A Homogeneous Method to Measure Nucleotide Exchange by α-Subunits of Heterotrimeric G-Proteins Using Fluorescence Polarization

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ABSTRACT

The mainstay of assessing guanosine diphosphate release by the α-subunit of a heterotrimeric G-protein is the [^{35}S]guanosine 5′-O-(3-thiotriphosphate) (GTPγS) radionucleotide-binding assay. This assay requires separation of protein-bound GTPγS from free GTPγS at multiple time points followed by quantification via liquid scintillation. The arduous nature of this assay makes it difficult to quickly characterize multiple mutants, determine the effects of individual variables (e.g., temperature and Mg^{2+} concentration) on nucleotide exchange, or screen for small molecule modulators of Gα nucleotide binding/cycling properties. Here, we describe a robust, homogeneous, fluorescence polarization assay using a red-shifted fluorescent GTPγS probe that can rapidly determine the rate of GTPγS binding by Gα subunits.

INTRODUCTION

Seven transmembrane-domain G-protein coupled receptors (GPCRs), along with their associated heterotrimeric G-proteins (Gα guanosine diphosphate [GDP]/Gβ/Gγ), serve to transduce signals from diverse extracellular stimuli, such as photons, tastants, hormones, and neurotransmitters, to the intracellular compartment.[1–3] Agonist binding to the GPCR elicits guanine nucleotide exchange factor (GEF) activity, resulting in receptor-catalyzed release of GDP by Gα and subsequent binding of guanosine triphosphate (GTP).[4] The GTP-bound Gα subunit is the active signaling species, yet has an intrinsic ability to hydrolyze GTP back to GDP, which can be accelerated by a family of regulators of G-protein signaling (RGS proteins[5]). Historically, both receptor-catalyzed and spontaneous nucleotide release by Gα subunits has been measured using the radioactively labeled, nonhydrolyzable nucleotide [^{35}S]guanosine 5′-O-(3-thiotriphosphate) (GTPγS).[6] These radionucleotide binding assays typically involve incubation of [^{35}S]GTPγS with the Gα subunit, followed by vacuum filtration, buffer washes, membrane dessication, and then quantification of protein-bound [^{35}S]GTPγS by liquid scintillation.[7] While producing reliable and accurate results, this method is tedious, generates radioactive waste, and is not easily amenable to automation. The use of membrane-immobilized scintillation proximity assay (SPA) beads has allowed several groups to develop high-throughput-screen (HTS)-compatible [^{35}S]GTPγS assays[8–10]; however, inherent to the use of radionucleotides is the generation of unwanted radioactive waste. In an attempt to develop a nonradioactive, HTS-compatible GTPγS binding assay, others have reported using an europium-labeled GTPγS probe either in time-resolved fluorescence resonance energy transfer (TR-FRET)[11–13] or in quenching resonance energy transfer (QRET).[14] The ability to use QRET in a homogenous format (i.e., without the need for separation of bound and unbound Eu-GTPγS) represents an advance over the earlier TR-FRET-based assays.[15] Additional, non-lanthanide-based fluorescent GTP analogs have also led to the establishment of nonradioactive assays to quantify the nucleotide cycling properties of G-proteins[16–18]; however, these alternate assays involve monitoring changes in the absolute intensity of the fluor as its local solvating environment changes upon binding or hydrolysis events. The need to measure absolute intensity change, coupled with the use of fluors in the green range, prevents these

ABBREVIATIONS: FP, fluorescence polarization; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GPCR, G-protein coupled receptor; GTP, guanosine triphosphate; GTPγS, guanosine 5′-O-(3-thiotriphosphate); HTS, high-throughput screen; QRET, quenching resonance energy transfer; SPA, scintillation proximity assay; TR, Texas Red; TR-FRET, time-resolved fluorescence resonance energy transfer.

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assays from being suitable for screening small molecule libraries for nucleotide-state modulators.[19,20] Advances in plate readers capable of detecting fluorescence polarization (FP) and the commercial availability of red-shifted fluorescent-GTP analogs recently allowed Evelyn *et al.*[21] to measure nucleotide exchange by small, Rho-subfamily GTPases using a BODIPY-Texas Red (TR)-GTPγS (Invitrogen). Here, we describe using this fluor-labeled GTPγS in a homogenous FP assay for measuring the rate of spontaneous nucleotide exchange by Gα subunits.

MATERIALS AND METHODS

BODIPY-TR-GTPγS was purchased from Invitrogen and all other chemicals were purchased from Sigma at the highest quality obtainable. Two human Gα subunits were each separately expressed in *Escherichia coli* and purified in their GDP-bound forms by affinity chromatography exactly as previously described[7,19]: namely, wild-type Gα_{i1} and a double-point-mutant Gα_{i1}(R178M/A326S) that we recently developed to have accelerated spontaneous GDP release and slowed GTP hydrolysis.[7] FP experiments were conducted on the POLARStar Omega plate reader (BMG Labtech) containing a dichroic mirror and a dual emission beam splitter to measure fluorescence intensity parallel ($F_{||}$) and perpendicular ($F_{\perp}$) to the excitation plane. Samples were excited at 584 nm (excitation filter range of 566–588 nm) and emission was detected at 630 nm (cutoff ± 5 nm). The photomultiplier tubes were calibrated so that 25 nM TR-GTPγS in assay buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 0.05% (v/v) NP40 alternative) had a polarization of ~35 mP. Polarization was calculated as $P = (F_{||} - F_{\perp})/(F_{||} + F_{\perp})$ and expressed as mP ("milliP" or 1000*P); fluorescence intensity was calculated as $I = F_{||} + 2F_{\perp}$. Trials were conducted at 26°C using Corning Black Polystyrene 96-well plates (cat# CLS3875; Sigma). Gα_{il} was diluted to 500 nM in assay buffer and plated at an initial volume of 180 µL/well. Experiments were initiated upon addition of 20 µL of 250 nM TR-GTPγS to each well (25 nM TR-GTPγS final concentration). All experiments were conducted at least in triplicate. Nonlinear regression was used to fit the data to a single exponential association curve without constraints to calculate the k_{obs} using Prism version 5.0c (GraphPad).

To compare results obtained by the FP assay with the traditional radioactivity-based assay, radionucleotide binding assays were performed in parallel as previously described.[22] Briefly, assays were initiated by addition of [^{35}S]GTPγS to 100 nM wildtype Gα_{i1} or Gα_{i1}(R178M/A326S) mutant, either in assay buffer or in assay buffer containing 100 µM GTPγS. At indicated time points, aliquots were filtered by vacuum through nitrocellulose membranes and washed with ice-cold buffer. Assays were conducted in duplicate and error bars represent standard error of the mean. Nonspecific binding was subtracted from all time points. Nonlinear regression and statistical analyses were performed in Prism version 5.

RESULTS

Using the TR-GTPγS FP assay, observed rates of GTPγS binding (k_{obs}) were determined to be 0.0013 s^{-1} (95% CI 0.0011–0.0015 s^{-1})

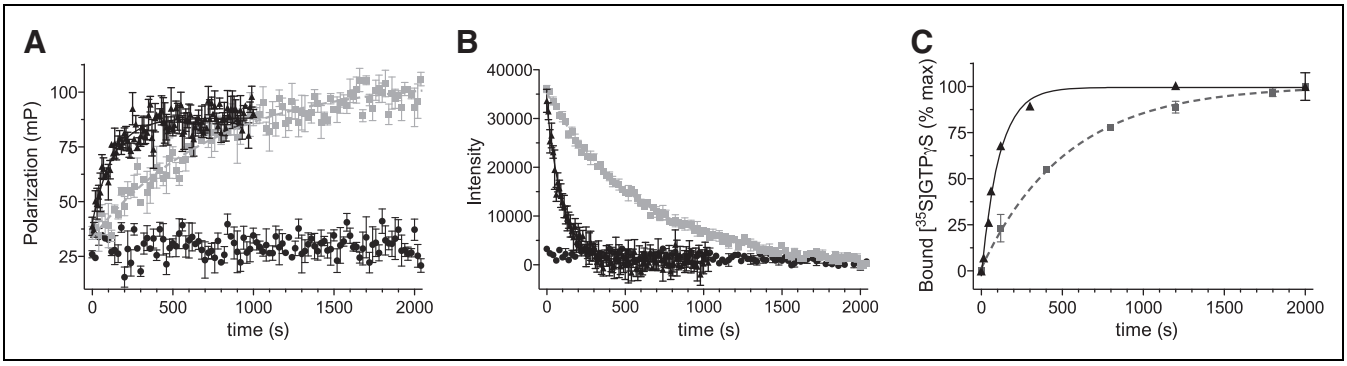

Fig. 1. Fluorescence-based Texas Red (TR)-guanosine 5′-O-(3-thiotriphosphate) (GTPγS) binding assay produces the same results as the gold-standard, [^{35}S]GTPγS radionucleotide binding assay. (**A**) Fluorescence polarization (FP) was measured at indicated time intervals upon addition of 25 nM TR-GTPγS (final concentration) to 500 nM of wildtype Gα_{i1} subunit (-■-, *light gray*), the rate-altered Gα_{i1}(R178M/A326S) mutant (-▲-, *black*), or buffer alone ("blank"; -●-, *dark gray*), all equilibrated at 26°C. FP assays were conducted in triplicate and error bars represent standard error of the mean. k_{obs} values were 0.0013 s^{-1} (95% CI 0.0011–0.0015 s^{-1}) and 0.0078 s^{-1} (95% CI 0.0063–0.0092 s^{-1}) for wildtype Gα_{i1} and the Gα_{i1}(R178M/A326S) mutant, respectively. (**B**) Fluorescence intensities were calculated from the same assay runs as shown in panel A by the equation: $\text{Intensity} = F_{||} + 2 \times F_{\perp}$. On the basis of the changes in total intensity (i.e., as subtracted from buffer only values; -●-, *dark gray*), k_{obs} values for wildtype Gα_{i1} (-■-, *light gray*) and Gα_{i1}(R178M/A326S) mutant (-▲-, *black*) were 0.0016 s^{-1} (95% CI 0.0015–0.0016 s^{-1}) and 0.010 s^{-1} (95% CI 0.009–0.011 s^{-1}), respectively. (**C**) Radionucleotide binding assays were performed as previously described [22]. k_{obs} values from the assay were 0.0019 s^{-1} (95% CI 0.0015–0.0023 s^{-1}) and 0.0090 s^{-1} (95% CI 0.007–0.0100 s^{-1}) for wildtype Gα_{i1} (-■-, *light gray*) and the Gα_{i1}(R178M/A326S) mutant (-▲-, *black*), respectively.

and 0.0078 s^{-1} (95% CI 0.0063–0.0092 s^{-1}) for wildtype $G\alpha_{i1}$ and the double-mutant $G\alpha_{i1}$(R178M/A326S), respectively (*Fig. 1A*); the greater k_{obs} for the latter Gα subunit is wholly consistent with its known increased rate of spontaneous GDP release and thus faster GTP binding.[7] The change in total intensity (*Fig. 1B*) was independently used to determine the k_{obs} for both $G\alpha_{i1}$ subunits: rates of GTPγS binding for wildtype $G\alpha_{i1}$ and $G\alpha_{i1}$(R178M/A326S) were determined to be 0.0016 s^{-1} (95% CI 0.0015–0.0016 s^{-1}) and 0.010 s^{-1} (95% CI 0.009–0.011 s^{-1}), respectively. Both the FP and fluorescence intensity results are consistent with the data obtained using the [^{35}S]-GTPγS radionucleotide binding assay (*Fig. 1C*), performed exactly as previously described.[22] Values of k_{obs} using [^{35}S]-GTPγS binding were determined to be 0.0019 s^{-1} (95% CI 0.0015–0.0023 s^{-1}) and 0.0090 s^{-1} (95% CI 0.007–0.0100 s^{-1}) for wildtype $G\alpha_{i1}$ and $G\alpha_{i1}$(R178M/A326S), respectively. While measuring fluorescence intensity and FP both allow monitoring of TR-GTPγS binding to an α-subunit of a heterotrimeric G-protein, measuring FP is considered superior given that it is a ratiometric measurement and thus less sensitive to interference from compounds that absorb or fluoresce in the same spectral region.

To validate the sensitivity of the TR-GTPγS FP assay with respect to changes in spontaneous GDP release, we used the $G\alpha_{i1}$ GDP-binding peptide AGS3Con, derived from the consensus of the 4 GoLoco motifs of AGS3 (TMGEEDFFDLLAKSQSKRMDDQRVDLAG; ref.[23]) and known to exhibit GDP dissociation inhibitory activity toward $G\alpha_i$ GDP subunits.[24,25] A dramatic decrease in the rate of TR-GTPγS binding was observed using FP upon addition of 10 μM AGS3Con peptide to wildtype $G\alpha_{i1}$, as expected (*Fig. 2*).[23] The rate of TR-GTPγS binding decreased from 0.0013 s^{-1} (95% CI 0.0012–0.0014 s^{-1}) for wildtype $G\alpha_{i1}$ alone, to 0.0004 s^{-1} (95% CI 0.0003–0.0005 s^{-1}) for $G\alpha_{i1}$ plus AGS3Con peptide. The addition of a 200-fold excess of nonfluorescent GTPγS was able to compete away completely any change in FP signal over the indicated time interval (*Fig. 2*), indicating that the TR-GTPγS does not bind nonspecifically to $G\alpha_{i1}$. To establish that this TR-GTPγS FP assay was not specific to a single plate reader, we also performed this assay on an EnVision Alpha HTS plate reader (Perkin-Elmer) and observed similar results (data not shown).

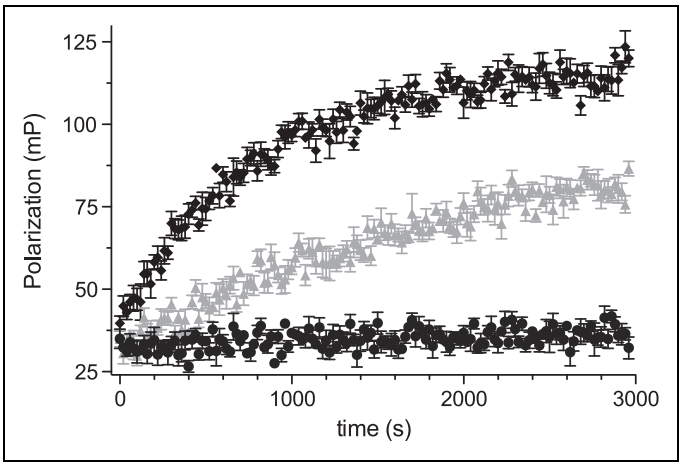

Fig. 2. The GoLoco motif peptide AGS3Con and unlabeled guanosine 5′-O-(3-thiotriphosphate) (GTPγS) both inhibit Texas Red (TR)-GTPγS binding to $G\alpha_{i1}$. AGS3Con peptide (10 μM; -▲-, *light gray*), unlabeled GTPγS (100 μM; -●-, *dark gray*), or buffer (-◆-, *black*) was incubated with wildtype $G\alpha_{i1}$ (500 nM) for 30 min at 37°C before the fluorescence polarization assay was initiated by addition of TR-GTPγS (25 nM final concentration). Using nonlinear regression, k_{obs} values were determined to be 0.0013 s^{-1} (95% CI 0.0012–0.0014 s^{-1}) for wildtype $G\alpha_{i1}$ alone versus 0.0004 s^{-1} (95% CI 0.0003–0.0005 s^{-1}) for $G\alpha_{i1}$ in the presence of the guanine nucleotide dissociation inhibitor peptide AGS3Con. No significant binding of TR-GTPγS was observed by $G\alpha_{i1}$ in the presence of excess, unlabeled GTPγS.

CONCLUSION

We have described a robust, "mix-and-measure," fluorescence-based assay system for measuring GTP binding activity by an α-subunit of a heterotrimeric G-protein. The TR-GTPγS FP assay should work universally for any Gα subunit, requires no radioactivity, and produces results consistent with radionucleotide binding assays. For researchers without the ability or desire to use radionucleotide binding assays, the TR-GTPγS FP assay offers a safer alternative. Additionally, performing this assay with the 96-well plate reader offers the advantage of automation (e.g., screening chemical libraries, profiling hits, and performing these assays on multiple Gα isoforms or multiple Gα point mutants). On the basis of its ability to detect differences in the rates of spontaneous GDP release by wildtype $G\alpha_{i1}$ versus the $G\alpha_{i1}$(R178M/A326S) mutant, we expect this assay to be readily utilized in assessing the GEF activity of GPCRs and nonreceptor Gα-GEFs such as Arr4 and RIC-8.[26,27]

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