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LEUKOTRIENE BLT2 RECEPTOR MONOMERS ACTIVATE THE G₁₂ GTP-BINDING PROTEIN MORE EFFICIENTLY THAN DIMERS

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Running title: G protein activation by monomeric and dimeric GPCRs

Accumulating evidence indicates that G protein-coupled receptors can assemble as dimers/oligomers but the role of this phenomenon in G protein coupling and signaling is not clear so far. We have used the purified leukotriene B₄ receptor BLT2 as a model to investigate the capacity of receptor monomers and dimers to activate the adenylyl cyclase inhibitory G₁₂ protein. For this, we overexpressed the recombinant receptor as inclusion bodies in the prokaryotic system *Escherichia coli*, using a human α_5 integrin as a fusion partner. This strategy allowed the BLT2 as well as several other G protein-coupled receptors from different families to be produced and purified in large amounts. The BLT2 receptor was then successfully refolded to its native state, as measured by high-affinity LTB₄ binding in the presence of the purified G protein G α_{12} . The receptor dimer, in which the two protomers displayed a well-defined parallel orientation as assessed by fluorescence resonance energy transfer, was then separated from the monomer. Using two methods of receptor-catalyzed GTP γ S binding assay, we clearly demonstrated that

monomeric BLT2 stimulates the purified G $\alpha_{12}\beta_1\gamma_2$ protein more efficiently than the dimer. These data suggest that assembly of two BLT2 protomers into a dimer results in reduced ability to signal.

G protein-coupled receptors (GPCRs), the largest family of integral membrane proteins (1-3), participate in the regulation of most physiological functions and are the targets of 30-50% of currently marketed drugs. In light of their biological and therapeutic importance, gaining detailed knowledge of their structural organization remains one of the most crucial task, but also, a great challenge facing modern biomedical research.

Dimerization/oligomerization is a common phenomenon in the GPCR superfamily (4), but its role in the structure, function and signaling of these receptors has still to be clarified. It is unambiguously evidenced that class C GPCRs exist and function as stable dimers (5). However, whether or not class A GPCR dimerization is necessary for G protein activation is still a crucial biological question (6). Indeed, for rhodopsin-like receptors, a role for monomers and dimers in signal transduction is still a matter of intense debate

and investigation (7, 8). Although evidence of GPCR dimerization is accumulating even in native tissues (9), perfect functionality in terms of G protein activation has been reported so far for four different monomeric GPCRs. Indeed, monomers of rhodopsin, β 2-adrenergic receptor (β 2AR), neurotensin NTS1 receptor and opioid μ receptor (MOR), efficiently activate their cognate G proteins, i.e. the transducin G_t (10, 11), the stimulatory G protein G_s of adenylyl cyclase (AC) (12), the stimulatory G protein G_q of phospholipase C (PLC) (13), and the inhibitory G protein G_i of AC (14), respectively.

Here, we investigated and compared the efficiency of isolated dimers and monomers of a prototypical GPCR to activate the purified G_{i2} protein. As a model, we used the G_i -coupled human leukotriene B_4 (LTB_4) BLT2 receptor (15, 16), which plays critical roles in inflammation and immunological diseases (17, 18). To produce sufficient amounts of pure and functional BLT2, isolate monomers from dimers and reconstitute receptor/G protein complexes, we have developed an original strategy. This approach is based first on the fusion of the receptor to an integrin fragment that allowed efficient overexpression in inclusion bodies (IBs) of the prokaryote *Escherichia coli* (*E. coli*), then on refolding and functional purification of the receptor, and finally on size exclusion chromatography (SEC) of the different species. Using the purified preparations of BLT2 monomers and dimers, we provide strong evidence that BLT2 monomers activate the G_{i2} protein more efficiently than dimers.

EXPERIMENTAL PROCEDURES

The construction, expression, and purification of the different GPCR fusions, as well as their thrombin cleavage and the subsequent purification of isolated GPCRs are described in the Supplemental Information section.

Refolding of the BLT2 receptor. The leukotriene BLT2 GPCR, purified by immobilized-metal affinity chromatography (IMAC) in elution buffer (20 mM Tris-HCl pH 8.00, 8 M urea, 0.2 % SDS, 150 mM NaCl, protease inhibitors (benzamidine (10 μ g/ml), leupeptine (5 μ g/ml), and phenylmethylsulfonylfluorid (PMSF; 10 μ g/ml), 100 mM imidazole), was dialyzed overnight at 20°C in dialysis buffer (25 mM

Tris-HCl pH 8.00, 50 mM NaCl, 0.4% SDS) to eliminate urea and imidazole using Slide-A-Lyser dialysis cassettes (Pierce, 10 KDa molecular weight cut-off). The quantity of BLT2 was determined by UV spectrophotometry using the Beer-Lambert law and calculation of molar extinction coefficients (19). Homogeneity of the preparation was checked by recording the scattering light and the fluorescence emission (20). We ensured that the concentration of the GPCR was 0.1-0.5 mg per ml. Refolding of the BLT2 was first developed using a miniaturized protocol allowing comparison of many parameters such as pH, ionic strength, temperature, detergents, concentration of detergents, lipids, additives like cholesterol. To determine whether exchange of SDS with detergents (Anatrace) and/or lipids (Fluka) could produce active receptors from the denatured purified samples, systematic ligand binding competency of the refolded fraction was measured using [3 H]- LTB_4 (22 Ci/mmol, PerkinElmer). Refolding was conducted at 20°C after binding of the 6xHis tag of the GPCR to the Ni-NTA superflow resin (Qiagen). Briefly, 0.5 ml of Ni-NTA superflow slurry was loaded onto a Qiaprep spin column (Qiagen) that had been equilibrated twice with the dialysis buffer. The GPCR sample (0.1-0.5 mg per ml) was loaded onto the Ni-NTA resin by a 3-step centrifugation at very low speed (30 x g) for 2 min. After this step, 75 to 100 μ g of the receptor was bound to the resin. Optimized refolding was achieved by low speed centrifugation using 4 x 0.5 ml of detergent buffer (25 mM Tris-HCl pH 8.00, 50 mM NaCl, n-dodecyl phosphocholine (DPC) and hexadecyl- β -D-maltoside (HDM) (1:1 DPC:HDM ratio), asolectin (1:15 protein:detergent and 1:5 detergent:lipid mass ratios), 0.02% cholesteryl hemisuccinate (CHS)). Elution of the refolded GPCR was performed with 2 x 0.5 ml of elution buffer (25 mM Tris-HCl pH 8.00, 50 mM NaCl, DPC/HDM/asolectin/CHS and 300 mM imidazole) using a final low-speed centrifugation step. The refolded GPCRs were kept on ice or at 4°C until use. Determination of the yield of the refolding step and quantification of the protein concentration were performed by UV spectrophotometry as described above (18).

Ligand-affinity purification of the BLT2 receptor. To isolate the active population

(corresponding to the fraction able to bind the ligand) of the BLT2 receptor, the IMAC-purified GPCR was dialyzed overnight at 20°C in 25 mM Tris-HCl pH 8.00, 50 mM NaCl, 0.4% SDS containing 5 mg/mL asolectin before refolding. Refolding was typically carried out at a protein concentration of 0.1-0.5 mg/ml. The unfolded protein was immobilized onto the Ni-NTA matrix as described above. The resin was then washed with 10 column volumes of a buffer of 12.5 mM Na-borate, 10 mM NaCl, 10% glycerol, pH 9.0 containing DPC and HDM as the detergents (1:1 DPC:HDM ratio), asolectin (1:15 protein:detergent and 1:5 detergent:lipid mass ratios), and 0.02% CHS. Elution of the refolded protein was carried out with the same buffer containing 0.3 M imidazole. All subsequent steps were carried out at 4°C. The IMAC-purified and refolded receptor was dialyzed overnight in a buffer containing 12.5 mM Na-borate pH 7.5, 10 mM NaCl, DPC/HDM/asolectin/CHS (see above for detergent and lipid concentrations). The unfolded aggregated fractions were removed through a gel filtration chromatographic step on a Sephacryl S200 HR column (1.5 x 100 cm, GE Healthcare) using the same buffer. Finally, the active receptor was ligand-affinity purified on a 5b α -bound affinity column. The 5b α antagonist molecule was immobilized through its free carboxylate moiety on an Affi-Gel 102 (Bio-Rad) matrix (21). Immobilization was carried out as described by the manufacturer. The refolded BLT2 receptor was recirculated (flow-rate 0.5 mL/min) on the matrix for at least 12 hours in the above buffer, and then washed with the same buffer. The receptor was then eluted with the buffer containing 0.1 mM 5b α . The antagonist was removed from the eluted protein by dialysis for 36 hours against buffer containing 12.5 mM Na-borate pH 7.5, 10 mM NaCl and DPC/HDM/asolectin/CHS. Following this step, the BLT2 concentration was in the 10⁻⁶-10⁻⁷ M range. When necessary, the protein preparation was concentrated by ultrafiltration (centricon 30 device, Amicon) and subsequently dialyzed to reequilibrate the detergent concentration.

Radiolabeled ligand binding assays. Binding of a radiolabeled ligand to the purified soluble BLT2 was done by equilibrium dialysis at 18°C for 24 hours. Dialysis cassettes from Dianorm (The Nest Group, Inc.) with two 250-

μ l cavities separated by high-permeability 10 KDa molecular weight cut-off dialysis membranes were used. Binding buffer containing 12.5 mM Na-borate pH 7.5, 10 mM NaCl and DPC/HDM/asolectin/CHS was used in all dialysis experiments, with protein concentrations in the 10⁻⁷ M range. 200 μ l of receptor and 200 μ l of tritiated ligand were put in each cavity of the cassettes at the beginning of the procedure. The cassettes were held in a multiequilibrium apparatus (Dianorm) in order to ensure constant stirring of the samples. To measure nonspecific binding, a series of control experiments was done in parallel in which an excess of unlabeled ligand was added together with the radiolabeled ligand on one side of the cassette. Another series was included in the experiment without the receptor at each concentration to control free diffusion of the radiolabeled ligand alone through the dialysis membrane. [³H]-LTB₄ was used as the radioligand. Affinity of the [³H]-LTB₄ was directly determined in saturation experiments, with concentrations ranging from 750 nM to 30 nM. Unlabeled LTB₄ was used in the control experiment at 100 μ M to measure nonspecific binding. At the end of the equilibrium dialysis, samples were recovered from each cavity of each cassette and the radioactivity determined by scintillation counting. The ligand binding data were analysed by nonlinear least-squares regression using the computer program Ligand (Elsevier-Biosoft).

Fluorescence-based ligand binding assays.

Saturation and competition ligand binding experiments with purified BLT2 monomers and dimers (see below) were performed using fluorescence anisotropy with LTB₄ labeled with AlexaFluor-568 (LTB₄-568) following the method described by Sabirsh *et al.* (22). Briefly, LTB₄-568 was produced using LTB₄-aminopropylamide (LTB₄-APA; Biomol International Inc.) and amine-reactive succinimidyl ester of AlexaFluor-568 (Invitrogen, Molecular Probes products). Binding buffer containing 12.5 mM Na-borate pH 7.5, 10 mM NaCl and DPC/HDM/asolectin/CHS was used for these experiments with BLT2 monomer or dimer concentrations at 10⁻⁷ M. Saturation assays were performed using up to 0.9 μ M fluorescent LTB₄ with or without 100 μ M LTB₄. For competition assays, fluorescence experiments

were carried out at a constant LTB₄-568 concentration of 350 nM. The different ligands were added at increasing concentrations and binding lasted for 30 minutes at 15°C. Fluorescence anisotropy associated with BLT2-bound LTB₄-568 was measured using a Cary Eclipse fluorometer equipped with an anisotropy device. Data were recorded as millianisotropy units as a function of competing ligand concentration and converted as of maximum.

G protein coupling assays. Two different types of experiments were carried out to demonstrate functional coupling of purified receptor monomers and dimers to the G_i protein.

First, a nucleotide exchange assay using the purified G α_{i2} subunit was carried out as described by Hamm and colleagues (23). G α_{i2} was prepared as already published (24). For measuring systematic activation of the G protein, the basal rate of GTP γ S binding was determined by monitoring the relative increase in the intrinsic fluorescence (λ_{exc} =300 nm, λ_{em} =345 nm) of G α_{i2} (200 nM of purified G α_{i2}) in the presence of BLT2 (20 nM, the final receptor to G protein molar ratio is 1:10) in buffer containing 10 mM MOPS (pH 7.2), 130 mM NaCl and 2 mM MgCl₂ for 40 min at 15 °C after the addition of 10 μ M GTP γ S. The detergent/lipid mix (DPC/HDM/asolectin/CHS) was kept constant for preserving receptor structure. Similarly, the receptor-catalyzed rate was measured under the same conditions in the presence of 50 μ M LTB₄. The data were normalized to the baseline (buffer contribution, 0%) and the fluorescence maximum obtained with BLT1 (20 nM in the presence of 1 μ M LTB₄, 100%). A negative control experiment was also carried out under the same conditions with the recombinant 5-HT_{4(a)} receptor. For kinetic studies, purified G $\beta_1\gamma_2$ subunits (500 nM) were added in the mixture. The $\beta_1\gamma_2$ subunits of the G protein were prepared as described previously (24). Effects on fluorescence changes were also recorded under the same conditions in the presence of 20 μ M of the BLT2 antagonist LY255283.

Second, we directly measured the amount of GTP binding in a more classical way using radiolabeled [³⁵S]GTP γ S. Incorporation of the non-hydrolyzable [³⁵S]GTP γ S (1250 Ci/mmol, PerkinElmer) was done in the buffer used for

monitoring the intrinsic fluorescence of G α_{i2} , in the presence of 10 mM MgCl₂ instead of 2 mM. [³⁵S]GTP γ S was used at ~2.5 nM (~1200000 dpm per reaction). The BLT2 (monomer, dimer or mixed populations) and the G α_{i2} were added at a 1:1 molar ratio (20 nM each), with an excess of G $\beta_1\gamma_2$ subunits (500 nM). The detergent/lipid mix (DPC/HDM/asolectin/CHS) was kept constant during the incubation which was done at 25°C for 10 min. For measuring the agonist-induced receptor-dependent activity of the G α_{i2} , 1 μ M of LTB₄ was added in the mixture. Nonspecific binding was determined in the presence of 100 μ M GTP γ S. The reaction was stopped by putting the tubes in a ice-cold bath. Specific [³⁵S]GTP γ S binding (separation of free and bound radiolabeled GTP analogue) was then quantified by equilibrium dialysis using dialysis cassettes and membranes equivalent to those described above for radiolabeled [³H]-LTB₄ binding studies. To ensure complete equilibration of labeled [³⁵S]GTP γ S and cold GTP γ S, the dialysis lasted for 4 hours and 30 min. Samples were then recovered from each cavity of each cassette and radioactivity was determined by liquid scintillation counting.

Separation of oligomeric states of BLT2 by size exclusion chromatography.

SEC experiments were carried out on a Superose 6 column (16x70 mm, GE Healthcare). The column was first equilibrated with the 12.5 mM Na-borate pH 7.5, 10 mM NaCl, DPC/HDM/asolectin/CHS buffer. The ligand affinity-purified BLT2 sample at a 10⁻⁶-10⁻⁷ M range concentration (1 mL in buffer 12.5 mM Na-borate pH 7.5, 10 mM NaCl, DPC/HDM/asolectin/CHS) was then loaded on the column and gel filtration was carried out with the equilibration buffer at a 0.2 mL/min flow rate. 0.3 mL fractions were collected. The oligomeric state of the receptor in the different fractions was then assessed using a chemical cross-linking approach with dithiobis(succinimidylpropionate) (dTSP), as previously described (25). Briefly, the different receptor fractions were submitted to cross-linking at room temperature, after addition of dTSP (125 mM stock solution in N,N-dimethylformamide) to a final protein-to-cross-linker molar ratio of 1:10. The optimal cross-linking time value was inferred from a time course analysis of the cross-linked species. The reaction was stopped by addition of glycine to

a final concentration of 50 mM. The cross-linked species were then submitted to SDS-PAGE analysis under non-reducing conditions and the intensity of the electrophoretic bands determined by densitometry (public domain NIH Image software). The BLT2 monomer and dimer receptor fractions were used to catalyze exchange of GDP for GTP γ S on G α_{i2} as described above in the presence of G $\beta_1\gamma_2$ at 1 μ M and increasing concentrations in G α_{i2} . The BLT2 receptor concentration was kept constant at 20 nM in the assays. The duration of the reaction was 15 min in all cases. GTP γ S binding in the absence of LTB $_4$ was subtracted from that in the presence of 1 μ M LTB $_4$ and the resulting data were analyzed by using a one-site binding equation (Prism software, GraphPad) to assess the K_m values for G α_{i2} saturation of receptor-catalyzed GDP/GTP γ S exchange. Data were normalized both to the fluorescence maximum observed for saturating G protein concentrations (in the 10 μ M range) and to BLT2 receptor concentration.

Fluorescence resonance energy transfer (FRET) studies. For the FRET experiments, the BLT2 receptor was labeled at the N- or C-terminus with AlexaFluor-488 or AlexaFluor-568 (Invitrogen, Molecular Probes products) as a donor and acceptor of fluorescence, respectively. N-terminal labeling was carried out as described for the BLT1 receptor (26), using a dye/protein molar ratio 10:1. Under the conditions used, a labeling of \sim 0.9 was achieved, as assessed by measuring the absorbance of the protein at 276 nm and that of the dye at its absorbance maximum. C-terminal labeling of the receptor was carried out by using the transglutaminase (TGase) approach described by Jäger *et al.* (27). For this, the TGase tag Pro-Lys-Pro-Gln-Gln-Phe-Met was appended at the C-terminal end of the protein (the construction of the expression plasmid is described in Supplemental Information section). The fusion protein α_5 I-BLT2 was thus purified as described for the wild-type receptor, dialyzed as indicated above and labeling carried out as described by Jäger *et al.* (27). Briefly, the protein (10 μ M) in a 20 mM Tris-HCl pH 8.0, 150 mM NaCl buffer was incubated with 1 mM Alexa dyes (dye/protein molar ratio 100:1) and 0.5 units of TGase (Sigma) for 24 hours at 25°C in the dark. The unreacted dye was then removed by gel filtration. Labeling yields of 85-90% were

achieved under these conditions, as assessed by the absorbance of the protein at 276 nm and that of the dye at its maximum. The fusion partner was then cleaved and removed as described for the wild-type receptor. For dimer assembly, receptor labeled either with the fluorophore donor or acceptor were mixed in equivalent amounts (molar ratio 1:1) before refolding. Then, refolding and purification was carried out as described above for the unlabeled BLT2. Fluorescence emission spectra were recorded at 25°C between 490 and 700 nm on a Cary Eclipse spectrofluorimeter with an excitation at 480 nm or 570 nm. Buffer contributions were systematically subtracted. The FRET ratio corresponded to the ratio of the acceptor emitted fluorescence at 603 nm following excitation at two different wavelengths, 480 and 570 nm (28).

Statistical analysis. All data are reported as group means \pm SEM. Statistical significance of the differences between independent groups were assessed by paired *t*-test.

RESULTS

Identification of new fusion partners for efficient GPCR overexpression in *E. coli* IBs.

In order to produce recombinant GPCRs in sufficient amounts to reconstitute, *in vitro*, receptor/G protein complexes, we used an approach that had been initially described for the olfactory OR5 receptor and which is based on the accumulation of the receptor target in *E. coli* IBs as a fusion protein and on subsequent *in vitro* refolding (29, 30). Although the concept of this approach has been set up for overexpression and functional refolding of the leukotriene BLT1 and the serotonin 5HT $_{4(a)}$ receptors (31, 32), a generic and reliable method for GPCR accumulation in IBs has not been reported so far (33). To this end, we explored an alternative based on the identification of novel original fusion partners able to produce full-length receptors in high amounts, and that would be applicable to most GPCRs. We thus defined three criteria: 1) the fusion partner has to be targeted to IBs and highly accumulated when expressed as an isolated protein; 2) based on statistical predictions which correlate targeting of the recombinant protein to IBs with its primary sequence and physico-chemical properties (34), both the partner and the fusion have to be classified as insoluble; 3) the length of the

fusion partner was limited to a maximum of 600 residues. We thus selected a non-specific PLC of *Bacillus cereus* (35), the glutamine phosphoribosyl pyrophosphatase amidotransferase (PurF) of *E. coli* (36), and a fragment of the extracellular β -propeller domain of the human α_5 integrin (α_5 I) (37). According to statistical predictions (34), the two most important parameters controlling inclusion body formation (or insolubility) are charge average and fraction of β -turn-forming residues. As indicated in Table 1, the PLC, the PurF and the α_5 I all possess a higher β -turn-forming residue fraction than the usual partner Glutathione-S-transferase (GST). Moreover, with respect to charge, both PurF and α_5 I contain much higher proportions of charged residues than PLC or GST. These observations suggested that PurF and α_5 I proteins may constitute the best potential candidates.

GPCR production and purification.

Both α_5 I and PurF appeared as very efficient partners for accumulating GPCRs in IBs, based on the data with the human arginine-vasopressin (AVP) V2 receptor used as a reference (see Supplemental Figure S1). In addition, the α_5 I allowed efficient overexpression in *E. coli* IBs and purification of several other class A GPCRs (rhodopsin-like) varying with respect to their length (from 337 to 472 amino acids), their physico-chemical properties, their G protein coupling selectivity and the nature of their specific ligand (Figure 1). Although with different efficiencies, high amounts of the catecholamine β 3AR, the hormone AVP V2 receptor and oxytocin (OT) receptor (OTR), the chemokine CCR5 and CXCR4 receptors or chemokine-like ChemR23 receptor, the cannabinoid CB1 receptor and the bioactive lipid leukotriene BLT2, CysLT1 and CysLT2 receptors, were overexpressed. The V2, β 3AR, ChemR23, BLT2, CysLT1, CysLT2, and CB1 receptors were produced and purified as simple α_5 I fusions (Figure 1A). The leukotriene BLT1 and the AVP V1b receptors were also accumulated and purified using this strategy (data not shown). In addition, the OTR, CXCR4, and CCR5 were only produced as complex α_5 I-V2 fusions (Figure 1B). Apparent molecular weight of each fusion was compatible with the corresponding calculated masses (70-75 kDa for the α_5 I-GPCR fusions, around 120 kDa for the α_5 I-V2-GPCR

fusions). Moreover, the integrity of the different fusions was confirmed both by N-terminal Edman sequencing and chemiluminescence detection of the GPCR C-terminal 6xHIS tag (data not shown).

For each fusion, the α_5 I partner was efficiently removed by thrombin cleavage and the isolated GPCRs purified using a second IMAC. Complex fusions (e.g. α_5 I-V2-OTR) required an additional gel filtration step before the IMAC purification for eliminating uncleaved proteins. As illustrated in Supplemental Figure S2, representative purified OTR, ChemR23, V2 and β 3AR, appeared as two major bands, corresponding to a monomer at 35-40 kDa and to a dimer at around 75 kDa. Identity of each receptor monomer and dimer was confirmed by direct N-terminal Edman sequencing. The quantity of each purified receptor was calculated as indicated in Experimental procedures: it varied from 0.2-0.5 mg (OTR for instance) to 2-3 mg (e.g. the V2 or the BLT2) from 100 ml of bacterial cell culture (equivalent to 0.6 g wet cells).

***In vitro* refolding, functional purification and binding properties of the ligand-competent BLT2 receptor.**

As stated above, the BLT2 receptor was efficiently accumulated in *E. coli* IBs and purified as a denatured protein in large quantities. Refolding conditions were subsequently explored as described for the BLT1 receptor (31). The BLT2 receptor was refolded to its native state in well-defined detergent/lipid mixed micelles (DPC/HDM/asolectin/CHS). Under such conditions, the ligand-competent fraction represented around 4% of the total receptor preparation, as determined by [³H]-LTB₄ binding. The ligand-competent fraction of the BLT2 was then purified through a ligand-immobilized affinity chromatography procedure using the LTB₄ antagonist 5b α (20). Homogeneity of the affinity-purified BLT2 was demonstrated by binding assays with [³H]-LTB₄ (Figure 2). The calculated linear Scatchard plot revealed the presence of a single population of binding sites and a stoichiometric ratio of 1:1 ligand molecule per receptor was calculated ($B_{max} = 1.06 \pm 0.02$; $n=3$). It has been previously shown with native BLT2 that a receptor molecule binds a single ligand (15). The occurrence of a 1:1 ligand:BLT2 molar ratio with our recombinant pure receptor therefore implies that all

receptors in the preparation are in a ligand-competent state.

It is to be noted that, despite this homogeneity, the [³H]-LTB₄ affinity for the BLT2 ($K_d = 232 \pm 29$ nM (n=3)) is significantly lower than that described for the receptor expressed in mammalian HEK293 cells, 22.7 nM (15). This could be due to the lack of interaction with stabilizing membrane lipids or protein partners such as G proteins, as it was demonstrated for the other LTB₄ receptor BLT1 (31) or the serotonin 5HT_{4(a)} receptor (32). Because BLT2 was demonstrated to couple to G_i protein (15-17), we reconstituted the affinity-purified receptor with the purified G_{i2} subunit. A significant increase in the affinity of BLT2 for the LTB₄ agonist was observed in the presence of the G_{i2} protein (Figure 2.); in that case, the measured K_d was 49 ± 5 nM (n=3). This value was much closer to that for BLT2 expressed in HEK293 cells, indicating that agonist high-affinity binding state of the refolded affinity-purified BLT2 is dependent on the presence of the G protein.

G protein coupling properties of the affinity-purified BLT2 receptor.

Functionality of the affinity-purified BLT2 was assessed by monitoring the relative increase in the intrinsic fluorescence of G_{i2} after addition of GTPγS to the purified G protein subunit reconstituted with the BLT2 in the absence or presence of LTB₄. A significant BLT2-catalyzed GTPγS binding occurred upon binding of the LTB₄ (Figure 3A). Moreover, the amount of GTPγS bound to G_{i2} was found similar to that induced by the LTB₄ stimulation of BLT1 receptor, as previously described (24). By contrast, a purified 5HT_{4(a)}, which is not naturally coupled to G_i, was unable to stimulate GTPγS binding (32). The kinetics of the G protein G_{i2}-BLT2 receptor coupling were recorded in the presence of Gβ₁γ₂ subunits (Figure 3B) or in their absence. As illustrated, the LTB₄-stimulated GTPγS incorporation was fast and saturated within 15 min (profile 2). It remained stable for longer periods of interaction. This result could be mimicked in the absence of the Gβγ subunits, but the rate of increase was slower and saturated within 40 min instead of 15 min (data not shown). As expected, the BLT2 specific antagonist LY255283 was unable to stimulate GTPγS binding (profile 3), and the intrinsic fluorescence signal was equivalent to that of

the basal condition (profile 1). Interestingly, the time-course and maximal incorporation of GTPγS measured in the presence of BLT2/G protein was equivalent to that recorded with the BLT1 receptor positive control (profile 4). These results confirmed that coupling of the affinity-purified BLT2 receptor to the G protein was functional as well in terms of kinetics, although Gβγ were necessary for rapid GTPγS binding.

Oligomeric state of the affinity-purified BLT2 receptor. We then analyzed the oligomeric state of the ligand affinity-purified BLT2 receptor using a SEC approach. As shown in Fig. 4A, a main peak was observed that was centered at about 70 mL. No peak was observed in the dead volume of the column indicating the absence of high molecular species that could correspond to aggregated receptor. As clearly shown in Fig.4A, the main peak displayed a well-defined shoulder at ca. 65 mL indicating the occurrence of different species in the eluted fractions.

To assess the oligomeric state of the different receptor populations in this elution peak, we carried out a series of chemical cross-linking experiments. The different fractions making the elution peak were pooled in three main fractions, labeled 1, 2 and 3 as a function of their elution volume. The proteins in these fractions were then submitted to chemical cross-linking using dTSP as a disulfide reagent, as previously described with the BLT1 receptor (31). The extent of cross-linking was finally assessed by SDS-PAGE under non-reducing conditions (see inset to Fig. 4A). A major band at ~66 kDa was observed for the first protein fractions eluted from the SEC column (fraction 1). This mass value is compatible with that of the homodimeric BLT2. However, in this case, a minor band with an electrophoretic mobility compatible with that of the receptor monomer was still observed that corresponded to less than 5% of the total protein, as assessed by densitometric analysis of the SDS-PAGE gel (not shown). The occurrence of this band after cross-linking could arise either from an incomplete chemical cross-linking or from the presence of a minor fraction of monomeric receptor. In contrast, the last eluting proteins (fraction 3) strictly correspond to protein species with an electrophoretic mobility at ~35 kDa compatible with that of the monomeric BLT2. This indicates that the receptor purified

following ligand-immobilized chromatography essentially corresponds to a mixture of monomeric and dimeric species that can be separated using SEC. As expected, the protein fractions at intermediate elution volumes, i.e. fraction 2, correspond to a mixture of monomer and dimer, as assessed by chemical cross-linking.

We finally determined the topological features of the BLT2 dimer, i.e. whether the two protomers in the dimeric assembly are in a parallel or antiparallel orientation. For this, we devised a FRET-based approach that consisted in labeling either the N-terminus or the C-terminus of the receptor with a fluorescence donor or acceptor and then measuring the transfer efficiency between these two probes to assess proximity (see Experimental procedures section). N-terminal labeling was carried out as described for BLT1 (26). To specifically label the receptor C-terminus, we introduced a transglutaminase recognition sequence that allows enzymatic modification of a reactive glutamine and incorporation of a fluorophore (27). The receptors labeled with the donor (AlexaFluor-488) and acceptor (AlexaFluor-568) probes were mixed in equivalent amounts before refolding and then were refolded and the dimers purified as described above. As expected, under such conditions, essentially equivalent amounts of donor- and acceptor-labeled protein were found in the dimeric fraction (fraction 1 as above), based on the UV absorption features of the proteins in this fraction (see Experimental procedures). Since labeling does not affect the dimerization properties of BLT2 (similar SEC profiles were obtained for both the labeled and unlabeled proteins; not shown), one can expect the final dimeric fraction to be composed of a mixture of dimers where both protomers are labeled with the donor or with the acceptor molecule, and dimers where each of the protomers is labeled either with the donor or with the acceptor. Moreover, the distance between the extreme N- and C-termini of the BLT2 is expected to be significantly larger than the R_0 value (~ 60 Å) of the fluorophore pair used in the experiments, based on the different GPCR crystal structures obtained so far and biophysical data published for the fluorescently-labeled $\beta 2AR$ (38, 39). Consequently, FRET was expected to arise only from the latter species. As shown in Figure 4B, a significant FRET signal was

observed when protomers were labeled either both at the N-terminus or both at the C-terminus. This strongly suggested that the N-terminal regions are in proximity in the dimeric assembly, as well as both C-termini. This is likely to be a specific effect of receptor dimerization since no signal was observed with the monomeric fractions (fraction 3 as above), ruling out possible collisional effects. In contrast, no signal was observed when one of the protomers was labeled at its N-terminus and the other at the C-terminus. All these data indicate that the two protomers in most, if not all dimers, are likely in a parallel orientation.

Pharmacology of the BLT2 monomer and dimer. To assess that both monomeric and dimeric BLT2 populations were functional, we first measured the ligand-binding properties of the different fractions obtained after SEC. A similar K_d value for [3H]-LTB₄ was measured for both species, i.e. 255.8 ± 35.2 nM (n=3) and 253.3 ± 38.7 nM (n=3) for the monomeric and dimeric fractions, respectively. This is in agreement with the K_d calculated for the refolded affinity-purified BLT2 (see above). Moreover, the stoichiometric ratio of ~ 1 obtained for the receptor dimer indicates that both protomers in the dimeric assembly are able to bind the LTB₄ agonist. We then checked whether the receptor monomer and dimer displayed different pharmacological profiles. For this, we carried out a series of saturation and competition experiments using fluorescence anisotropy and the LTB₄ derivative LTB₄-568 as a fluorescent probe. Both this probe and the fluorescence anisotropy approach for monitoring ligand binding to the LTB₄ receptors have been described previously (22). We first determined, by saturation fluorescence anisotropy binding assays, that affinity of the LTB₄-568 for BLT2 monomers and dimers was in the same range than that defined with [3H]-LTB₄ for dimers and monomers, respectively. Indeed, LTB₄-568 affinity was calculated to be $K_d = 310$ (n=2) for monomers and 297 nM (n=2) for dimers. We then assessed the pharmacological profile of the monomer and dimer in competition assays using LTB₄-568 as the fluorescent tracer. As shown in Figure 5, the two BLT2 populations displayed very similar pharmacological profiles, i.e. they both bound the LTB₄ and 12-HHT agonists as well as the BLT2-specific LY255283 antagonist. In contrast, the BLT1-specific U75302 agonist

did not significantly displaced LTB₄-568 binding, considering either the monomer or the dimer. Such a pharmacological profile is fully compatible with what has been reported for the BLT2 receptor expressed in neutrophils or transfected cell membrane fractions [15-18, 22, 40]. Moreover, as shown in Table 2, similar IC₅₀ values were obtained for all the ligands whether the monomer or the dimer are considered, indicating that dimerization of BLT2 has not a significant impact in its ligand binding properties.

Monomer- and dimer-catalyzed G protein activation.

We subsequently analyzed whether the BLT2 monomer and dimer could efficiently activate the purified G_{α₁₂β₁γ₂} protein. Using the fluorescence-based assay, we first assessed LTB₄-catalyzed GTPγS incorporation to the G₁₂ protein with the BLT2 monomers and dimers in the presence of increasing concentrations of the agonist. As shown in Figure 6A, both fractions were able to trigger G₁₂ activation. Half-saturation of LTB₄-catalyzed GTPγS binding was reached at comparable agonist concentrations with monomers and dimers.

We then analyzed the kinetic aspects of G protein activation triggered by the BLT2 monomer and dimer still using the fluorescence-based assay. Interestingly, when normalized to the total number of LTB₄ binding sites, the reaction was approximately two times faster using the monomer compared to what was observed with the dimer (Fig. 6B, t_{1/2} = 3.6 min and 7.5 min for the monomer and the dimer, respectively). This suggests that the monomeric state of the receptor would represent the most active form of BLT2. To further investigate this observation on an experimental basis, we analyzed G₁₂ saturation of BLT2-catalyzed GDP/GTPγS exchange with the two protein fractions, i.e. the monomeric and dimeric ones. As shown in Figure 6C, the BLT2 monomer in the presence of saturating LTB₄ concentrations was completely able to trigger GDP/GTPγS exchange at the level of the G₁₂ subunit with a K_m value of 44.6 ± 7.6 nM (n=3). This clearly indicated that the receptor monomer is fully competent in terms of G protein activation. Interestingly, when the experiment was carried out with the protein fraction in which the receptor dimer was the major species, the calculated apparent K_m value was significantly

different by around 2 fold (82.0 ± 7.3 nM (n=3)). This suggests that for an equivalent number of bound agonists, the BLT2 dimer is less efficient than the monomer in terms of G protein activation.

We finally confirmed the signaling reduced ability of the BLT2 dimer by directly measuring the receptor-dependent GTP binding activity of the G_i protein using radiolabeled [³⁵S]GTPγS. To demonstrate that the receptors (monomers and dimers) are fully capable of G protein activation, each population of BLT2 and the G₁₂ were added at a 1:1 molar ratio. As shown in Figure 6D, interestingly, the amount of specifically-bound [³⁵S]GTPγS to the G₁₂ following ligand stimulation of BLT2 monomer was significantly higher than that obtained with the BLT2 dimer (545766 ± 29740 dpm (n=4) *versus* 428547 ± 34200 dpm (n=4), p<0.05). For comparison, the incorporation of the radiolabeled nucleotide in the absence of ligand-receptor complexes (basal activity of the G₁₂ protein) was much lower (131991 ± 16430 dpm (n=6)). The one measured in the presence of the mixed population (BLT2 receptor before SEC separation) was intermediate (501026 ± 61580 dpm (n=4)).

DISCUSSION

We analyzed and compared here the capacity of GPCR dimers and monomers to activate their cognate G protein using the purified BLT2 receptor as a model. We showed that BLT2 monomer catalyzes GTPγS binding at the level of the G₁₂ protein with higher affinity than the corresponding homodimer and with faster kinetics. We confirmed this result by directly measuring specific [³⁵S]GTPγS incorporation to G₁₂ in presence of either the BLT2 monomer or dimer with a saturating concentration of the LTB₄ agonist. These results strongly suggest that for an equivalent number of ligand-occupied binding sites, BLT2 monomers activate G₁₂ protein more efficiently than dimers.

Our data indicate that the minimal BLT2 unit for G₁₂ activation is the monomer and are in agreement with accumulating evidence showing that monomeric GPCRs are functional. Indeed, several studies demonstrated that monomeric rhodopsin is capable of full coupling to transducin G_t (11, 41, 42). The NTS1 neurotensin receptor monomer was also shown to activate the G_q

protein better than its corresponding homodimer, although activation of G_q by monomeric and dimeric receptors has not been evaluated in the same conditions (13). Finally, the $\beta 2AR$ and the MOR were reconstituted into high-density lipoprotein particles at one receptor per particle and were shown to efficiently activate their selective G protein G_s and G_i , respectively (12, 14). Our data with BLT2 confirm that like G_t , G_q and G_s , G_{12} can be activated by a GPCR monomer, indicating that this is likely to be a common feature for all G protein subtypes. It is thus clear that although accumulating data reinforce the idea that GPCRs oligomerize in heterologous expression systems and in native cells (4, 9), the receptor monomer has *per se* all molecular determinants necessary for G protein activation.

Since the BLT2 monomer appears as the minimal unit for G protein activation (at least in detergent/lipid mixed micelles), this raises the question of what mechanism is responsible for the reduced capacity of receptor dimers to activate G proteins. Different interpretations have been proposed so far.

First, Bayburt *et al.* (41) have suggested that a lower efficiency of rhodopsin dimers to activate transducin could be the consequence of rhodopsin reconstitution into lipid nanodiscs that would result in a random orientation of the protomers in the dimer with two equal populations, parallel and anti-parallel, the latter being unable to activate transducin. In addition, Banerjee *et al.* have indeed demonstrated the occurrence of anti-parallel GPCR dimers incorporated in nanoscale apolipoprotein-bound bilayers using electron microscopy of nanogold-labeled rhodopsin (43). However, this is not the case here for BLT2 since we directly demonstrated using an original FRET-based approach that the receptor dimer is essentially composed of a single population with both protomers in a parallel orientation. In this context, our observation means that, although established in a detergent environment far from that of a native membrane, protein:protein contacts in the BLT2 dimeric entity are of sufficient specificity and lead to correctly folded and assembled dimers.

Second, as proposed for the NTS1 receptor (13), steric hindrance between the two G protein binding sites in the dimer would be responsible for the reduced efficacy of the

BLT2 dimer to activate G_{12} . Such a model has also been proposed for rhodopsin for which steric constraints could prohibit interaction with more than one transducin at a time and explain why a rhodopsin dimer is less efficient than the monomer for transducin activation (41). Steric hindrance effects that would lead to G protein competition may also explain why activation by BLT2 dimers is less efficient. Although we have not so far any direct evidence for the stoichiometric features of the BLT2: G_{12} complex, this would favor, as suggested for rhodopsin, NTS1 or BLT1 receptors (25), a complex where the BLT2 dimer would efficiently interact with essentially a single G_{12} protein.

A third possibility would be to consider a model recently proposed whereby dimerization could serve as a “desensitization” mechanism (44), rapidly suppressing G protein-mediated signaling when there are too many active receptors. In agreement with this hypothesis, dimerization could constitute a way to modulate G protein-mediated signaling. A reduced coupling efficiency of dimeric receptors to their cognate G protein, as observed in this work as well as for the neurotensin NTS1 or rhodopsin, would be consistent with this idea. Although, as proposed above, steric constraints could affect accessibility of one of the two protomers to the G protein and be responsible for this “desensitization” process, another possibility would be to consider a negative allosteric mechanism between protomers through direct trans-conformational changes within the receptor dimer. In this case, agonist-induced conformational changes in one protomer, compatible with a complete activation of its cognate G protein, would trigger an inhibitory trans-conformational change of the second protomer. Such inhibitory cross-conformational changes have been recently proposed to occur in a μ -opioid: α_{2A} adrenergic receptor dimer (45). This study is consistent with asymmetric roles for GPCR subunits in receptor dimers. Examples of asymmetry for different class A GPCR families in terms of G protein activation have been reported (13, 24, 41, 46). In addition, the asymmetric nature of GPCRs has also been elegantly analyzed with class C GPCRs. Very importantly, studies with metabotropic glutamate receptors mGlu₁ and mGlu₅ also support the conclusion that a G protein needs just one active protomer, and that

two protomers in the active conformation impede signaling (47, 48).

It has to be strongly emphasized here that all the data presented on the differential activation of G_{i2} protein by BLT2 monomers and dimers have been obtained because of the possibility to produce the receptor in high amounts in a functional state. This highlights the importance of the strategy we have developed that combines an original and efficient way to overexpress the receptor in *E. coli* IBs to the purification and refolding steps previously described (27, 29, 30). Indeed, improving the expression method allowed to obtain sufficient amounts of functional protein even with

moderate refolding yields. Fusing the receptor to α_5I led to high expression levels for all GPCRs tested, without any optimization of either the cell culture conditions or the extraction/purification procedures. This constitutes a significant improvement over what has been described to date with respect to expression levels in bacteria and therefore represents an important breakthrough for *in vitro* studies aimed at understanding the molecular bases of the function of class A GPCRs and, possibly, of other membrane proteins.

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ABBREVIATIONS

AC, adenylyl cyclase; AVP, arginine-vasopressin; BLT2, leukotriene B₄ receptor subtype 2; *E. coli*, *Escherichia coli*; FRET, fluorescence resonance energy transfer; GPCRs, G protein-coupled receptors; G protein, Guanosine triphosphate-binding protein; G α , G protein α subunit; GST, Glutathione S-transferase; α_5 I, α_5 integrin; IBs, inclusion bodies; IMAC, immobilized metal affinity chromatography; LTB₄, leukotriene B₄; PLC, phospholipase C; PurF, glutamine phosphoribosylpyrophosphatase amidotransferase; SEC, size exclusion chromatography; V2, AVP receptor subtype 2.

FIGURE LEGENDS

Fig. 1: Overexpression and purification of α_5 I-GPCR fusions.

Samples were loaded onto 12% SDS-polyacrylamide gels and proteins stained with Coomassie Blue. To directly compare the purification yield, 10 μ l of eluted fusions were put into each well. A) Schematic representation of α_5 I-GPCR fusions and comparison of the corresponding purified proteins: V2, β 3AR, ChemR23, BLT2, Cys-LT1, Cys-LT2, or CB1 receptor (lanes 1 to 7). B) Schematic representation of α_5 I-V2-GPCR fusions and detection of the corresponding purified proteins. The entire α_5 I-V2 fusion was used as a new partner for expressing another GPCR: OTR, CXCR4, CCR5 (lanes 1 to 3).

Fig. 2: Binding of [³H]-LTB₄ to the refolded affinity-purified BLT2 receptor.

Three series of ligand-binding experiments were carried out by equilibrium dialysis in the absence (closed circles) or presence (open circles) of the purified G α_{i2} protein (Inset: corresponding scatchard plots). The binding data are presented as a plot of the binding degree \bar{X} as a function of the ligand concentration. \bar{X} is defined by bound mole of LTB₄ per mole of BLT2 (49). The experiments shown are representative of 3 independent trials, each performed in duplicate. K_d of [³H]-LTB₄ was calculated as explained in Experimental Procedures. Mean values \pm SEMs are given in Results. The statistics of the illustrated fits were as follow. The calculated K_d in the absence of G proteins was 217.8 \pm 47.7 nM (22% error) with a binding ratio of 1.07 \pm 0.08 (7.5% error). The calculated K_d in the presence of G proteins was 58.1 \pm 18.9 nM (29% error) with a binding ratio of 1.09 \pm 0.07 (6.5% error).

Fig. 3: G protein coupling properties of the refolded affinity-purified BLT2 receptor.

(A) BLT2-catalyzed GTP γ S binding assessed by changes in the fluorescence of G α_{i2} . Experiments were carried out with the refolded BLT2 (20 nM) in the absence or presence of saturating concentrations of LTB₄ (50 μ M). For comparison, BLT1 and 5HT_{4(a)} were also used at 20 nM and their ligands at 1 and 10 μ M, respectively. Mean values \pm SEMs are shown. Statistics are given: ***, p < 0.01. (B) Time course of the relative increase in the intrinsic fluorescence of G α_{i2} upon addition of GTP γ S. The fluorescence was monitored as described in Experimental Procedures in the presence of the purified BLT2 receptor in the absence of ligand (profile 1), in the presence of the LTB₄ agonist (profile 2) or in the presence of the LY255283 antagonist (profile 3). The data were normalized to the changes induced by the purified BLT1 receptor in the presence of LTB₄ (profile 4). The experiment illustrated here is representative of 3 independent assays.

Fig. 4: Separation and characterization of monomeric and dimeric species of BLT2 receptor.

(A) Separation of monomeric and dimeric species of BLT2 by SEC. The affinity-purified BLT2 preparation was loaded onto a Superose 6 column and the separation of the different species was carried out as described in the Experimental Procedures. The proteins eluted from the superose 6 column were pooled in three fractions labeled 1, 2, and 3 as a function of their retention time, as indicated in the elution profile, and submitted to chemical cross-linking. Inset: SDS-PAGE analysis of the protein content in fractions 1, 2 and 3 after chemical cross-linking. (B) FRET ratio measured between alexaFluor-488- and alexaFluor-568-labeled BLT2 protomers. The species considered in each case are schematically represented below, where (☆) is the fluorescence donor and (★) the acceptor. The upper position represents N-terminal labeling, the lower position corresponds to C-terminal

labeling. FRET ratios were calculated as indicated in Experimental Procedures (28). The experiments shown in the figure were repeated three times. Results are given as mean values \pm SEM.

Fig. 5: Pharmacological profile of the BLT2 monomers and dimers. Fluorescence anisotropy-monitored competition experiments were carried out using the fluorescent LTB₄-568 and the BLT2 monomer (A) or dimer (B) as described in Experimental Procedures (100 nM of monomers or dimers). Data are presented as fluorescence anisotropy (% of maximum, defined in the absence of displacing ligand) as a function of ligand concentration. Closed squares, LTB₄; closed triangles, LY255283; closed circles, U75302; and open triangles, 12-HHT. The values are means from triplicates measured in an experiment representative of three independent assays, each done in triplicate.

Fig. 6: Activation of the G protein G α_{i2} $\beta_1\gamma_2$ by monomeric and dimeric BLT2 receptors. (A) LTB₄ saturation of monomer (open circles) and dimer (closed circles) catalyzed GDP/GTP exchange. Data are presented as the percentage of maximal GTP γ S binding as a function of LTB₄ concentration. Results are given as mean values \pm SEM calculated from three independent experiments. (B) time-dependent activation of G α_{i2} catalyzed by the LTB₄-saturated form of the BLT2 monomer (closed circles) or dimer (open circles). Data are expressed as the percentage of maximal GTP γ S binding as a function of time. The experiment shown is representative of three independent assays. In A and B, the BLT2 concentration was 20 nM, that of G α_{i2} was 200 nM and those of G $\beta_1\gamma_2$ were 500 nM. (C) G α_{i2} saturation of GDP/GTP γ S exchange triggered by the BLT2 monomers (closed circles; fraction 3 in Fig. 4) and dimers (open circles; fraction 1 in Fig. 4). The contribution of the basal exchange (around 20% of the maximal receptor-catalyzed exchange) in the absence of agonist was systematically subtracted. The BLT2 concentration was 20 nM, those of G $\beta_1\gamma_2$ were in excess, 500 nM. Data are expressed as the percentage of maximal GTP γ S binding normalized to BLT2 receptor concentration. The experiments shown in the figure were repeated three times. Results are given as mean values \pm SEM. (D) [³⁵S]-GTP γ S binding to the G α_{i2} in the presence of LTB₄-stimulated BLT2 monomers and dimers. BLT2 receptor preparations and G α_{i2} were added at an equimolar ratio (20 nM each) in the presence of 500 nM G protein $\beta_1\gamma_2$ subunits. Data are expressed as specific dpm incorporated to the G α_{i2} . Mean values \pm SEMs are shown. Statistics are given: ***, $p < 0.0001$; *, $p < 0.05$. These experiments have been repeated at least three times, each done in triplicates.

Table 1: Physico-chemical properties of the fusion partners.

	Charge average	fraction of β -turn forming residues (%)	length (amino acid residues)
GST	-2	18.7	224
PLC	-2	22.7	258
PurF	-14	21.4	504
α_5 I	-19	31.6	285

The physico-chemical parameters of each potential fusion partner were calculated according to Wilkinson and Harrison (34). The combination of a high fraction of both charged residues and β -turn forming residues is critical for targeting a recombinant protein to *E. coli* IBs.

Table 2: Ligand binding properties of the BLT2 monomer and dimer fractions.

	LTB ₄	LY255283	U75302	12-HHT
BLT2 monomer	404 ± 12	158 ± 13	n.m.	61 ± 3
BLT2 dimer	344 ± 9	151 ± 11	n.m.	73.5 ± 5

The data indicated in the table are IC₅₀ (nM) values inferred from the fluorescence anisotropy competition binding experiments reported in Figure 5. These values are means ± SEM calculated from three distinct experiments carried out from two independent BLT2 monomer and dimer preparations. n.m., not measurable.

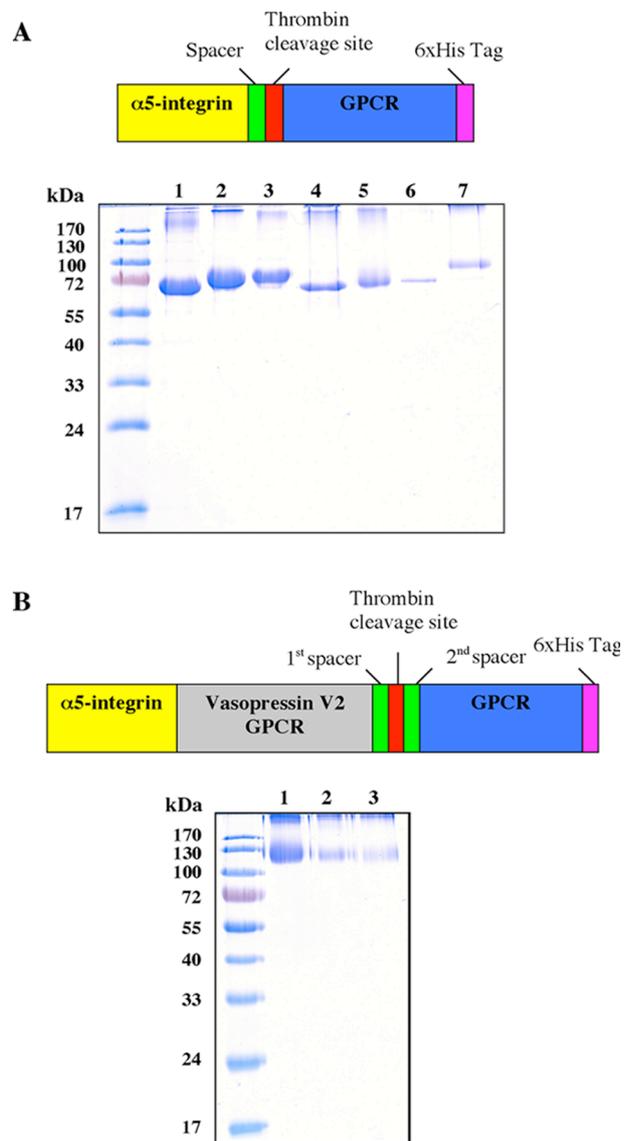


Figure 1

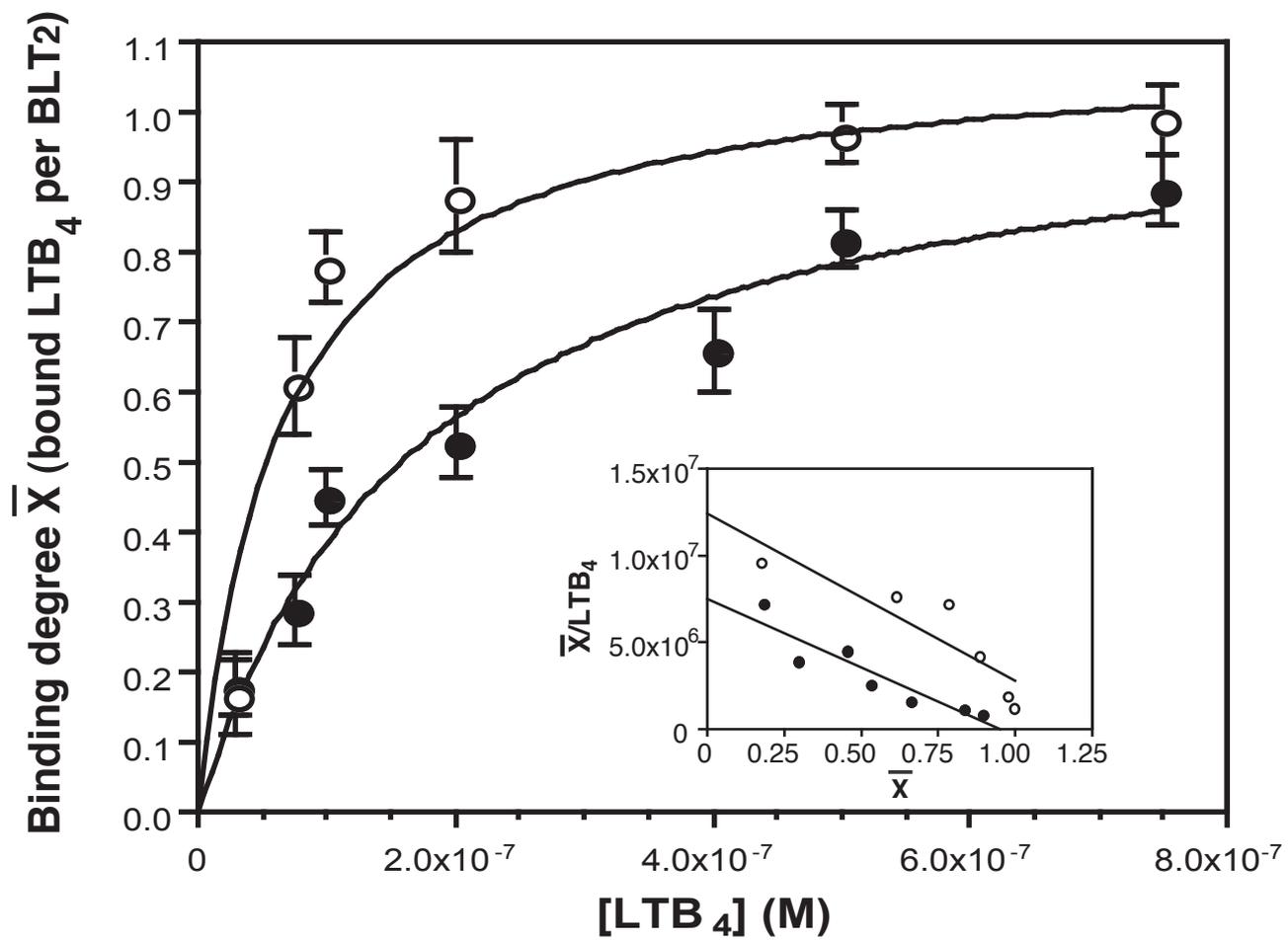


Figure 2

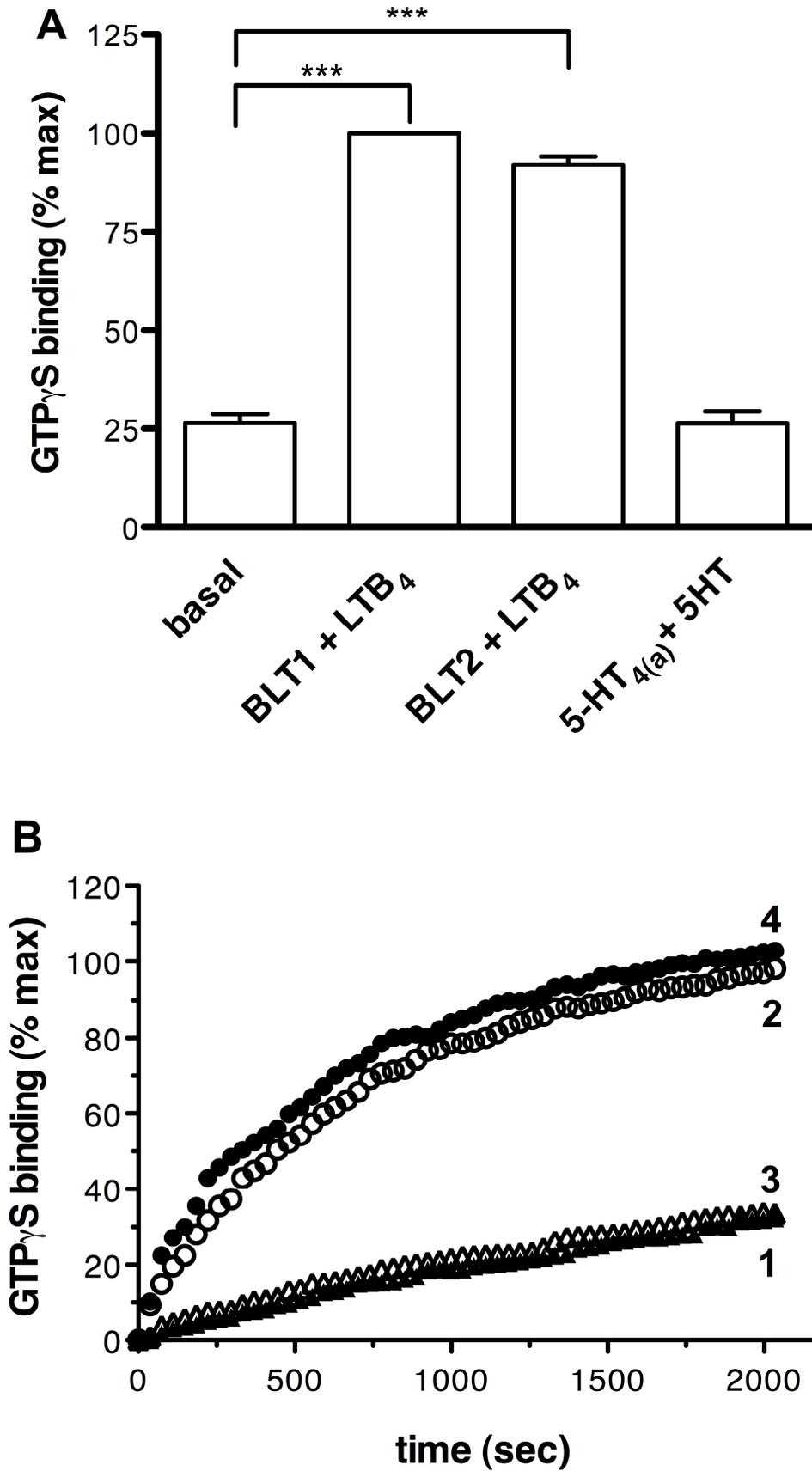


Figure 3

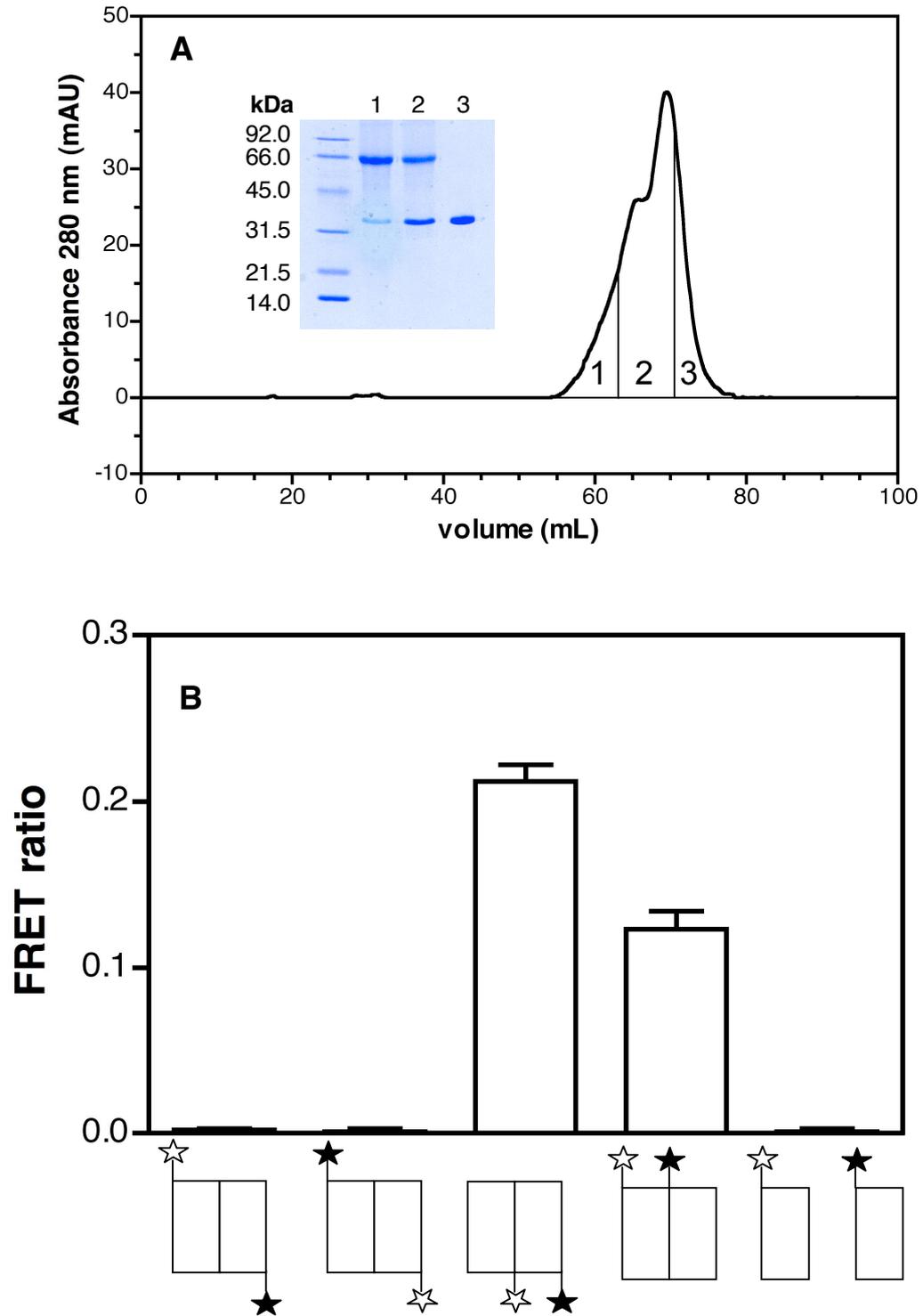


Figure 4

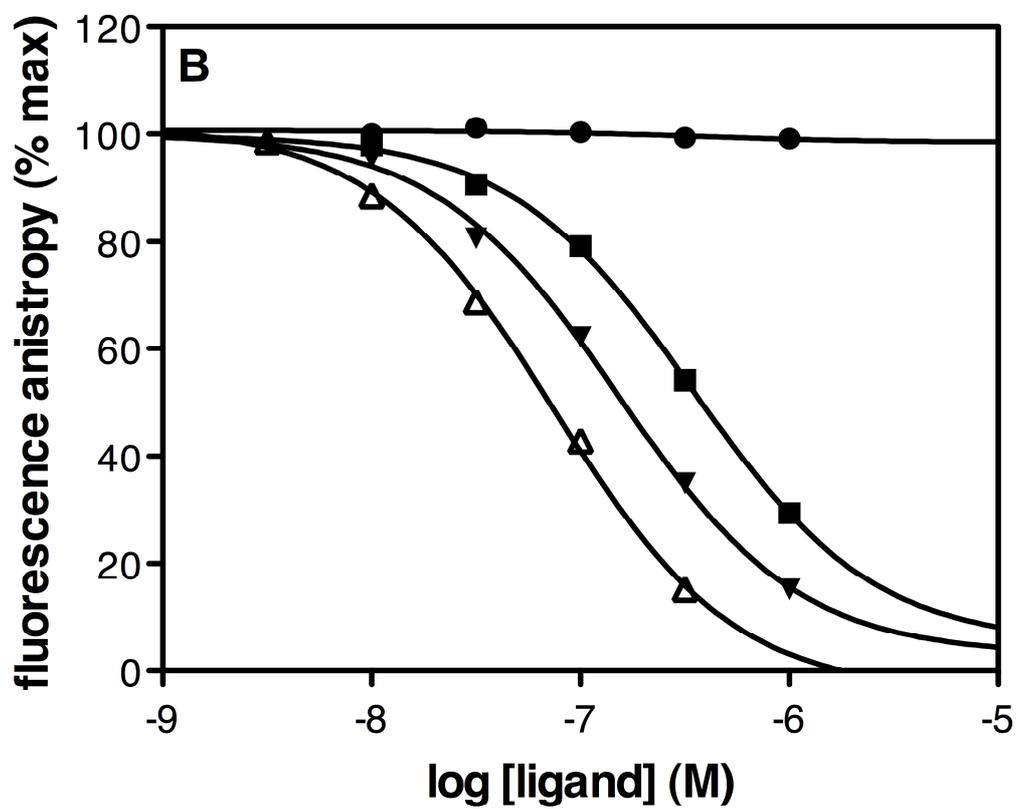
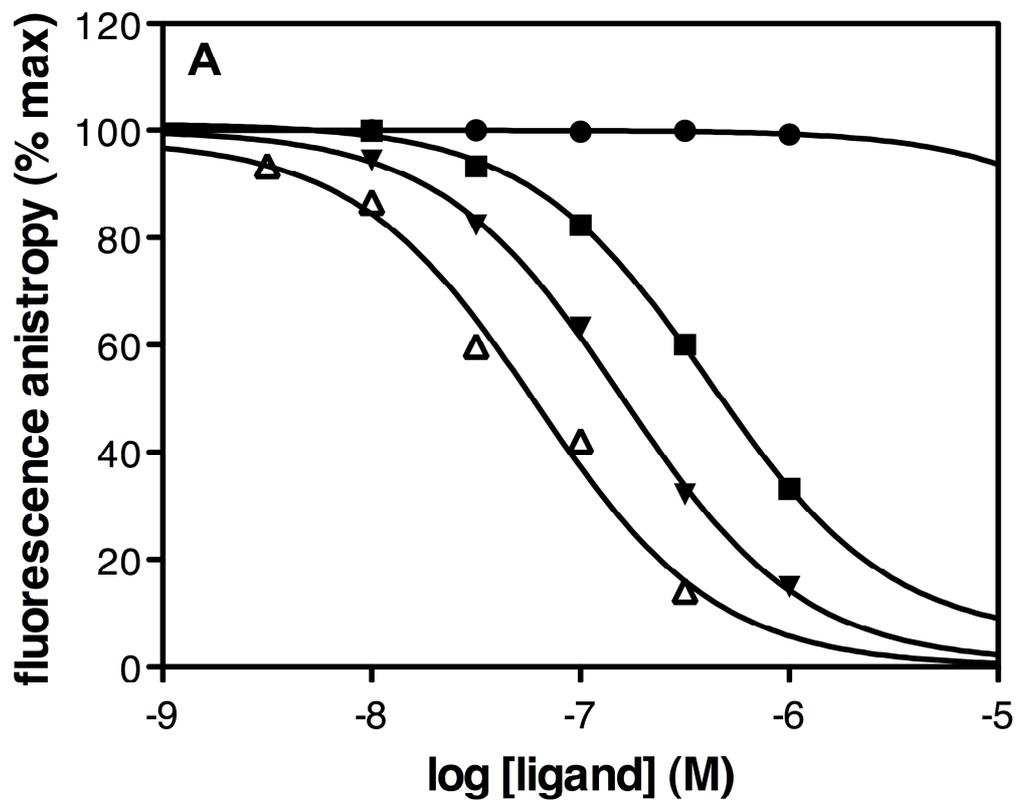


Figure 5

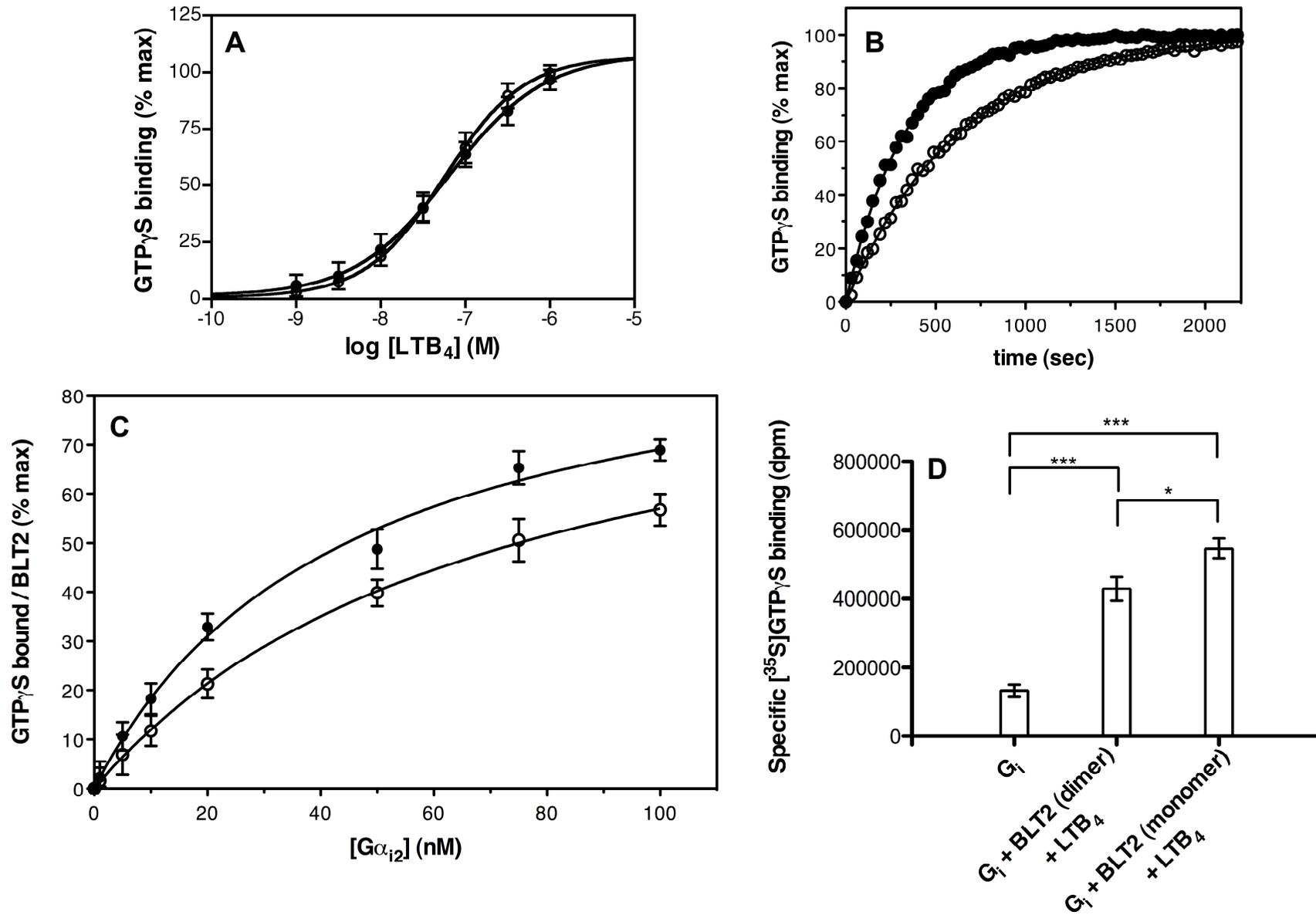


Figure 6