from protein aggregation due to cell lysis and detergent solubilization, we used BRET to assess directly the occurrence of GBR1b and GBR2 homo- and heterodimers in living cells. For this purpose, Myc-GBR1b and HA-GBR2 were fused at their carboxy terminus to the energy donor Rluc or the energy acceptor GFP10 and transiently co-expressed in HEK-293 cells. Since the luciferase substrate, Coelenterazine, readily penetrates the cells, BRET can be used to

follow protein–protein interactions in all compartments without disrupting cell integrity.

Confocal immunofluorescence microscopy was used to verify that the addition of the Rluc or GFP10 to the carboxy tail of the receptors did not affect their subcellular localization. As shown in Figure 2, the addition of either Rluc or GFP10 was without any effect on the distribution pattern of GBR1b and GBR2. Indeed, as for the Myc-GBR1b, the expression of Myc-GBR1b-Rluc and Myc-GBR1b-GFP10 fusion proteins was restricted to the inside of the cell and could only be detected after permeabilization (Figure 2A). For their part, the HA-GBR2-Rluc and HA-GBR2-GFP10 fusion proteins were targeted to the plasma membrane where they could be detected in non-permeabilized cells (Figure 2B). The ability of the Rluc- and GFP10-fused HA-GBR2 to promote cell-surface trafficking of Myc-GBR1b (Figure 2D) as well as the capacity of Myc-GBR1b-Rluc and Myc-GBR1b-GFP10 to be targeted to the plasma membrane on co-expression with HA-GBR2 (Figure 2C) were also confirmed. The functionality of the fusion proteins was verified by measuring their ability to stimulate ERK activity. As shown in Figure 3, the addition of the Rluc or GFP10 moieties to GBR1b and GBR2 did not affect their ability to confer a GABA_B-mediated ERK activation.

The formation of GBR1b and GBR2 homo- and hetero-dimers was then assessed by determining the extent of energy transfer between receptor-Rluc and receptor-GFP10 constructs. As shown in Figure 4, significant BRET occurred between Myc-GBR1b and HA-GBR2 independent of the identity of the receptor harbouring the energy donor or acceptor, confirming that heterodimerization readily occurred between the two receptor subtypes. BRET ratios of a similar magnitude were also observed when a BRET pair of the same receptor subtype (i.e. Myc-GBR1b-Rluc/Myc-GBR1b-GFP10 or HA-GBR2-Rluc/HA-GBR2-GFP10) were co-expressed, indicating that GBR1b and GBR2 can form homo-dimers in living cells. To exclude the possibility that the signal observed could reflect spurious interactions resulting from the overexpression of the partners, the GBR-Rluc fusions were co-expressed with either an unrelated GPCR (CCR5), a single transmembrane protein (hTfR), both fused to GFP10, or the soluble GFP10. As shown in Figure 4, the BRET signals observed between GBR-Rluc and any of the negative controls were negligible compared with those obtained between GBRs at similar GFP10/Rluc ratios, confirming the selectivity of the GBR homo- and hetero-dimerization.

To assess whether homo- and hetero-dimerization could occur with similar relative affinities, BRET titration curves [17] were performed for all four GBR homo- and hetero-dimer combinations. As seen in Figure 5, all GBR pairs tested gave similar hyperbolic titration curves indicative of specific interactions. When considering BRET₅₀ (GFP/Rluc ratio required to reach 50% of the maximal BRET signal), similar values obtained for each curve indicate that both homo- and hetero-dimerization can occur at relative expression levels that are not drastically different, suggesting that the homodimers are not a consequence of overexpression.

Subcellular distribution of homodimers and heterodimers

Since BRET can detect protein–protein interactions in all compartments, we turned to FRET confocal microscopy to assess the subcellular distribution of the homo- and hetero-dimers. In these experiments, the GBR1a isoform was used instead of the GBR1b to confirm that both isoforms can homodimerize. The GBR subtypes were fused at their carboxy terminus to either CFPs or YFPs.

The subcellular distribution of each of the construct expressed alone was assessed. As seen in Figure 6(A), fusion of the CFP and YFP moieties did not affect the subcellular distribution of the

Figure 2 Immunofluorescence analysis of the Rluc- and GFP10-fused GBR

HEK-293 cells were transiently transfected with Myc-GBR1b, Myc-GBR1b-Rluc or Myc-GBR1b-GFP10 alone (**A**) or in combination with HA-GBR2 (**C**) or with HA-GBR2, HA-GBR2-Rluc or HA-GBR2-GFP10 alone (**B**) or in combination with Myc-GBR1b (**D**). Cells were permeabilized (+) or not (-) 48 h after transfection, and receptors were detected using anti-Myc and/or anti-HA antibodies. Immunoreactivity was revealed using appropriate secondary antibodies coupled with Texas Red to monitor Myc-GBR1 and either Oregon Green or Alexa633 to monitor HA-GBR2. Labels in boldface indicate the visualized receptors. Insets show bright fields (Myc-GBR1 and Myc-GBR1-Luc) or direct fluorescence of GFP10 (Myc-GBR1-GFP10) to confirm the presence of cells in the selected field. Experiments using confocal microscopy were performed using a Leica DM IRBE microscope with an oil immersion $\times 100$ objective. The results shown are representative of at least three independent experiments.

Figure 3 Functional analysis of the Rluc- and GFP10-fused GBRs

The ability of the GBRs, fused or not fused to Rluc or GFP10, to stimulate ERK1 and ERK2 phosphorylation was assessed in HEK-293 cells. GBR1b/GBR2, GBR1b-Rluc/GBR2-GFP10 or GBR1b-GFP10/GBR2-Rluc were transfected in HEK-293 cells and stimulated with 10 mM baclofen or 10% FBS for 5 min. Cell lysates were then resolved by SDS/PAGE and the ERK activity detected using anti-phospho ERK1/ERK2 antibodies. The total ERK loaded in each lane was controlled by using an anti-ERK1/ERK2 antibody. These results are representative of three independent experiments.

constructs. Whereas both CFP- and YFP-tagged GBR1a showed a diffused intracellular labelling, the GBR2 fused to either fluorophore displayed distinctive plasma-membrane labelling. Then, the distributions of homo- and hetero-dimers were examined in cells co-expressing the different constructs pairwise. In each case, the expression of the CFP- and YFP-tagged constructs was verified by detecting the fluorescence emitted by each fluorophore after excitation at the appropriate wavelength. Exciting the CFP but acquiring the images using the YFP emission filter then

Figure 4 Interaction between GBR1b and GBR2 assessed by BRET in living cells

HEK-293 cells were transiently co-transfected with GBR1b-Rluc (left panel) or GBR2-Rluc (right panel) and either GBR1b-GFP10, GBR2-GFP10, CCR5-GFP10, hTIR-GFP10 or soluble GFP10. Luminescence, fluorescence and energy transfer (BRET) were measured 48 h after transfection. BRET ratios are expressed as the means $\pm$ S.E.M. for three to four independent experiments performed in duplicate. The relative expression level of the Rluc and GFP10 constructs obtained under each condition is expressed as an average GFP/Rluc ratio and presented at the bottom of each bar.

Figure 5 Homo- and hetero-dimerization assessed by BRET titration in living cells

HEK-293 cells were transiently co-transfected with a constant amount of receptor-Rluc construct (GBR1b in **A** and GBR2 in **B**) and an increasing amount of DNA of either GBR1b-GFP10 or GBR2-GFP10. Total luminescence and fluorescence as well as energy transfer (BRET) were measured 48 h after transfection. BRET ratios are represented as a function of the GFP10/Rluc ratios used as an index for the relative concentration of receptor-GFP10 expressed. The curves were fitted using a non-linear regression equation assuming a single binding site (GraphPad Prism).

assessed the occurrence of FRET. As shown in Figure 6(B), FRET was detected for the GBR2-CFP/GBR1a-YFP heterodimeric pair as well as for the GBR1a-CFP/GBR1a-YFP and GBR2-CFP/GBR2-YFP homodimeric pairs. The specificity of the FRET signals was confirmed by the absence of fluorescence detected with the FRET setting, when either CFP- or YFP-tagged receptors were expressed alone (Figure 6A and results not shown). For GBR2-CFP/GBR1a-YFP and GBR2-CFP/GBR2-YFP pairs, FRET images indicate the formation of dimers both inside the cells and at the cell surface. In contrast, FRET between GBR1a-CFP and GBR1a-YFP could be detected only inside the cell with no apparent labelling of the plasma membrane, in agreement with the proposed ER retention of this receptor subtype. These results also confirm that homodimerization is not sufficient to permit cell-surface trafficking of the GBR1 isoform.

Localization of GBR1b and GBR2 in ER and Golgi

Several studies suggested that the lack of cell-surface expression of GBR1 is a consequence of its retention in the ER [7–9]. To determine whether GBR1 is restricted to the ER or can enter the Golgi in the absence of GBR2, confocal microscopy analyses were performed. For each GBR subtype expressed alone or in combination, co-localization with specific markers of the ER (Calnexin), ERGIC (ER–Golgi intermediate compartment p58) and TGN [*trans*-Golgi network; γ -AP1 (γ -adaplin 1)] was assessed.

As shown in Figure 7, both Myc-GBR1 and HA-GBR2, expressed alone (Figure 7A) or in combination (Figure 7B), showed a marked co-localization with the ER marker Calnexin. Surprisingly, however, both receptors expressed either individually (Figure 7C) or in combination (Figure 7D) also showed a good co-localization with p58. This indicates that, unlike what had been proposed, GBR1 can escape the ER and enter the early Golgi compartment even in the absence of GBR2. Differences in the co-localization patterns between the two receptor subtypes emerged when considering the TGN marker. Indeed, as seen in Figure 7(E), only modest co-localization between Myc-GBR1 and γ -AP1 was observed after the expression of this subtype alone. This weak co-localization detected only at the periphery of the TGN is in contrast with the strong and complete co-localization between γ -AP1 and GBR2 (Figure 7E), thus indicating that GBR1 did not readily enter the TGN when expressed alone. Co-expression of the two receptor subtypes (Figure 7F) led to a strong co-localization of both GBR1 and GBR2 with γ -AP1, consistent with the notion that association with GBR2 allows the efficient transport of GBR1 into the TGN.

DISCUSSION

Occurrence of both homo- and hetero-dimers in living cells

Using a combination of co-immunoprecipitation, BRET and FRET approaches, the present study demonstrates that, in addition to the formation of heterodimers, both GBR1 and GBR2 can engage in the homodimerization within living cells. Whereas both GBR1/GBR2 heterodimers and GBR2 homodimers can traffic to the plasma membrane, the GBR1 homodimer was found to be retained intracellularly in the ER and early Golgi. The results therefore indicate that dimerization is not sufficient to promote the release of GBR1 from the ER–Golgi compartments and that specific interaction with GBR2 is required.

Although functional GBRs were first identified as obligatory heterodimers formed by the GBR1 and GBR2 subtypes [6], the finding that each of the subtypes can also self-associate is not surprising in the light of the fact that many GPCRs have been shown to form homodimers [20,21]. In particular, functionally important homodimerization of other members of family 3 GPCRs, such as the metabotropic glutamate receptor subtypes and the calcium-sensing receptor, has been documented [22–25]. Interestingly, several receptors belonging to family 1 GPCRs have also been shown to engage in both homo- and heterodimerization, leading in some cases to distinct functional properties [21]. The finding that GBR1 and GBR2 can form homodimers and heterodimers with similar propensity therefore raises the question of potential distinct roles for the homodimers.

A potential role for GBR1 or GBR2 homodimers should be considered in the context of the results obtained from mice in which either of the two receptor-subtype genes was invalidated. Analysis of knockout mice lacking either GBR1 [26–28] or GBR2 [29] revealed a complete lack of classical GBR responses, clearly indicating that heterodimerization is essential for the well-known GBR-mediated functions. Whereas GABA_B-mediated responses were completely absent from GBR1^{−/−} mice, an atypical response in which the GABA agonist baclofen inhibited instead of activating K⁺ channels was observed in GBR2^{−/−} animals [29]. Whether GBR1 homodimers such as those reported in the present study are needed for such atypical GBR1-mediated response remains to be investigated. In that context, our observation that GBR1 homodimers cannot be trafficked to the plasma membrane and are retained in the ER and early Golgi is intriguing and could suggest that another interacting partner is required for the proper trafficking of GBR1 to the plasma membrane where it could be active. Consistent with this idea, a recent study suggested that the

Figure 6 Interaction of GBR1a and GBR2 in cells assessed by FRET

(A) HEK-293 cells were transiently transfected with GBR1a-CFP, GBR2-CFP, GBR1a-YFP or GBR2-YFP and the receptor detected by confocal fluorescence microscopy. CFP-tagged constructs were detected after excitation (ex) at 450 nm and emission (em) at 488 nm, whereas YFP-tagged constructs were detected after excitation at 490 nm and emission at 550 nm. As a negative control for FRET, the GBR2-CFP construct expressed alone was excited at 450 nm and the emission detected at 550 nm. Emission was not detected at 550 nm when a YFP-tagged receptor was excited at 450 nm (results not shown). (B) HEK-293 cells were transiently co-transfected with GBR1a-CFP or GBR2-CFP and either GBR1a-YFP or GBR2-YFP. Each of the CFP- and YFP-tagged receptors were detected as in (A) and the FRET measured by exciting the CFP-tagged receptors at 450 nm and detecting the emission of the YFP-tagged receptors at 550 nm. Arrows point to cells where FRET signals originating from the cell surface can be distinguished.

γ 2S subunit of the GABA_A receptor can interact with GBR1 and promote its transport to the cell surface, thus offering a possible alternative for novel GBR1 functions [30]. Possible roles for GBR1 assemblies, in the absence of GBR2, are further supported by the fact that GBR1 has a more widespread tissue distribution when compared with GBR2 [31–35].

When considering the GBR2 homodimers, the fact that GBR2 is not sufficient to sustain any GBR response in GBR1^{-/-} is not surprising given the fact that this receptor subtype, unlike GBR1, cannot bind GABA or any known GABA agonists [36]. This however does not exclude the possibility that GBR2 homodimers could have a function unrelated to GABA-stimulated signalling. Even though GBR2 does not seem to be expressed in the absence

of GBR1 within adult tissues, the possibility that GBR2 could have non-GABA-related functions independent of GBR1 may still deserve further investigation when considering the fact that the relative level of expression of each receptor subtype varies in different brain regions and that GBR2 can even be detected in the absence of GBR1 within some regions of the developing rodent brain [35].

Although the possibility that GBR1 or GBR2 could form distinct receptor entities either on their own or in interaction with other partners remains highly speculative, our finding that GBR1 and GBR2 can exist as homodimers with distinct cellular distribution sheds new light on their macromolecular organization. The proper cell-surface trafficking of the GBR1/GBR2 heterodimers is believed to result from the masking of two ER retention/

Figure 7 Subcellular localization of GBR1b and GBR2

HEK-293 cells were transiently transfected with Myc-GBR1b or HA-GBR2 individually (**A, C, E**) or co-transfected with the two receptors (**B, D, F**). The subcellular localization of each receptor was determined by assessing its co-localization with specific markers of the ER (Calnexin; **A, B**), ERGIC (p58; **C, D**) and TGN (γ -AP1; **E, F**). Detection was performed using anti-Myc or anti-HA antibodies for the receptors and the anti-Calnexin, anti-p58 and anti- γ -AP1 antibodies for the organelle markers. Immunoreactivity was revealed using appropriate secondary antibodies coupled with Texas Red (red), Oregon Green (green) and Alexa633 (blue). The co-localization of a receptor with a subcellular compartment, results in the apparition of a third colour in the overlay image: yellow for red and green co-localization, turquoise for blue and green co-localization and fuchsia for red and blue co-localization. Confocal microscopy was performed using a Leica DM IRBE microscope with an oil immersion $\times 100$ objective. Results shown are representative of at least three independent experiments.

retrieval signals (RXR and RR, where R is arginine and X is any amino acid) through the coiled-coil assembly of their carboxy tails [7–9]. It could therefore be hypothesized that the ER retention/retrieval signals remain exposed within the GBR1 homodimers.

Our finding that GBR1 expressed alone can be readily detected in the ER and early Golgi but not in the TGN is entirely consistent with the model proposed for the retention/retrieval of incompletely assembled proteins. Indeed, the incompletely or

inappropriately assembled GBR1 monomers or homodimers would be largely retained in the ER. Those that escape ER retention and enter the Golgi by default would then be efficiently retrieved from post ER compartments through a retrograde transport flow [37] that involves the binding of the COPI (coat protein complex I) complex to the RXR and RR dibasic motifs [38].

Taken together, our results clearly demonstrate that GBR1 and GBR2 can assemble in living cells to form both homo- and heterodimers with distinct subcellular distribution. This obviously raises questions about the potential existence and physiological roles of GBR homodimers in native tissues.

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