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# Measurement of heterotrimeric G-protein and regulators of G-protein signaling interactions by time-resolved fluorescence resonance energy transfer

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**Abstract**

G-protein-coupled receptors transduce their signals through G-protein subunits which in turn are subject to modulation by other intracellular proteins such as the regulators of G-protein signaling (RGS) proteins. We have developed a cell-free, homogeneous (mix and read format), time-resolved fluorescence resonance energy transfer (TR-FRET) assay to monitor heterotrimeric G-protein subunit interactions and the interaction of the Gα subunit with RGS4. The assay uses a FRET pair consisting of a terbium cryptate chelate donor spectrally matched to an Alexa546 fluor acceptor, each of which is conjugated to separate protein binding partners, these being $G\alpha_{i1}$:$\beta_4\gamma_2$ or $G\alpha_{i1}$:RGS4. Under conditions favoring specific binding between labeled partners, high-affinity interactions were observed as a rapid increase (>fivefold) in the FRET signal. The specificity of these interactions was demonstrated using denaturing or competitive conditions which caused significant reductions in fluorescence (50–85%) indicating that labeled proteins were no longer in close proximity. We also report differential binding effects as a result of altered activation state of the $G\alpha_{i1}$ protein. This assay confirms that interactions between G-protein subunits and RGS4 can be measured using TR-FRET in a cell- and receptor-free environment.


*Keywords:* G-protein; RGS; TR-FRET

Many sensory, hormonal, and neurotransmitter signaling pathways trigger important physiological responses via the family of G-protein-coupled receptors (GPCRs)[1]. Signal transduction occurs when such ligand-activated receptors interact with guanine-nucleotide-binding proteins (reviewed in [1–3]). When inactive, the G-protein subunits Gα, Gβ, and Gγ are associated as a heterotrimer with GDP bound to the guanine-nucleotide-binding site of the Gα subunit. Receptor–agonist binding activates the receptor and G-proteins through conformational changes which promote the exchange of GDP for GTP on the Gα subunit. This in turn causes conformational rearrangements in the "switch regions" of the Gα subunit resulting in further changes which could include rearrangement [4] or dissociation [5] of the Gα subunit from the Gβγ dimer. The Gα and Gβγ subunits are then available to interact with downstream effectors such as adenylyl cyclase and $K^+$ or $Ca^{2+}$ channels to transmit the signal and produce a biological response to the agonist. The Gα subunit contains an intrinsic GTPase activity which hydrolyses bound GTP to GDP, terminating signaling with reformation of the inactive

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[1] *Abbreviations used:* $\alpha_{2A}$-AR, $\alpha_{2A}$-adrenergic receptor; AMP-PNP, adenylylimidodiphosphate; DTT, dithiothreitol; GDP, guanosine diphosphate; GPCR, G-protein-coupled receptor; GTP, guanosine triphosphate, [$^{35}$S]GTPγS, radiolabeled isotope $^{35}$S-conjugated to 5′-*O*-(3-thiotriphosphate); Ni–NTA, $Ni^{2+}$–nitrilotriacetic acid; RGS, regulator of G-protein signaling; TR-FRET, time-resolved fluorescence resonance energy transfer; BRET, bioluminescence resonance energy transfer.

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heterotrimer. The rate of GTP hydrolysis by the Gα subunits in the $G\alpha_i$ and $G\alpha_o$ G-protein families can be significantly increased by interaction with intracellular proteins such as the family of regulators of G-protein signaling (RGS proteins) [6]. The discovery of RGS proteins has partially explained how many reactions mediated by G-protein signaling are terminated at rates considerably faster than the rate of intrinsic GTP hydrolysis by Gα subunits [7].

RGS proteins appear to be involved in a variety of physiological functions including modulating cell proliferation, differentiation, responses to neurotransmitters, membrane trafficking, and embryonic development [8]. While the pharmaceutical importance of GPCRs with regard to existing and future drug targets is well recognized by the significant market share of therapeutics that target such receptors [9], G-proteins and other regulatory proteins of GPCR-mediated signaling (such as the RGS family) are attracting interest as potential drug targets to increase selectivity of responses that cannot be mediated through receptors [10–14]. Indeed, it has been suggested that ligands directly targeted to G-proteins could potentially modulate individual effector pathways, alter signaling arising from particular G-protein classes or subclasses, and/or modify the kinetics of G-protein signaling [10]. Although there is significant potential to modulate individual effector pathways by interactions targeted at this level, no specific therapeutic compounds appear to be presently available.

Fluorescence resonance energy transfer (FRET) is a well-established technology for measuring the spatial interactions of proteins important in biological systems. Energy transfer from donor to acceptor fluorophores depends on appropriate spectral overlap and the relative distance between the fluorophores [15]. FRET has been used extensively in relation to defining GPCR and G-protein interactions, mainly in cell-based assay systems [4,16,17]. In this regard, FRET and the closely related bioluminescence resonance energy transfer (BRET) technologies have mainly been used with one binding partner having a large genetic modification to include a fluor such as green fluorescent protein or variants thereof [16,18]. Lanthanide ions, particularly europium ($Eu^{3+}$) and terbium ($Tb^{3+}$), have been shown to offer many advantages as donors in FRET [19] and have been used to study binding partners [20–22] and to develop various assay platforms [19]. Furthermore, they possess characteristic, sharp, and spectrally well-separated excitation and emission peaks which reduce spectral cross talk and long emission lifetimes, allowing for time-resolved (TR) measurements which can significantly increase the signal-to-noise ratio by reducing the background fluorescence at the time of signal measurement.

This study used TR-FRET to characterize the interaction of heterotrimeric G-protein subunits both between themselves and with RGS4, by using a terbium cryptate chelate donor spectrally matched to an Alexa546 fluor acceptor, which are conjugated to different protein binding partners. We show rapid, specific, high-affinity binding between the G-protein subunits and between the $G\alpha_{i1}$ subunits and the RGS4 protein, in addition to differential binding effects resulting from the various activation states of the $G\alpha_{i1}$ protein. This fluorescence-based assay is cell free, homogeneous (i.e., mix and read), and adaptable to a high-throughput format. As such, it offers potential to identify inhibitors and potentiators of the Gα:Gβγ interaction and RGS protein binding, which may have therapeutic applications.

## Materials and methods

### *Materials*

Baculovirus stocks [hexa-histidine-tagged $G\alpha_{i1}$, $\gamma_2$, and $\alpha_{2A}$-adrenergic receptor, ($\alpha_{2A}$AR)] and recombinant pQE60 containing RGS4 cDNA were obtained from Professor R. Neubig, University of Michigan, USA and $G\beta_4$ baculovirus stock was obtained from Dr J. Garrison, University of Virginia, USA. The fluorescent probes Alexa546 fluor $C_5$ and CS124-DTPA-EMCH-terbium were purchased from Invitrogen (Victoria, Australia). Radiolabeled isotope $^{35}S$-conjugated to 5′-*O*-(3-thiotriphosphate) ([$^{35}S$]GTPγS) was from Perkin–Elmer; 5′-adenylylimidodiphosphate (AMP-PNP), GTPγS, and all other reagents of the highest quality grade were purchased from Sigma–Aldrich (NSW, Australia).

### *Cell culture*

*Spodoptera frugiperda* (*Sf*9) cells in SF900II media (Invitrogen) were grown in suspension at 28 °C with shaking (138 rpm in an orbital shaker). Baculovirus stocks were filtered (using 0.2 μm syringe filter or vacuum filtration Millipore Stericup) and added to *Sf*9 cells at $1–2 \times 10^6$ cells/ml to infect at a multiplicity of infection of 2. Infected cells were grown for 48–72 h at 28 °C with shaking (138 rpm) prior to harvesting of cells and purification of recombinant protein.

### *$\alpha_{2A}$-Adrenergic receptor membrane preparation*

*Sf*9 cells (up to 1.5 L) infected with the $\alpha_{2A}$AR baculovirus were centrifuged at 1000*g* for 10 min and resuspended in 125 ml ice-cold lysis buffer (50 mM Hepes, pH 8.0, 0.1 mM EDTA, 3 mM $MgCl_2$, 10 mM β-mercaptoethanol with protease inhibitors, 0.02 mg/ml phenylmethylsulfonyl fluoride, 0.03 mg/ml benzamidine, 0.025 mg/ml bacitracin, and 0.03 mg/ml lima bean trypsin inhibitor). Cells were subjected to $N_2$ cavitation at 500 psi (3400 kPa) for 15 min, followed by sedimentation of nuclei and unbroken cells (750*g*, 10 min). Membranes were isolated by centrifugation of the supernatant at 100,000*g* for 30 min at 4 °C. The membrane pellet was then resuspended (50 ml per 1 L of original infected *Sf*9 cell culture) in incubation buffer (250 mM sucrose, 10 mM Tris, pH 8.0, 3 mM $MgCl_2$) containing 7 M urea and protease inhibitors (as above) at 4 °C. After 30 min incubation on ice, membranes were diluted to

contain 4 M urea with incubation buffer (containing protease inhibitors) and centrifuged at 100,000*g* at 4 °C for 30 min. The urea-treated membrane pellet was washed twice in incubation buffer. The final urea-treated membrane pellet was resuspended to approximately 1–3 mg/ml protein and aliquots were rapidly frozen in liquid $N_2$ and stored at −80 °C until use.

### *[$^{35}$S]GTPγS membrane reconstitution assay*

Functionality of fluorophore-labeled and non-fluorophore-labeled G-proteins was measured using a modification of the [$^{35}$S]GTPγS binding technique [23]. A reconstitution mix consisting of 0.1 mg/ml $\alpha_{2A}$AR membranes, 5 μM GDP, 10 μM AMP-PNP, appropriate Gα and Gβγ proteins (±fluorescent conjugates as prepared below) at 20 nM, and 0.2 nM [$^{35}$S]GTPγS in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) was prepared on ice. The reactions (100 μl final volume) were initiated by addition of either buffer (control) or 10 μM UK14304 (an $\alpha_{2A}$AR agonist; Sigma–Aldrich). Then 100 μM yohimbine ($\alpha_{2A}$AR antagonist) was added (where indicated) to determine receptor-induced signaling specificity. Samples were incubated at 27 °C with gentle mixing for 90 min and then filtered over Whatman GF/C filters. Filters were washed with 3 × 4 ml washes of ice-cold TMN buffer and dried. Bound [$^{35}$S]GTPγS:G$\alpha_{i1}$ was determined by liquid scintillation counting.

### *G-protein expression and purification*

*Sf*9 cells (up to 2 L) were infected with the recombinant baculoviruses G$\alpha_{i1}$ (hexahistidine tag), $\beta_4$, and $\gamma_2$ for 72 h and were subsequently harvested by centrifugation at 1000*g* for 10 min. All further operations were performed on ice, using a modification of published methods [24]. The cell pellet was then gently resuspended in ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4) and centrifuged at 1000*g* for 10 min. The pellet was resuspended in ice-cold lysis buffer containing 10 μM GDP. Cells were lysed by loading the cells into a nitrogen cavitation chamber and subjecting them to 500 psi for 15 min. The lysate was centrifuged at 750*g* for 10 min at 4 °C to remove any intact cells and nuclei. The supernatant (containing the crude membrane fraction) was centrifuged at 100,000*g* for 30 min. The membrane pellet was then resuspended in wash buffer (50 mM Hepes, 3 mM $MgCl_2$, 50 mM NaCl, 10 mM β-mercaptoethanol, 10 μM GDP, containing protease inhibitors as for lysis buffer, pH 8.0) using a large Dounce homogenizer. The membranes were centrifuged at 100,000*g* for 30 min before resuspension in a small volume of wash buffer to maintain total protein concentration of approximately 5 mg/ml. Protein concentration was determined using the Bradford protein assay [25]. Suspensions in appropriate-sized aliquots were then rapidly frozen in liquid nitrogen and stored at −80 °C. Subsequently, frozen *Sf*9 membranes (at ⩾5 mg/ml protein) containing G$\alpha_{i1}$, $\beta_4$, and $\gamma_2$ were thawed and diluted to 5 mg/ml protein with wash buffer containing fresh protease inhibitors and 1% (w/v) cholate (final concentration). Membranes were stirred on ice for 1 h to extract. The sample was then centrifuged at 100,000*g* for 40 min and the supernatant collected (termed membrane extract). The membrane extract containing G-proteins was diluted fivefold with buffer A (20 mM Hepes, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5% (w/v) polyoxyethylene-10-lauryl ether, and 10 μM GDP, pH 8.0) prior to column loading. The samples were loaded onto nickel–nitrilotriacetic acid (Ni–NTA) beads (Qiagen) to allow selective binding of histidine-tagged proteins. The column containing G-proteins was washed with 50 ml of buffer A containing 5 mM imidazole and 300 mM NaCl to remove proteins not specifically bound to the column via $Ni^{2+}$–histidine interaction. All washing procedures were carried out at 4 °C.

### *Labeling of G-proteins*

Following the capture of histidine-tagged G-proteins on Ni–NTA beads, washing proceeded using buffer A in the absence of β-mercaptoethanol to eliminate interference to subsequent thiol conjugation of fluorophores using a maleimide linker. The Alexa546 maleimide dye was solubilized in anhydrous dimethyl sulfoxide and the terbium cryptate maleimide conjugate in 50 mM Hepes (pH 8.0), to concentrations of 4.8 and 1.1 mM, respectively. The desired label (i.e., Alexa546 maleimide or terbium cryptate maleimide conjugate) was then added to purified G-protein (Gαβγ heterotrimer) preparations (on column) at an approximate fivefold molar excess. The columns were incubated at room temperature for 3 h (terbium) or 2–3 h (Alexa546) as recommended by the manufacturer's protocol, with occasional mixing. Following incubation, the column containing labeled G-proteins was washed with 50 ml buffer A containing 5 mM imidazole and 300 mM NaCl (pH 8.0) to remove unbound Alexa546 until the eluent containing nonconjugated fluorescent probes produced fluorescence similar to that of buffer alone. Fluorescence was monitored by measuring the emission at 550 nm for the terbium conjugate and 572 nm for the Alexa546 conjugate, after excitation at 340 or 545 nm, respectively (Hitachi Fluorescence Spectrophotometer 650-10S). This was performed to verify that column-bound, labeled heterotrimeric G-proteins had been thoroughly washed to remove any nonconjugated fluorescent probe. The labeled $G\beta_4\gamma_2$ subunits (containing either Alexa546 or terbium labels) were eluted with 750-μl volumes of buffer E (20 mM Hepes, pH 8.0, 50 mM NaCl, 10 mM β-mercaptoethanol, 10 μM GDP, 1% (w/v) cholate, 50 mM $MgCl_2$, 5 mM imidazole, 10 mM NaF, and 30 μM $AlCl_3$ ($AlF_4^-$). A pure sample of the fluorescently labeled $G\beta_4\gamma_2$ protein subunits was therefore obtained in the absence of its His-tagged G$\alpha_{i1}$-protein binding partner. The remaining labeled (with either Alexa546 or terbium) G$\alpha_{i1}$ proteins were eluted from the nickel column with buffer E containing

150 mM imidazole. Aliquots of each elution fraction were then run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Thus, each labeling reaction provided a labeled Gα subunit sample (e.g., $G\alpha_{i1}Tb$) and a separate labeled Gβγ subunit sample (e.g., $G\beta_4\gamma_2Tb$). The pooled elution fractions (to ⩽3 ml) were dialyzed with 4 × 200 ml buffer F (20 mM Hepes, pH 8.0, 3 mM $MgCl_2$, 10 mM NaCl, 10 mM β-mercaptoethanol, 1 μM GDP, and 0.1% (w/v) cholate) and overnight against 200 ml buffer F using a Slide-a-lyzer (Pierce Chemical Co., Rockford, IL, USA) to remove remaining $AlF_4^-$, thus restoring Gα subunits to the inactive (GDP-bound) state. Following dialysis, the purified G-protein subunits were rapidly frozen in liquid $N_2$ and stored at −80 °C in buffer F or in 50% (v/v) glycerol in buffer F.

*RGS4 labeling with Alexa546*

Competent M15[pREP4] *Escherichia coli* (Qiagen) were transformed by heat shock with recombinant pQE60 plasmid (Qiagen) containing an RGS4 insert. Correct insertion of the RGS4 cDNA was confirmed by DNA sequencing, providing for expression of RGS4 with a C-terminal hexa-histidine tag. Isolated transformed *E. coli* colonies were cultured in YT broth (8 g/L tryptone, 5 g/L yeast extract, and 2.5 g/L NaCl) containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin and incubated at 37 °C with vigorous shaking. To express RGS4, 50–200 ml cultures ($OD_{600nm}$ ~0.5) were induced with 1 mM isopropyl β-D-thiogalactoside for 2–3 h prior to harvesting. Cell pellets were then stored frozen at −80 °C in preparation for purification following a modification of a previously published method [26]. Cell pellets containing RGS4 were resuspended in 10 ml of TBP buffer (50 mM Tris, pH 8.0, 10 mM β-mercaptoethanol, 0.02 mg/ml phenylmethylsulfonyl fluoride, 0.03 mg/ml benzamidine) and then lysed using 0.2 mg/ml lysozyme, 0.01 mg/ml DNaseI, and 5 mM $MgCl_2$. A 20% (w/v) cholate solution [50 mM Na-Hepes, pH 8.0, 3 mM MgCl2, 50 mM NaCl, and 200 g/L cholic acid ($Na^+$)] was then added to obtain a final cholate concentration of 1% w/v cholate. The sample was stirred on ice for 1 h to extract and then centrifuged at 100,000*g* for 40 min. The supernatant was preincubated with Ni–NTA beads for 10 min (in TBP buffer) prior to running through a Ni–NTA column under gravity. The column containing (His-tagged) RGS4 was washed with TBP buffer containing 200 mM NaCl and 10 mM imidazole to remove nonspecifically bound proteins. RGS4 was then labeled (i.e., RGS4-Alexa546) with the Alexa546 fluorophore in a manner similar to that for the G-proteins except RGS4-Alexa546 was eluted from the column using TBP buffer containing 100 mM NaCl and 250 mM imidazole.

*Protein concentrations*

Final protein concentration was determined by SDS–PAGE of G-protein subunits or RGS4 (using bovine serum albumin standards), followed by laser scanning densitometry (LKB Ultroscan XL, Enhanced Laser Densitometer, Sweden) or Bradford protein assay [25].

*Determination of labeling efficiency*

Labeling efficiency was determined by comparing the fluorescence emission intensity of Alexa546 or terbium in solution against G-protein- or RGS4-conjugated label (subtracting any background fluorescence for unlabeled protein). Calibration curves of terbium and Alexa546 labels in solution were generated and then measured using a Victor$^3$ multilabel plate reader (Perkin–Elmer Corp., Boston, MA, USA).

*TR-FRET assays*

Fluorescence measurements were obtained using a Victor$^3$ multilabel plate reader fitted with a 1500-V xenon flash light source. Experiments were carried out in black 96-well plates. The required amounts of proteins labeled with acceptor or donor fluors were aliquoted onto either side of a well so that mixing did not occur until required. Reactions were initiated by addition and mixing of TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) in the well (to give a final assay volume of 100 μl). To utilize the long emission lifetime of the Tb chelate donor and the time-resolved emission of the acceptor fluorophore, the measurement was gated for a short time period to minimize background emission from the assay components. Time-resolved fluorometric measurements were conducted with the following instrument settings: excitation 340 nm, emission 572 nm, delay 50 μs, and counting duration 900 μs. Readings were taken until the fluorescence emission stabilized. At this time, other components such as unlabeled $G\alpha_{i1}$, RGS4, or Gβγ were added, and fluorescence readings were then continued until fluorescence again stabilized. The Gα subunit can exist in various states of activation which have been shown to significantly alter the conformation of the switch regions of this subunit [27]. These various states of activation which include the inactive, active, and transition states can be induced by the presence of GDP, GTPγS, or $AlF_4^-$, respectively. The Gα subunits used in these experiments had been dialyzed with buffer containing GDP and were therefore in the inactive (GDP-bound) state. Therefore, unless otherwise stated, the $G\alpha_{i1}$ subunits used throughout these experiments had GDP bound to the GDP/GTP binding sites. Experiments using $AlF_4^-$ were conducted by dispensing TMND buffer containing 10 mM NaF and 30 μM $AlCl_3$ or adding these components at the specified time to the appropriate final concentration. Experiments with GTPγS were performed by preincubating the Gα subunit with an excess of GTPγS for >1 h and then adding the appropriate amount of mixture required to achieve the desired final concentrations. Background fluorescence was determined by the addition of the appropriate

concentration of terbium-labeled protein to TMND buffer and exposure to the same TR-FRET conditions. Background fluorescence was subtracted from all data to enable kinetic parameters to be measured.

*Data and statistical analysis*

$K_d$ values for the saturation curves were determined by nonlinear least-squares analysis fitted to a single hyperbolic binding function (GraphPad Prism V4.0, San Diego, CA, USA). Statistical comparison was performed by one-way ANOVA.

## Results

Terbium labeling efficiency of Tb:G-protein ranged from 1:4 to 1.6:1. Alexa546 labeling efficiency ranged from 1:4 to 1.2:1 for functional labeled G-proteins. RGS4 was labeled with Alexa546 at a ratio of 1 mole of Alexa546 per 4–6 moles of RGS4. Signaling activity of G-proteins labeled with terbium or Alexa546 was measured by specific binding of [$^{35}$S]GTPγS (final concentration 0.2 nM) induced by activation of the $\alpha_{2A}$AR with the specific agonist UK14304 in the presence of 20 nM unlabeled (Fig. 1A) or labeled (Fig. 1B) G-proteins. [$^{35}$S]GTPγS binding increased from approximately 0.02 fmol (basal) to 0.14 fmol (stimulated; see Fig. 1) for labeled proteins, reflecting the GDP–GTP exchange following activation of $G\alpha_{i1}$ by the agonist-bound receptor. UK14304-stimulated signaling activity was almost completely inhibited by the $\alpha_{2A}$AR antagonist yohimbine for both fluorescently conjugated and nonconjugated G-proteins. This indicated that fluorescent labeling did not significantly affect specificity of receptor activation of the G-proteins.

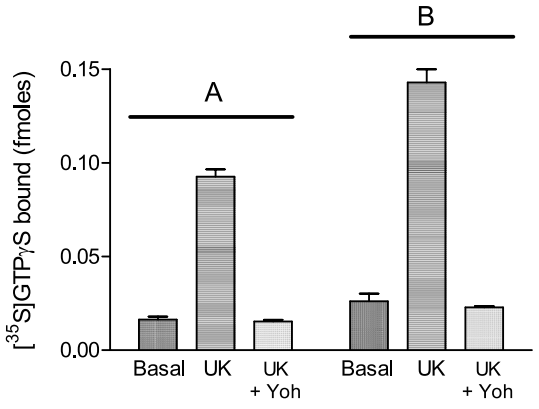


Fig. 1. [$^{35}$S]GTPγS reconstitution assay. Reconstitution of $\alpha_{2A}$AR with (A) $G\alpha_{i1} + G\beta_4\gamma_2$ or (B) $G\alpha_{i1}$Alexa546 + $G\beta_4\gamma_2$Tb. G-protein subunits (20 nM) were combined with 0.1 mg/ml of $\alpha_{2A}$AR membranes, 2 μM GDP, 10 μM AMP-PNP ("reconstitution mix"), 0.2 nM [$^{35}$S]GTPγS, and 100 μM yohimbine (where shown) in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT). The reaction was started by addition of UK14304 (10 μM final concentration). The reaction was incubated for 90 min at 27 °C with shaking. The final volume was 100 μl and the entire reaction mix was filtered over a GF/C filter and washed with 3 × 4 ml ice-cold TMN buffer. Data shown are mean ± SE ($n = 3$). UK, UK14304, Yoh, yohimbine.

TR-FRET was successfully used to show specific protein–protein interactions. To obtain the greatest fluorescence increase, the plate reader parameters for flash intensity, count time, and gating period were optimized, with the gating period making the only significant difference where a 50-μs delay generated an optimal signal-to-noise ratio (data not shown). Upon mixing of potential binding partners (Tb–cryptate-conjugated $G\alpha_{i1}$ [$G\alpha_{i1}$Tb) and Alexa546-conjugated $G\beta_4\gamma_2$ ($G\beta_4\gamma_2$Alexa546)], an increase in acceptor fluorescence emission intensity (3- to 6-fold, at 572 nm) was observed over background emission due to the terbium chelate alone. This is indicative of heterotrimer association, thus bringing donor and acceptor fluors into sufficient proximity for TR-FRET to occur (Fig. 2A). Experiments were conducted in the presence of various concentrations of GDP to ensure that the Gα subunit was maintained in an inactive conformation. Results showed that the 1 μM concentration of GDP present in the dialysis buffer (buffer F) adequately occupied the GDP binding sites of the $G\alpha_{i1}$ samples, as increasing concentrations of GDP showed no effect on the TR-FRET measurements of the G-protein interactions. While terbium chelate labeling of $G\alpha_{i1}$ and Alexa546 fluor labeling of $G\beta_4\gamma_2$ subunits were the predominant labeling formats, reciprocal labeling also proved successful, with TR-FRET occurring upon mixing of the G-protein subunits (Fig. 2B). Indeed, the half-maximal binding times for $G\alpha_{i1}$Tb:$G\beta_4\gamma_2$Alexa546 and $G\beta_4\gamma_2$Tb:$G\alpha_{i1}$Alexa546 were 0.65 and 0.71 min, respectively. This suggested that either combination of labeled G-proteins could be used for the FRET studies.

The term "bystander BRET" has been used to describe nonspecific interactions observed in GPCR dimerization studies of cell membranes using bioluminescence resonance energy transfer technology [18], where models of BRET resulting from random collisions have been estimated to increase in conjunction with high receptor density in the membrane. The phenomenon that we have termed "bystander FRET," where random collisions result in respective FRET spectral pairs coming within sufficient proximity for FRET to occur, was investigated in the present study. Random collisions between the FRET donor (conjugated to a protein subunit) and the FRET acceptor (free in solution) increased bystander FRET as a function of the fluorophore concentrations in solution. The ratio of FRET donor (Tb) to FRET acceptor (Alexa546) represented in the G-protein/RGS interaction assay was determined by considering those proteins that were calculated as being labeled based on labeling efficiency. The results showed that artifacts arising from the production of bystander FRET occur exclusively at concentrations of fluorescent partners significantly higher than those used in these FRET assays (ratio donor:acceptor >1:60) (Fig. 3). Stringent washing of unbound fluor after conjugation further limited any free fluor associated with the labeled protein sample used in the TR-FRET assays.

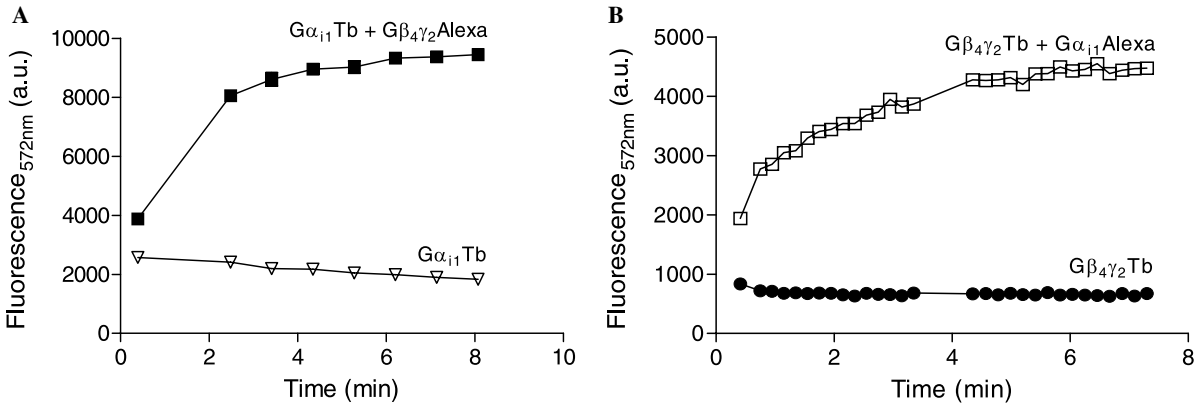


Fig. 2. $G\alpha_{i1}$ interactions with $G\beta_4\gamma_2$ were demonstrated by TR-FRET (A) TR-FRET between 50 nM $G\alpha_{i1}Tb$ + 50 nM $G\beta_4\gamma_2$Alexa546 (■) in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) was observed as an increase in fluorescence at 572 nm (fivefold) over background emission from 50 nM terbium-labeled subunit alone (▽). (B) When subunits were reciprocally labeled, TR-FRET between 50 nM $G\beta_4\gamma_2Tb$ and 50 nM $G\alpha_{i1}$Alexa546 (□) was observed as an increase in fluorescence with time above background levels (●) (representative data).

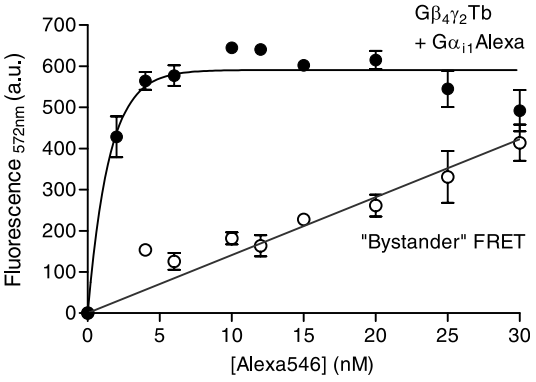


Fig. 3. Contribution of "bystander" TR-FRET. Steady state TR-FRET between 10 nM $G\beta_4\gamma_2Tb + G\alpha_{i1}$Alexa546 (labeled protein concentration indicated on *x* axis) in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) (●). TR-FRET between 10 nM $G\beta_4\gamma_2Tb$ (500 pM labeled G-protein) and Alexa546 free in solution, termed bystander FRET(○). All data shown are mean ± SE ($n = 3$). All samples were diluted in TMND buffer.

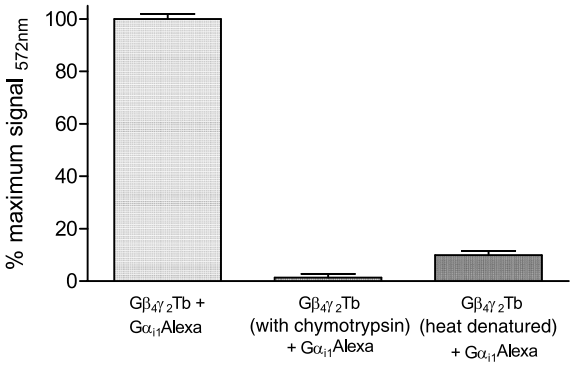


Fig. 4. Functional proteins were required for TR-FRET. Column 1 shows the TR-FRET signal from 10 nM $G\alpha_{i1}$Alexa546 and 10 nM $G\beta_4\gamma_2Tb$ subunits in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) after a 5-min incubation period. Columns 2 and 3 show significantly decreased signals when $G\beta_4\gamma_2Tb$ was treated with chymotrypsin or heat-denatured, respectively, suggesting that degraded $G\beta_4\gamma_2Tb$ is unable to bind to $G\alpha_{i1}$Alexa546. All data shown are mean ± SE ($n = 3$).

When fully functional labeled G-protein preparations were substituted for protease (chymotrypsin)- or heat-treated G-protein subunits, TR-FRET was decreased by >90%, indicating that the TR-FRET signal was dependent on specific high-affinity interaction of the heterotrimeric G-proteins (Fig. 4). Furthermore, the protease or heat-denaturing treatments had no effect on the background signal generated by $G\beta_4\gamma_2Tb$ alone (data not shown), indicating that this effect was not due to the conjugation of the label to the protein or the disruption of the protective cryptate structure of the terbium.

To further determine whether the FRET signal was indicative of a specific interaction between heterotrimeric G-protein binding partners, excess unlabeled binding partners were added to the formed heterotrimer. The addition of increasing concentrations of unlabeled $G\alpha_{i1}$ progressively and rapidly decreased the TR-FRET signal by a maximum of approximately 70% in a concentration-dependent manner, with a $t_{1/2}$ of approximately 0.9 min (Fig. 5A) and an $IC_{50}$ of ~54.5 ± 19.3 nM (Fig. 5B). Decreases in steady state fluorescence could also be achieved following the addition of a molar excess (10-fold) of unlabeled $G\beta_4\gamma_2$ subunits (Fig. 5C). Fluorescence measured simultaneously at the second peak of terbium emission (545 nm) arising from the $^5D_4 \rightarrow {}^7F_5$ electronic transition [28] increased upon subunit dissociation (data not shown), while a corresponding decrease in Alexa546 emission (observed at 572 nm) is consistent with increased distance between labeled proteins and is therefore a corresponding decrease in the extent of FRET. It was also shown that when $AlF_4^-$ was added following a steady state level of TR-FRET between $G\alpha_{i1}Tb$ and $G\beta_4\gamma_2$Alexa546, fluorescence rapidly decreased to approximately 50% of the original signal (Fig. 6).

Under the same conditions used to observe association between $G\alpha_{i1}Tb$ and $G\beta_4\gamma_2$Alexa546, an association between $G\alpha_{i1}Tb$ and RGS4-Alexa546 was also observed.

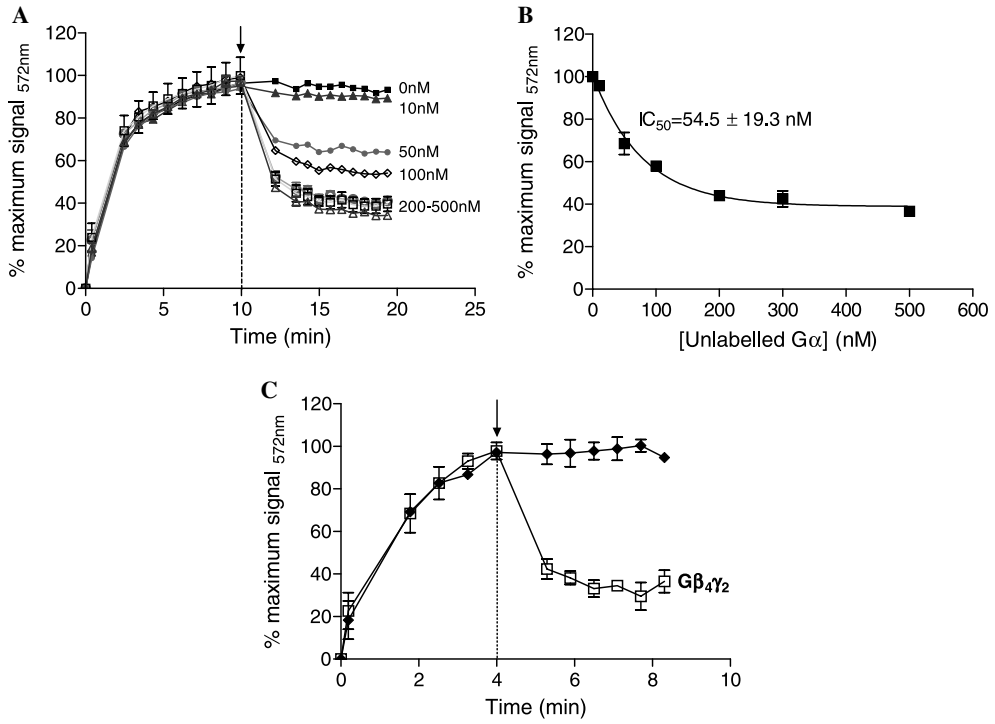


Fig. 5. Unlabeled binding partners compete for binding to $G\alpha_{i1}Tb$ or $G\beta_4\gamma_2$ causing a decrease in TR-FRET. (A) TR-FRET between 50 nM $G\alpha_{i1}Tb$ + 50 nM $G\beta_4\gamma_2$Alexa546 in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) was reduced in a dose-dependent manner upon addition of increasing amounts of unlabeled $G\alpha_{i1}$, which exhibited an $IC_{50}$ of 54.5 nM as shown in (B). (C) Addition of a 10-fold excess of unlabeled $G\beta_4\gamma_2$ (final concentration of 200 nM), indicated by the arrow, caused a 70% reduction in TR-FRET between 20 nM $G\alpha_{i1}Tb$ + 20 nM $G\beta_4\gamma_2$Alexa546. Data shown are mean ± SE ($n = 3$).

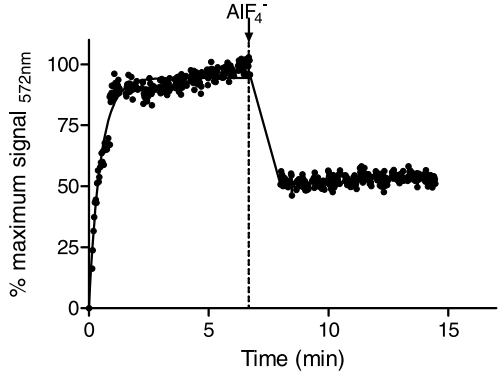


Fig. 6. Addition of $AlF_4^-$ caused a reduction in TR-FRET between $G\alpha_{i1}Tb$ and $G\beta_4\gamma_2$Alexa. TR-FRET between 15 nM $G\alpha_{i1}Tb$ + 15 nM $G\beta_4\gamma_2$Alexa546 in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) was rapidly decreased upon the addition of $AlF_4^-$ (10 mM NaF followed by 30 μM $AlCl_3$) (representative data).

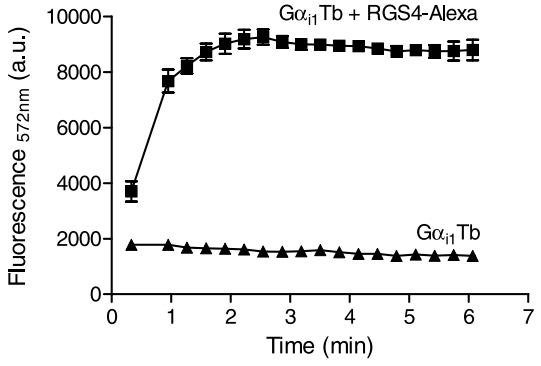


Fig. 7. $G\alpha_{i1}Tb$ interactions with RGS4-Alexa546 demonstrated by TR-FRET. TR-FRET between 50 nM $G\alpha_{i1}Tb$ + 150 nM RGS4-Alexa546 (■) in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) was observed as an increase (4.5-fold) above the background emission of the terbium-labeled subunit alone (▲). All data shown are mean ± SE ($n = 3$).

These labeled proteins produced increases in acceptor fluorescence three- to sixfold above background levels, which reached equilibrium after approximately 2.5 min (Fig. 7). This interaction was shown to be specific by the addition of excess amounts of unlabeled RGS4 or $G\alpha_{i1}$ which were able to compete for their respective binding partners to induce a rapid decrease in TR-FRET (70–80%) between $G\alpha_{i1}Tb$ and RGS4-Alexa546 (Figs. 8A and B).

It was also observed that decreases in steady state TR-FRET between $G\alpha_{i1}Tb$ and $G\beta_4\gamma_2$Alexa546 could be achieved following the addition of a molar excess of unlabeled RGS4 (Fig. 9A). Likewise, the addition of unlabeled $G\beta_4\gamma_2$ to steady state TR-FRET between $G\alpha_{i1}Tb$ and RGS4-Alexa546 caused a rapid decrease in fluorescence (Fig. 9B). However, the decrease in fluorescence observed upon addition of unlabeled $G\beta_4\gamma_2$(~70%) was greater than that achieved by unlabeled RGS4 (~50%) although a higher final concentration of labeled RGS4 was used.

Saturation binding curves were generated by titrating increasing amounts of $G\beta_4\gamma_2$Alexa546 or RGS4-Alexa546

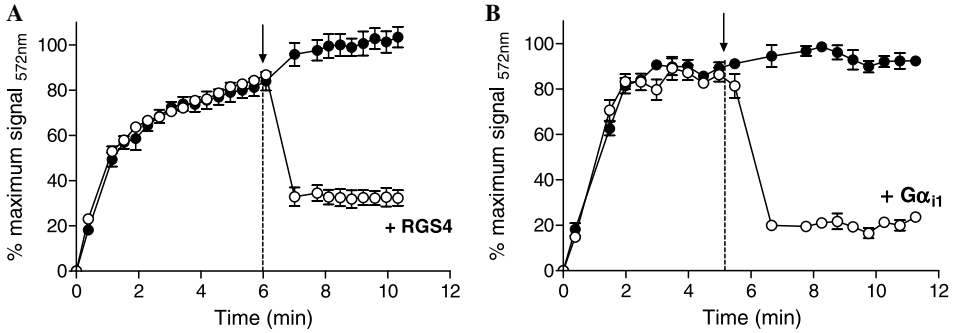


Fig. 8. Unlabeled binding partners compete for $G\alpha_{i1}$Tb and RGS4-Alexa546 binding causing a decrease in TR-FRET. (A) TR-FRET between 50 nM $G\alpha_{i1}$Tb + 150 nM RGS4-Alexa546 was rapidly decreased (70%) following the addition of a 10-fold excess of unlabeled RGS4 at the indicated time. (B) Steady state TR-FRET between 40 nM $G\alpha_{i1}$Tb + 70 nM RGS4-Alexa546 in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) was rapidly decreased (80%) upon the addition of a 16-fold excess of unlabeled $G\alpha_{i1}$ subunits at the indicated time. All data shown are mean ± SE ($n = 3$).

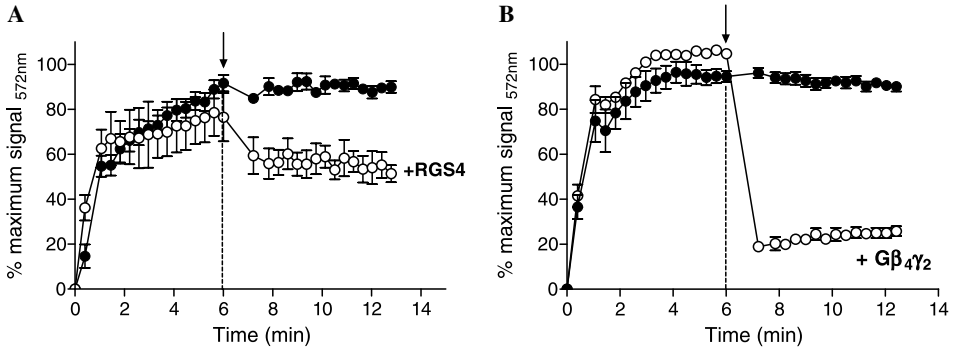


Fig. 9. RGS4 can compete with $G\beta_4\gamma_2$ for binding to $G\alpha_{i1}$. (A) Addition of a 35-fold excess of unlabeled RGS4 (final concentration of 1.75 μM) at the indicated time caused a 40% reduction in steady state TR-FRET between 50 nM $G\alpha_{i1}$Tb + 50 nM $G\beta_4\gamma_2$Alexa546 in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT). (B) Steady state TR-FRET between 50 nM $G\alpha_{i1}$Tb + 150 nM RGS4-Alexa546 in TMND buffer was rapidly decreased (75%) upon the addition of excess unlabeled $G\beta_4\gamma_2$ subunits (at the indicated time) to a final concentration of 900 nM. All data shown are mean ± SE ($n = 3$).

with a constant concentration of $G\alpha_{i1}$Tb. From these, an apparent dissociation constant ($K_d$) of 2.4 nM for the G-protein heterotrimer was observed. When the concentration of $G\alpha_{i1}$Tb was increased from 3 to 15 nM, the degree of TR-FRET increased, however, the $K_d$ did not significantly change (data not shown). A $K_d$ of 14.6 nM for $G\alpha_{i1}$ binding to RGS4 was observed (Fig. 10), suggesting that $G\alpha_{i1}$ has a greater affinity for $G\beta_4\gamma_2$ than RGS4.

To observe whether changes in the interaction of $G\alpha_{i1}$ with RGS4 occurred due to conformational changes caused by the state of activation of the Gα subunit, TR-FRET was conducted with $G\alpha_{i1}$ bound to either GDP, GTPγS (by preincubation), or $AlF_4^-$. The results showed that the steady state TR-FRET signal in the presence of $AlF_4^-$ was significantly increased (approximately twofold) compared to the Gα subunit present with either GTPγS or GDP, both of which had similar maximum fluorescent signals (Fig. 11A). Furthermore, the rate constants generated on association of the proteins varied significantly depending on the activation state of the subunit. The rate of association ($K_{obs}$) of RGS4-Alexa546 and $G\alpha_{i1}$Tb in the presence of GTPγS (active state) was

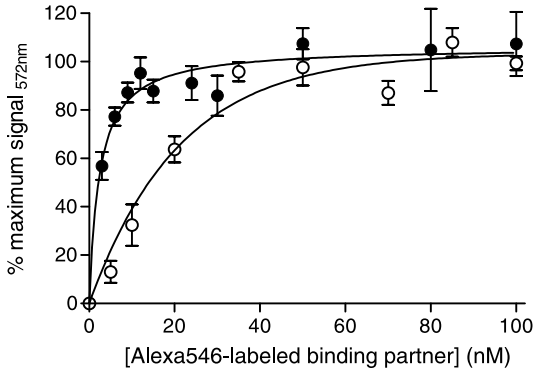


Fig. 10. $G\beta_4\gamma_2$ has a higher affinity for $G\alpha_{i1}$ than RGS4. TR-FRET between 5 nM $G\alpha_{i1}$Tb and various concentrations of $G\beta_4\gamma_2$Alexa546 (●) in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) generated an apparent $K_d$ of 2.4 nM. TR-FRET between 50 nM $G\alpha_{i1}$Tb and various concentrations of RGS4-Alexa546 (○) produced an apparent $K_d$ of 14.6 nM. All data shown are mean ± SE ($n = 3$).

$0.80 \pm 0.07\ min^{-1}$, which was significantly slower than that in the GDP-bound state (inactive state) with an association rate of $1.36 \pm 0.13\ min^{-1}$ ($p < 0.05$). Additionally, $AlF_4^-$

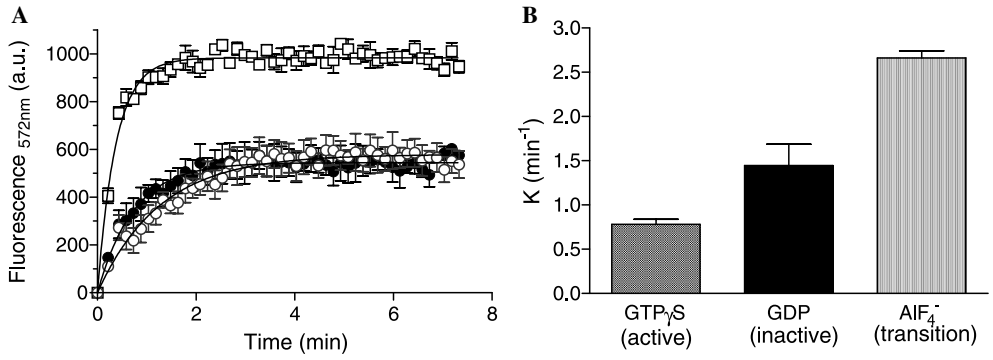


Fig. 11. Activation state of Gα alters interaction with RGS4. (A) TR-FRET between 40 nM inactive (+GDP) (●), active (+GTPγS) (○), or transitional state $+AlF_4^-$ (□) $G\alpha_{i1}$Tb with 70 nM RGS4-Alexa546 in TMND buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT). (B) Comparison of the rate constants ($K$) of association between 40 nM $G\alpha_{i1}$Tb in the various states of activation with 70 nM RGS4-Alexa546. All data shown are mean ± SE ($n = 3$).

(transition state) significantly ($p < 0.05$) increased the rate of Gα:RGS4 binding to $2.67 \pm 0.12\ min^{-1}$ compared with the GTPγS- or GDP-bound states (Fig. 11B).

## Discussion

Specific, high-affinity binding within the G-protein heterotrimeric unit Gαβγ and between $G\alpha_{i1}$ and RGS4 was demonstrated using a novel TR-FRET assay. Terbium cryptate (chelate) and Alexa546 were chosen as donor and acceptor fluorophores, respectively, and these were covalently conjugated to prospective binding partners via maleimide linkage to exposed available cysteine (Cys) residues. Upon binding of labeled proteins, a signal which was three- to sixfold above background fluorescence was generated. This increase in fluorescence was observed over a period of minutes before an equilibrium value which could be maintained for at least another 15 min was reached. To establish that this phenomenon was a result of specific protein interactions, the possibility of bystander FRET between fluors alone was investigated and shown to require significantly higher concentrations of the fluors to be present for equivalent TR-FRET signals to be achieved (Fig. 3). Functional proteins were shown to be necessary for significant TR-FRET increases, with heat- or protease-treated proteins producing significantly reduced TR-FRET responses. Furthermore, competition with excess unlabeled G-protein subunits also significantly reduced the TR-FRET signal. Together, these data indicate that specific protein interactions were occurring to bring the fluors within the critical distance for TR-FRET to allow energy transfer from terbium to Alexa546, measured as an increase in fluorescence at the Alexa546 emission wavelength of 572 nm.

Conjugation of terbium and Alexa546 to available Cys residues did not significantly influence the ability of the G-protein heterotrimeric complex to sustain a signaling response when coupled to agonist-activated $\alpha_{2A}$AR and measured by the well-established [$^{35}$S]GTPγS binding assay. This indicates that the high affinity ($K_d = 2.4$ nM) heterotrimeric G-protein subunit interactions observed in this study using TR-FRET were not likely to have been significantly perturbed by the addition of the fluorescent probes. However, the efficiency of labeling rarely exceeded 50% and would more commonly yield a value of between 5 and 40% of the protein subunits being successfully conjugated to a fluorescent molecule. $G\alpha_{i1}$, $\beta_1$, and $\gamma_2$ subunits contain 8, 14, and 1 Cys residue/s, respectively [29]; however, not all of these residues are in a suitable position or orientation for conjugation to occur. Yang and colleagues [30] have reported the successful labeling of the surface-exposed Cys residues of a recombinant $G\alpha_t/G\alpha_{i1}$ subunit with lucifer yellow. Limited trypsinolysis and mutational analysis of recombinant $G\alpha_t/G\alpha_{i1}$ demonstrated that two specific Cys residues are the major fluorescent labeling sites: $Cys^{210}$, located in the switch II region, and $Cys^{347}$, located at the C terminus. The major α/β interface covers switch II of the α subunit [29]. Similarly, the $G\alpha_{i1}$ structure obtained from the RDSB protein databank (Protein Explorer program, RDSB Protein Databank http://www.rcsb.org/pdb/index.html.) [31] as the space-filled model of the G-protein heterotrimer (PDB: 1GP2) displayed two surface-exposed Cys residues, $Cys^{305}$ and $Cys^{325}$ (perhaps the latter with limited accessibility), with $Cys^{214}$ located in the Gα/βγ interface region. $Cys^{271}$ (and $Cys^{204}$ and $Cys^{25}$ to a limited extent) appear to be the only accessible Cys residues on the $G\beta_1$ subunit of the $G\beta_1\gamma_2$ dimer. Five Cys residues on the $\beta_1$ subunit are present in regions that are included in the hydrophobic core at the α/βγ interface [29] and are not likely to be available for fluor labeling. $\beta_4$ and $\beta_1$ subunits have high amino acid sequence similarity (90%) and all but one of the Cys residues are spatially conserved ($Cys^{233}$ of $\beta_1$ is not present in $\beta_4$). As the described labeling procedure for the Gα subunit was carried out in the presence of the Gβγ dimer to protect the Gα/Gβγ interface, the Cys residues involved in this binding region would be expected to be unavailable for labeling, which could potentially explain the low labeling efficiency in purified G-proteins.

The RGS4 protein structure obtained from the RDSB protein databank shows that only 4 of 10 Cys residues present in RGS4 are potentially available for labeling, of which $Cys^{71}$ and $Cys^{132}$ appear exposed at the surface of the

protein. A previous study labeling RGS4 with amine-reactive probes has described a 1:1 molar ratio of protein to label [32]. Since amine-containing residues such as lysine are over twice as abundant as Cys residues in RGS4, it is expected that lower labeling efficiencies would be achieved using thiol-reactive probes. If, in this study the respective FRET pairs conjugated to Cys residues were located on $Cys^{305}$ (Gα subunit) and $Cys^{271}$ (Gβ subunit), the fluors would be approximately 3.8 nm apart, while for the RGS/Gα interaction, the fluors located on $Cys^{305}$ (Gα subunit) and $Cys^{71}$ (RGS4) or $Cys^{132}$ (RGS4) would be approximately 5.7 or 3.5 nm apart, respectively. In this case, the fluors are well within their Forster radii, allowing FRET to occur efficiently. Finally, it is of significance to note that heterotrimeric G-protein subunit interactions could be observed when thiol-conjugated donor ($Tb^{3+}$) and acceptor (Alexa546) FRET pairs were swapped between the respective binding partners.

Structural studies have suggested that RGS4 binds to regions on the Gα subunit that overlap with the Gα/βγ binding interface [33]. Further to this, other studies have shown Gβγ subunits competing with RGS4 for binding to a Gα subunit, using resonance energy transfer methods [32,34]. In agreement, our results show rapid decreases in TR-FRET upon the addition of unlabeled binding partners such as Gα, RGS4, or Gβγ. This indicates competitive binding by the unlabeled protein species (reduced number of donor and fluor molecules within the Forster radius) which reduced the acceptor fluorescence emission at equilibrium. It was observed that addition of a higher concentration of RGS4 was less effective than addition of a significantly lower concentration of Gβγ at competing for subunit binding. The $K_d$ for $G\alpha_{i1}$ and Gβγ has been reported to be 2.9 nM [35] and further demonstrated here with TR-FRET as 2.4 nM. This is much lower than the $K_d$ of 14.6 nM found in this study for the interaction of RGS4-Alexa546 and $G\alpha_{i1}$Tb. It would therefore be expected that Gβγ would compete much more efficiently for binding to the Gα subunit than RGS4 at lower concentrations and this may partly explain why RGS4 is not normally interacting with the heterotrimer under basal conditions.

$AlF_4^-$ induces the transitional conformation change in the Gα subunit by occupying the position on the Gα-bound GDP nucleotide normally occupied by the γ-phosphate of GTP. The fluorine atoms assume a square planar configuration about the central aluminum atom and the complex is coordinated to a phosphate oxygen of GDP [27]. $AlF_4^-$ has been shown previously to dissociate the Gα and Gβγ complex [36]. TR-FRET allowed measurement of the association of G-protein subunits $G\alpha_{i1}$ and $G\beta_4\gamma_2$ and subsequent dissociation of the heterotrimer upon addition of $AlF_4^-$ (formed by the addition of 10 mM NaF and 30 μM $AlCl_3$). Decreases in TR-FRET of 50–85% were routinely measured following the addition of $AlF_4^-$; however, fluorescence emission of Alexa546 did not completely return to background values (i.e., resulting from fluorescence emission produced by $G\alpha_{i1}$Tb alone). This phenomenon could be due to incomplete dissociation of $G\alpha_{i1}$Tb upon $AlF_4^-$ activation with some residual affinity of $AlF_4^-$-bound Gα subunits for Gβγ, which has been reported previously [35]. Alternatively, an incomplete saturation of the Gα subunits with $AlF_4^-$ may leave some inactive Gα subunits in the assay with residual affinity for the Gβγ dimer. The addition of unlabeled competitors also did not completely abolish the FRET signal and may be explained by residual binding of labeled partners maintaining a low level of FRET-induced fluorescence. It also cannot be ruled out that the decrease in emitted acceptor fluorescence could be attributed to an increase in the average distance between the fluors due to subunit rearrangement rather than subunit dissociation, i.e., the fluors could remain close enough to maintain a lower efficiency of fluorescence resonance energy transfer. Heterotrimer rearrangement has been demonstrated in previous cell-based studies [4,37]. However, recent observations [5] indicate that this is unlikely in a cell-free environment and it is more likely that there is a change in the steady state activity of the G-proteins due to the $AlF_4^-$-induced decrease in subunit affinity, leading to dissociation of the heterotrimer.

Although there was a decrease in TR-FRET between $G\alpha_{i1}$Tb and $G\beta_4\gamma_2$Alexa546 in the presence of $AlF_4^-$, an increase in TR-FRET was observed with RGS4-Alexa546 as the binding partner of $G\alpha_{i1}$Tb in the presence of $AlF_4^-$. RGS4 has been widely reported to have a higher affinity for the $AlF_4^-$-bound conformation [6,27,38,39] and increased fluorescence could therefore be due to increased affinity of RGS4 for the transitional state Gα subunit. Alternatively, this could be a result of acceptor and donor fluors being brought into closer proximity upon the change in conformation of the Gα subunit with a subsequent increase in the efficiency of energy transfer producing a higher TR-FRET signal. $AlF_4^-$ also increased the rate at which steady state TR-FRET was achieved compared to inactive or active $G\alpha_{i1}$. This provides further evidence for the transitional state $G\alpha_{i1}$ being the preferential binding partner for RGS4 since it is proposed that RGS4 stabilizes the transition state so that GTP hydrolysis can proceed more rapidly [27]. However, in contrast to the results reported in previous studies [6,39], an RGS4 interaction was also observed with both active (GTPγS-bound) and inactive (GDP-bound) $G\alpha_{i1}$ subunits. Interactions between RGS4 (or other RGS proteins belonging to the same family) and GDP-bound or GTPγS-bound states of $G\alpha_{i1}$ have also been reported to show lowered affinity when in these conformations [38], suggesting that stable complexes form independent of activation state [40–42]. Interactions between both activated and inactive $G\alpha_q$ and RGS4 have also been reported in a cell-free environment using coumarin–dabcyl SE resonance energy transfer [32]. This study also found that Gβγ was able to compete with RGS4 for binding to the Gα subunit while also being able to act as a binding partner for RGS4.

Studies of the kinetics of G-protein subunit association have been carried out using a fluorescein-labeled Gα and

an eosin-labeled Gβγ dimer, the latter acting as a quencher upon association [43], or RGS4 and $G\alpha_q$ conjugated to coumarin and dabcyl SE, respectively [32]. However, the resolution of the signals in these studies was minimal (e.g., being about 15% for the latter study). Chimeric Gα and RGS proteins fused with cyan or yellow-fluorescent protein, respectively, were used to overcome problems of inefficient labeling of proteins and allowed incorporation of the fluor in a position that is predetermined [44]. While FRET was observed in that study, the method was limited since time resolution was not possible. Only a modest signal was produced and relatively minor changes occurred on disruption of the Gα-RGS4 complex. By contrast, in this study using TR-FRET technology, the signal (when high-affinity association of protein binding partners was induced) was well over 300% above background fluorescence. It is likely that this improvement in signal resolution has been achieved, first, by careful choice of the FRET partners with regard to donor emission/acceptor excitation spectral overlap, second, by the use of time-resolved (or gated) collection of the emission signal of the acceptor fluor thereby markedly reducing the background fluorescent signal component, and, third, by choosing the emission peak of terbium that has the strongest signal (highest excitation coefficient), i.e., the second emission peak at 545 nm representing the $^5D_4 \rightarrow {}^7F_5$ electronic transition state [19]. In addition, the small size of the fluors used here may be more efficient for detecting small conformational changes and are also less likely to alter protein function.

The TR-FRET assay exhibits high sensitivity enabling measurements to be recorded at low protein concentrations of 3–50 nM. The ability to rapidly detect interactions between heterotrimeric G-proteins using the TR-FRET assay described in this study could provide a screen for potential modulators of such binding, with the cell-free, homogeneous (mix and read) format giving the added dimension of testing compounds that may interact with one of the many permutations of α, β, and γ G-protein subunits that are possible. In a similar manner, compounds that potentiate or inhibit RGS–G-protein interactions could be identified using a high-throughput modification of the assay described in this study and, importantly, provide a level for therapeutic intervention independent of the GPCR itself.

## Acknowledgments

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