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Europium-labeled GTP as a general nonradioactive substitute for [^{35}S]GTPγS in high-throughput G protein studies

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ABSTRACT

[^{35}S]GTPγS, the nonhydrolyzable radioactive GTP analog, has been a powerful tool in G protein studies and has set the standards in this field of research. However, its radioactive nature imposes clear limitations to its use in regular laboratory practice and in high-throughput experimentation. The europium-labeled GTP analog (Eu-GTP) has been used as an alternative in the analysis of G protein activation by G protein-coupled receptors in cellular membrane preparations. Here we expand the usage of Eu-GTP and show that it can be applied in other types of assays where [^{35}S]GTPγS has been previously utilized. We demonstrate the applicability of the modified Eu-GTP binding technology to analysis of heterotrimeric and monomeric G proteins of natural and recombinant sources, from different organisms, in assays with soluble proteins and membrane-containing assays of a high-throughput format. The deci-nanomolar K_D of Eu-GTP for the tested G proteins is similar to that of other fluorescent-modified GTP analogs, while the sensitivity achieved in time-resolved fluorescence analysis of Eu-GTP exceeds that of the radioactive measurements. Overall, the results of our modified Eu-GTP binding assay present Eu-GTP as a general nonradioactive alternative for G protein studies, especially attractive in high-throughput experiments.

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The usage of radioactively labeled nonhydrolyzable GTP-analog [^{35}S]GTPγS to investigate the G proteins has been pioneered by Gilman and co-workers in 1982 to study Gs, the heterotrimeric G protein regulating adenylate cyclase [1]. Since then [^{35}S]GTPγS has been widely used to study heterotrimeric G proteins in solution [2], as well as their activation by G protein-coupled receptors (GPCRs)[2] in membrane preparations [3,4]. As GPCRs represent the biggest animal receptor family [5] and the richest target group of current and future medications [6,7], the [^{35}S]GTPγS-binding assay has been employed in pharmacological investigations and drug discovery [8–10]. Additionally, members of different subgroups of small (monomeric) G proteins have been studied with the help of [^{35}S]GTPγS [11–13].

However, the [^{35}S]GTPγS-binding technology has clear limitations. The traditional format of this assay is very low throughput; additional obstacle to the usage of [^{35}S]GTPγS in a high-throughput format is the handling of radioactive compounds and waste, which often becomes undesirable in the modern laboratory practice. Certain advantages in this regard are provided by fluorescent GTP analogs, such as BODIPY-FL-GTPγS [14,15]. Unfortunately, the BODIPY analogs are far less sensitive than [^{35}S]GTPγS, resulting in the high consumption of the BODIPY-FL-GTPγS and G protein reagents. Another complication is the strong reduction in the affinity of certain G proteins to GTPγS on addition of the bulky BODIPY label [16].

Europium-labeled GTP analog (Eu-GTP) developed by PerkinElmer seems to combine the strong sides of the [^{35}S]GTPγS and the fluorescent assays [17]. This poorly hydrolyzable GTP analog was reported to have a slightly lower affinity for G proteins as compared with [^{35}S]GTPγS, but the unique fluorescent properties of the europium chelate allowed the application of the time-resolved fluorometry (TRF) protocol. In TRF, emission of the fluorescent label having a long decay time is recorded with a delay of about 0.4 ms after excitation, when the nonspecific fluorescence has died out, resulting in an excellent signal-to-noise ratio and thus extreme sensitivity, comparable to or even exceeding that of the radioactive compounds [17]. The high-throughput format of the TRF assay makes the Eu-GTP-binding assay ideal for GPCR-targeted drug discovery [18] and has been applied on membrane preparations containing various GPCRs such as α_{2A}-adrenergic [17], neuropeptide [19], dopamine [20], muscarinic [21], and chemokine receptors [22]. The current protocols involve retention of membrane-containing particles and washing out of nonbound Eu-GTP through hydrophilic macropore filters and are thus not applicable to solubilized G proteins and receptors.

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[2] *Abbreviations used:* DTT, dithiothreitol; Eu-GTP, europium-labeled GTP analog; GPCRs, G protein-coupled receptors; IPTG, isopropyl-1-thio-D-galactopyranoside, PMSF, phenylmethylsulfonyl fluoride; TRF, time-resolved fluorometry.

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Here we expand the Eu-GTP-binding assay to the solution-based format. For the first time we directly compare the Eu-GTP and [^{35}S]GTPγS affinities for various heterotrimeric and monomeric G proteins. Our results demonstrate the applicability of the Eu-GTP assays to the broad range of experimentation where [^{35}S]GTPγS was preferentially used so far.

Materials and methods

Materials

Drosophila melanogaster rab5 cDNA (clone GH24702) and pGEX-dCdc42-WT (barcode 1259) were from the *Drosophila* Genomics Resource Center. Recombinant GST-tagged human Ras1 (Cat. No. 553325), anti-Gβγ antibodies (Cat. No. 371821), and imidazole were from Merck. Complete protease inhibitor cocktail was from Roche. GDP, GTPγS, Thesit, lysozyme, mastoparan, and serotonin were from Sigma. PMSF and isopropyl-1-thio-D-galactopyranoside (IPTG) were from Roth. Oxotremorine-M was from Tocris Bioscience. BODIPY-FL-GTPγS was from Invitrogen. [^{35}S]GTPγS was from Hartmann Analytical. Eu-GTP was from the DELFIA Eu-GTP binding kit (PerkinElmer Life Sciences). The 36-mer GoLoco1 domain peptide corresponding to the *Drosophila* Pins protein (see [23]) was synthesized by Pepscan Presto BV (Lelystad, Netherlands) and freshly dissolved in water before each experiment.

Isolation of membranes from porcine brains

Halved brains from freshly slaughtered 1- to 2-year-old pigs were kept in 5 vol 25 mM ice-cold aqueous Tris-HCl (pH 8.0) for ⩽1 h. Further procedures were performed at 4 °C. After surgical removal of major portions of the white matter, the gray matter from ⩾4 brains was passed through a blender in 5 vol of 25 mM Tris-HCl. Most of the crude homogenate was used for isolation of proteins (see below), while a small aliquot (10–20 ml) was immediately supplied with protease inhibitors and further treated with 20 strokes in a Dounce homogenizer. Cell debris and nuclei were removed by centrifugation (10 min, 3200*g*). Membranes from the supernatant were pelleted by ultracentrifugation (1 h, 40,000 rpm) in Beckman Ti 50.2 rotor, resuspended in the storage buffer (50 mM Hepes-NaOH, pH 8.0, 150 mM NaCl, protease inhibitors) to 10 mg/ml total protein (measured by the Bradford assay), and stored in aliquots at −80 °C.

Isolation of trimeric G proteins from porcine brains

Trimeric G proteins were isolated from porcine brains essentially as previously described for bovine brains [24] with the following modifications: phenyl-Sepharose (GE Healthcare) was used instead of heptylamine-Sepharose, and the concentration of NaCl in the binding buffer was increased to 1 M. Gα activity in fractions was detected using BODIPY-FL-GTPγS [14], while Gβγ was detected by SDS-PAGE followed by Western blotting. Purest fractions containing Gα and Gβγ were pooled, concentrated to 500 µl on Amicon Ultracel-10 (Millipore) and separated in the gel-filtration buffer (50 mM Hepes, 150 mM NaCl, 0.1% Thesit) on a Superose 200 column (GE Healthcare). Fractions containing Gαβγ were pooled; the final preparation of 3 mg protein/ml contained different G protein subunits of ca. 95% homogeneity; Gαo constituted ca. 80% of all Gα-subunits as judged by Coomassie staining.

Isolation of Gβγ subunits from porcine brains

The Gβγ complex elutes as a broad peak on Ultrogel AcA34 gel filtration [24]. Whereas the forward part of this peak contains also the peak Gα activity and those fractions were pooled for trimeric G protein isolation, retained fractions containing mainly Gβγ were separately collected and resolved on phenyl-Sepharose followed by Superose 200 under the conditions described above. Purest fractions were pooled, resulting in ca. 95% homogeneity of 36- and 35-kDa Gβ and 8-kDa Gγ subunits in 1:1 molar proportion with less than 5% of residual Gα activity (as accessed by the Eu-GTP assay, see below).

Expression and purification of His_6-Gαo and -Gαi3

Hexahistidine-tagged *Drosophila* Gαo was purified on Ni–NTA agarose (Qiagen) and phenyl-Sepharose as described [23]; alternatively, His_6-Gαo of a similar purity was prepared through a single-step isolation on Ni–NTA agarose through elution with 250 mM imidazole after washing with 150 mM imidazole. His_6-Gαi3 was prepared as described [25].

Expression and purification of His_6-Rab5

Drosophila Rab5 cDNA was PCR-amplified with the primers, sense CGAACTGATCATGGCAACCACTCC, antisense GGACAGATATC GAGCGTAATGG, and cloned in pCR2.1-TOPO (Invitrogen). The inserted fragment was isolated with BclI – EcoRV and cloned in pQE32 (Qiagen) into the BamHI-EcoRV sites. The resulting plasmid pQE32-Rab5 was sequence-verified prior to bacterial expression of His_6-Rab5. *Escherichia coli* M15 pREP4 (Qiagen) was transformed with pQE32-Rab5 and grown in LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin at 37 °C to OD_{600} = 0.5 before induction with 1 mM IPTG and additional growth for 5–6 h, followed by harvesting by centrifugation. All subsequent procedures were performed at 4 °C. Cell pellets were resuspended in the isolation buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM PMSF, 1 mM DTT) supplemented with 10 mM imidazole and incubated for 30 min, followed by lysis with 5 mg/ml lysozyme for 30 min and sonication. Debris was removed by centrifugation at 20,000*g*/30 min. The supernatant was applied to the Ni–NTA resin preequilibrated in same buffer. The Ni–NTA resin was then washed 8 times/10 min with 10 column volumes of the isolation buffer supplemented with 20 mM imidazole. After elution by 250 mM imidazole in the isolation buffer, proteins were brought into the HKB buffer (10 mM Hepes, 135 mM KCl, 10 mM NaCl, 2 mM EGTA) supplemented with 1 mM DTT, 0.5 mM $MgCl_2$, and 0.05% CHAPS using Amicon Ultracel-10.

Expression and purification of GST-Cdc42

pGEX-dCdc42 was transformed into the *E. coli* M15 pREP4 strain. The protein was expressed and purified by affinity on glutathione-Sepharose beads (GE Healthcare) as recommended by the manufacturer. The protein was eluted with 10 mM reduced glutathione, buffer-exchanged to HKB + 1 mM DTT/0.5 mM $MgCl_2$ using Amicon Ultracel-10, and stored at 4 °C.

Measurements of specific activities of purified G proteins

Specific activities of G proteins were measured with [^{35}S]GTPγS as described [23], except for small G proteins which were incubated in the presence of 5 mM EDTA instead of 2.5 mM $MgCl_2$. Specific activities of recombinant proteins were as follows: His_6-dGαo, 92%; His_6-mGαi3, 37%; GST-dCdc42, 24%; His_6-dRab5, 22%; GST-hRas1, 20%.

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Eu-GTP-binding assay of porcine brain membranes

Assay was performed generally as suggested by the manufacturer for the DELFIA Eu-GTP binding kit (PerkinElmer) and a previous publication [17]. Briefly, components were mixed in appropriate volumes in the AcroWell 96 GHP filter plate (Pall) wells. Final concentrations were 50 mM Hepes, 50 mM NaCl, 1 mM $MgCl_2$, 0.1 μM GDP, 50 μg/ml Saponin, 100 μg/ml of membrane protein, and 50 μM of agonist. Reaction was started by the addition of Eu-GTP to a final concentration of 2.5 nM. After a 30-min incubation on a horizontal shaker (150 rpm) at room temperature, reaction mixtures were directly filtered on a Millipore Multiscreen HTS Vacuum Manifold and washed thrice with 150 μl of ice-cold washing solution (25 mM Tris-HCl, 100 μM $MgCl_2$). Plates were immediately measured in a Victor^3 Multilabel counter (PerkinElmer) using the standard protocol for Eu TRF. A protocol with a detailed step-by-step description of the Eu-GTP assay in the membrane-containing format is provided in the Supplementary Materials (Protocol 1).

Radioactive GTP-binding assay of porcine brain membranes

To allow comparison, radioactive assay was performed under conditions identical to those of Eu-GTP (see above) with the exceptions that GDP was included at a concentration of 10 μM to achieve maximal activation level [26] and reactions were performed in 96-well polypropylene plates (Greiner). For detection, the reaction mixtures were diluted to 1 ml with ice-cold washing solution and immediately transferred one-by-one onto 2.5-cm-wide 0.22-μm nitrocellulose filters (Millipore) for filtration using Millipore Sampling Manifold 3025. Unbound [^{35}S]GTPγS was washed 3 times with 2 ml of the washing solution and the filters were immediately submerged into 3 ml of Quicksafe scintillation liquid (Zinsser Analytics). After overnight extraction, signals were measured in Beckman LS6500 counter using built-in protocol for ^{35}S.

Solution-based Eu-GTP binding assay

All manipulations were performed in a manner similar to those for the membrane-based assay described above. Reactions conditions were 50 mM Hepes-KOH, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$ for experiments with trimeric G proteins; 50 mM Hepes, 100 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$ for recombinant Gαo; and HKB supplemented with 11 mM $MgCl_2$/10 mM EDTA/1 mM DTT for small G proteins. For experiments utilizing less than 10 nM of G proteins, 10 μg/ml BSA was added to avoid vessel absorption. Proteins and Eu-GTP were added in concentrations indicated in the text and figure legends. The total volume of reaction mixtures was 100–200 μl. After all components were added into 96-well polypropylene plates, reactions were started by addition of Eu-GTP. Meanwhile, nitrocellulose filters of the 96-well Acrowell BioTrace NT nitrocellulose plates (Pall) were prewetted by filtration of 100 μl 20% ethanol, followed by 2× 100 μl of the washing solution. The reaction mixtures were incubated in the polypropylene plates for the indicated time periods, transferred to the nitrocellulose plates, and immediately filtered and washed thrice with 150 μl of the washing solution, followed by immediate Eu TRF measurement as above. A protocol with a detailed step-by-step description of the Eu-GTP assay in the solution-based format is provided in the Supplementary Materials (Protocol 2).

Solution-based [^{35}S]GTPγS-binding assay

To allow comparison, [^{35}S]GTPγS assay reactions were performed for all cases under conditions identical to those of Eu-GTP, followed by filtration and detection as described above for brain membranes.

Measurements of K_D of interaction of G proteins with GTP analogs

Increasing concentrations of G proteins were incubated for 1 h with 1–10 nM [^{35}S]GTPγS or Eu-GTP in the solution-based assay as described above. Resulting values of [^{35}S]GTPγS or Eu-GTP retained on the filters were plotted using KaleidaGraph 4.0 (Synergy Software) with the hyperbolic equation $y = a + \frac{bx}{c+x}$, where c provided the EC_{50} of the nucleotide binding with G proteins. Dissociation constant was then calculated as $K_D = EC_{50} - 0.5C$, where C is the total concentration of [^{35}S]GTPγS or Eu-GTP used in the experiment.

Stimulation of trimeric G-proteins with mastoparan

Reaction conditions were as described for the solution-based assay, and additionally 0.1 and 10 μM GDP were included for the Eu-based and the radioactive assay, respectively. G-proteins and GTP nucleotides were included in concentrations of 50 and 2.5 nM, respectively; mastoparan was added at the indicated concentrations. Reaction time was 30 min.

GDI activity

A 1.7 μM Gβγ and 5 μM GoLoco1 peptide [23] were added to recombinant Gαo used at an effective concentration of 20 nM. Curves corresponding to Gβγ and GoLoco1 alone (without added recombinant Gαo) were measured separately and then subtracted. Reactions were initiated with addition of 2.5 nM Eu-GTP. All other procedures were performed as described above.

Results

[^{35}S]GTPγS and Eu-GTP record similar GPCR activation in natural membranes

To begin our analysis, we wanted to directly compare Eu-GTP and [^{35}S]GTPγS for their capacities to monitor activation of GPCRs in natural membranes. We purified porcine brain membranes (see Materials and methods) and stimulated them with two agonists, serotonin and oxotremorine-M. These agents activate various metabotropic serotonin receptors (5-HT1a, 5HT2c, etc.) and muscarinic receptors, respectively, which in turn activate G proteins of the Gq and Gi/o groups [21,27]. First, the conventional protocols were employed to monitor G protein activation in these membrane preparations using [^{35}S]GTPγS [3]. These agents both elicited ca. 30% G protein activation (Fig. 1A, left). Next, we utilized Eu-GTP in an identical assay, and found a similar stimulation of porcine brain membranes (Fig. 1A, right). G protein activation in porcine brain membranes that we observed on utilization of [^{35}S]GTPγS or Eu-GTP after stimulation with serotonin and oxotremorine-M agrees with that reported previously for other membrane preparations [21,28]. Thus, we conclude that in the natural plasma membranes stimulated by different agonists, Eu-GTP and [^{35}S]GTPγS, show a similar G protein activation.

Activation of heterotrimeric G proteins in solution can be monitored using [^{35}S]GTPγS and Eu-GTP similarly

The major heterotrimeric G protein of the brain tissue is Go [24]; Gi, Gs, and Gq represent less abundant species. Heterotrimeric G proteins can be efficiently studied in the solution-based format using [^{35}S]GTPγS [2]. We modified the initial Eu-GTP-binding assays to the solution-based format by the usage of the nitrocellulose filters in 96-well plates (see Materials and methods; also see Supplementary Materials, Protocol 2). This permitted the

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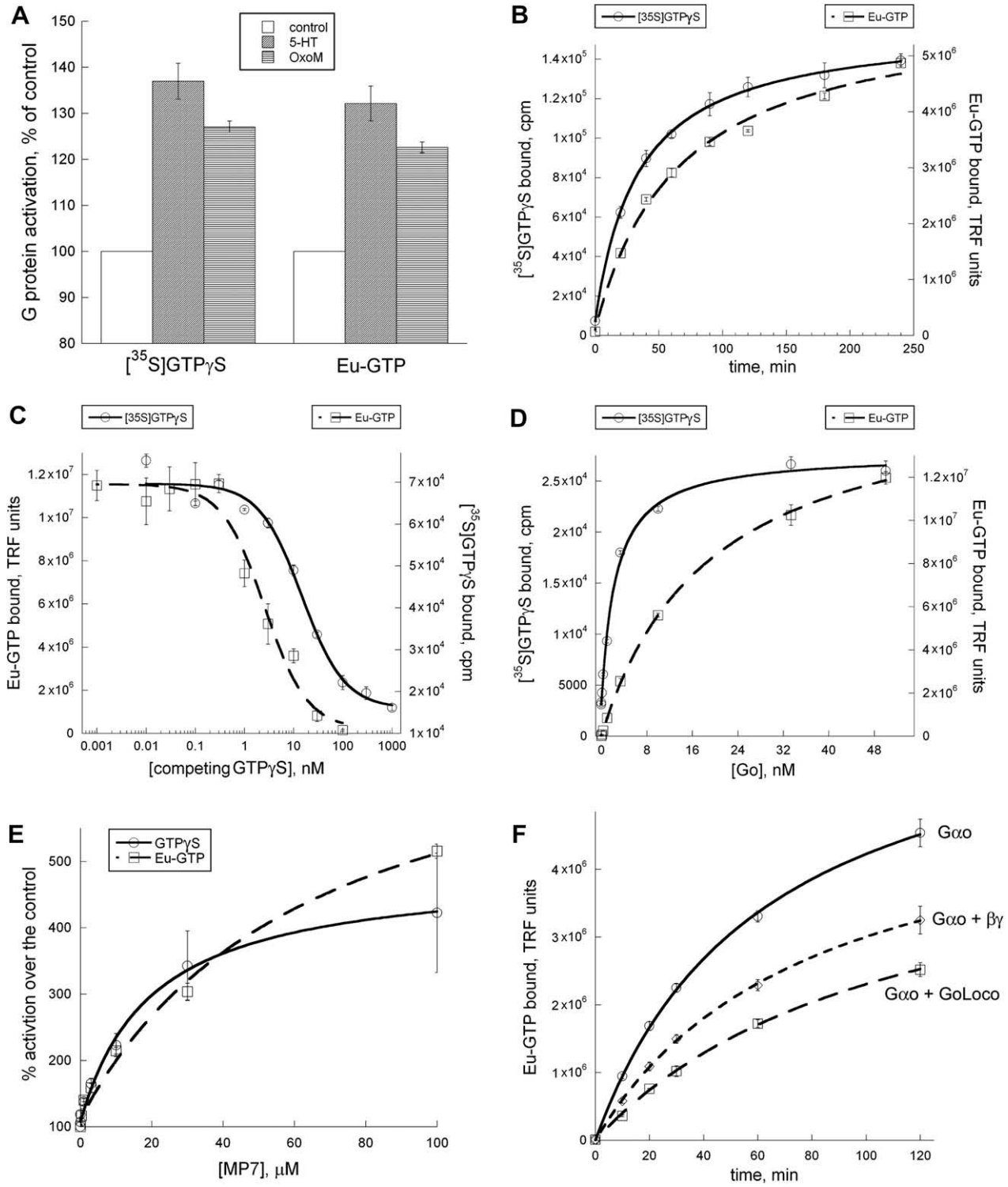

Fig. 1. Eu-GTP as fluorescent label in membrane-containing and solution-based GTP-binding assays. (A) Similar levels of G protein activation in porcine brain membranes stimulated by serotonin (5-HT) and oxotremorine-M (OxoM) are detected using the Eu-GTP and [^{35}S]GTPγS binding assays. (B) Kinetics of activation of 50 nM trimeric Go protein with 2.5 nM Eu-GTP or [^{35}S]GTPγS. (C) Competition binding assays. 10 nM trimeric Go protein was incubated with 10 nM Eu-GTP or [^{35}S]GTPγS and increasing concentrations of cold GTPγS. (D) Saturation binding curves of different concentrations of the trimeric Go protein to 1 nM Eu-GTP/[^{35}S]GTPγS after a 2-h incubation. (E) Dose-dependent activation of trimeric Go protein by the GPCR mimetic mastoparan (MP-7) measured with Eu-GTP or [^{35}S]GTPγS. (F) GDI activity of the βγ-subunits and GoLoco1 peptide derived from the Pins protein toward recombinant *Drosophila* Gαo as measured with Eu-GTP. All data are presented as mean±SEM, $n \geqslant 3$.

use of Eu-GTP in essentially the same way as [^{35}S]GTPγS was used before, but in a high-throughput format. Heterotrimeric G proteins were purified from porcine brains almost to homogeneity (see Materials and methods). Further on, we call this porcine brain G protein isolation as Go, since it is consists by ca. 80% of this G protein [24]. The kinetics of Go activation by GTP analogs in the presence of high concentrations of $MgCl_2$ was studied using [^{35}S]GTPγS and Eu-GTP, and revealed a similar profile (Fig. 1B). Next we compared the affinities of the two GTP nucleotides to Go using competition titrations. When 10 nM Go was incubated

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with 10 nM [35S]GTPγS and increasing concentrations of cold GTPγS, the concentration of GTPγS achieving a 50% reduction in the [35S]GTPγS radioactivity absorbed by nitrocellulose filters (IC_{50}) was found to be 14.7 nM (Fig. 1C). Reciprocally, when 10 nM Go and 10 nM Eu-GTP were titrated with increasing concentrations of GTPγS, IC_{50} was found to be 2.7 nM (Fig. 1C). These experiments suggest that the affinity of Eu-GTP for purified Go is ca. 5-fold lower than that of [35S]GTPγS, and agree with the estimation provided using α_{2A}-adrenergic receptors and G proteins of CHO cell membranes [17].

However, we also wished to directly measure the K_D of the interaction of Go with the two nucleotides. To this end, we titrated 1 nM [35S]GTPγS or Eu-GTP with increasing concentrations of purified Go (Fig. 1D). The resulting EC_{50} was 2.5 nM for [35S]GTPγS and 19.6 nM for Eu-GTP, which (see Materials and methods) yielded the K_D of the interaction of Go with [35S]GTPγS of 2.0 nM and with Eu-GTP of 19.1 nM (Table 1). This calculation provides the first direct measurement of the K_D of Eu-GTP/G protein interaction and is higher than the previous estimations (see above and [17]). However, this K_D is still in the low deci-nanomolar range and is comparable to those reported for Gαo and the fluorescently labeled GTP analogs [14,15].

To show the utility of Eu-GTP to further investigate heterotrimeric G proteins *in vitro*, we studied activation of Go by the GPCR peptide mimetic mastoparan [29] using [35S]GTPγS and Eu-GTP. We found again a similar profile of Go activation using the two methods (Fig. 1E).

Eu-GTP is useful in the analysis of purified Gα-subunits of heterotrimeric G proteins

In the experiments described so far, we showed the utility of Eu-GTP in the analysis of heterotrimeric G proteins. To prove that Eu-GTP can be efficiently used for the analysis of purified Gα-subunits of heterotrimeric G proteins, we expressed and purified from bacteria *Drosophila* Gαo and mouse Gαi3. First, we directly measured the K_D of the interaction of dGαo and mGαi3 with Eu-GTP and [35S]GTPγS. These measurements revealed that, similar to the results presented above for the heterotrimeric Go purified from porcine brains, these purified Gα-subunits interacted with Eu-GTP with a high affinity, which was, however, lower than their affinity for [35S]GTPγS (Table 1).

As an example of the utility of the Eu-GTP assay in the analysis of purified Gα-subunits, we studied the GDI (guanine nucleotide *d*issociation *i*nhibitor) activity of the βγ-subunits and of the GoLoco peptide derived from the Pins protein [23] against Gαo. As shown on Fig. 1F, preloading of Gαo with guanine nucleotides can be efficiently studied using Eu-GTP. As is well-established in [35S]GTPγS assays [30,31], addition of purified βγ-subunits slows the rate of this reaction (Fig. 1F). Similarly, GoLoco peptide derived from the Pins protein exerts the GDI activity (Fig. 1F), whose extent is similar to that previously measured using the fluorescent BODIPY-FL-GTPγS and [35S]GTPγS [23]. Of note, 50 times less protein and 400 times less nucleotide are used in this Eu-GTP experiment, as compared with the BODIPY-FL-GTPγS experiment (see Fig. 1 and [23]).

Eu-GTP has a reduced affinity for monomeric G proteins

To investigate whether small G proteins can be studied using Eu-GTP, we prepared small G proteins of different families (Table 1). Rab5 was a representative of the Rab family, Ras1 of the Ras family, and Cdc42 of the Rho family small G proteins. We also selected G proteins from different organisms—human and *Drosophila*. Direct measurements of the K_D of these G proteins to [35S]GTPγS produced the values of 9–60 nM, while the K_D to Eu-GTP was 20 to 50 times higher (Table 1).

Discussion

In the present article we demonstrate the utility of the Europium-labeled GTP analog, Eu-GTP, as a general substitute of the conventionally used radioactive [35S]GTPγS in various formats of analysis of GPCR signaling and G protein activity. Eu-GTP is found to be applicable in membrane-containing assays, as well as in solution-based assays to study heterotrimeric G proteins, purified Gα-subunits, and monomeric G proteins of different types and organisms. We show that Eu-GTP can be used in the same type of experiments as those traditionally utilizing radioactive nucleotides, with the same results. The range of experimental conditions, such as sample preparation, magnesium, and other ion concentrations, is essentially identical for the two assays. The main advantage Eu-GTP offers over [35S]GTPγS is the easiness of high-throughput measurements. Furthermore, Eu-GTP allows diverting from the handling of radioactivity and instead uses the highly sensitive time-resolved fluorescence protocol, which is a standard mode of measurements included in most microplate fluorescence readers (several dozens of readers of this type from numerous companies are currently on the market).

For the first time we directly measure the affinity of Eu-GTP for various G proteins, in parallel with the affinity of [35S]GTPγS. The results obtained (Table 1) show that the affinity of heterotrimeric G proteins for Eu-GTP is reduced 2- to 10-fold as compared to that of GTPγS, while the affinities of small G proteins are reduced 20- to 50-fold. These data suggest that the Eu-chelate added to GTP affects binding of the modified nucleotide to the small G proteins more than to the heterotrimeric G proteins we investigated.

The resulting K_D values for the heterotrimeric G proteins are similar to those measured for other fluorescently labeled GTP analogs [14,15]. Unlike these other analogs, Eu-GTP is detected through the time-resolved fluorescence measurements, achieving far-better sensitivity similar or even superior to that of the radioactive detection. This advantage results in consumption of the nucleotide, G protein, and GPCR in amounts by orders of magnitude lower than those required, e.g., for the BODIPY-FL-GTPγS experiments.

Table 1
Summary of K_D values for [35S]GTPγS and Eu-GTP for various G proteins.

G protein	Organism	Purification source	K_D for [35S]GTPγS, nM	K_D for Eu-GTP, nM	Fold
Go, heterotrimer	Pig	Brain	2.03 ± 0.20	19.12 ± 2.16	9.4
Gαo	*Drosophila*	Recombinant, *E. coli*	3.60 ± 0.97	33.77 ± 7.89	9.4
Gαi3	Mouse	Recombinant, *E. coli*	121.2 ± 3.5	247.4 ± 107.2	2.0
Ras1	Human	Recombinant, *E. coli*	49.5 ± 6.2	>1000*	>16
Rab5	*Drosophila*	Recombinant, *E. coli*	9.18 ± 1.00	218 ± 47	23.7
Cdc42	*Drosophila*	Recombinant, *E. coli*	16.9 ± 3.7	826 ± 85	48.9

* Ras1 titration curve did not reach saturation.

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Development of the solution-based assay to study G proteins utilizing Eu-GTP opens new avenues in investigations of soluble nonmembrane effectors and modulators of G proteins, including synthetic peptides and low-molecular substances. This solution-based assay is ideal also for experiments involving *in vitro* reconstituted G protein systems, e.g., with solubilized or purified GPCRs, especially from recombinant sources. Highly sensitive G protein assays utilizing purified components, previously only possible with radioactive GTPγS, now can be performed with Eu-GTP.

In contrast to [^{35}S]GTPγS, Eu-GTP is easily amenable to the high-throughput analysis. We routinely perform our experiments in 96-well plates; utilization of 384-well plates in Eu-GTP experiments is also possible. Overall, we present Eu-GTP as a general solution for various types of GPCR and G protein analysis in membrane-containing and solution-based setups. Its advantage over other fluorescent GTP analogs is its extreme sensitivity. Its advantage over [^{35}S]GTPγS is the high-throughput format and the nonradioactivity. We believe Eu-GTP will become the standard in high-throughput investigations of GPCRs and G proteins.

Detailed step-by-step protocols for the usage of Eu-GTP in the membrane-containing and solution-based formats are provided in the Supplementary Materials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.10.028.

References

[1] J.K. Northup, M.D. Smigel, A.G. Gilman, The guanine nucleotide activating site of the regulatory component of adenylate cyclase. Identification by ligand binding, J. Biol. Chem. 257 (1982) 11416–11423.
[2] D.J. Carty, R. Iyengar, Guanosine 5'-(gamma-thio)triphosphate binding assay for solubilized G proteins, Methods Enzymol. 237 (1994) 38–44.
[3] T. Weiland, K.H. Jakobs, Measurement of receptor-stimulated guanosine 5'-*O*-(gamma-thio)triphosphate binding by G proteins, Methods Enzymol. 237 (1994) 3–13.
[4] R.T. Windh, D.R. Manning, Analysis of G protein activation in Sf9 and mammalian cells by agonist-promoted [35S]GTP gamma S binding, Methods Enzymol. 344 (2002) 3–14.
[5] K.L. Pierce, R.T. Premont, R.J. Lefkowitz, Seven-transmembrane receptors, Nat. Rev. Mol. Cell. Biol. 3 (2002) 639–650.
[6] P. Nambi, N. Aiyar, G protein-coupled receptors in drug discovery, Assay Drug Dev. Technol. 1 (2003) 305–310.
[7] M.C. Lagerstrom, H.B. Schioth, Structural diversity of G protein-coupled receptors and significance for drug discovery, Nat. Rev. Drug Discov. 7 (2008) 339–357.
[8] C. Harrison, J.R. Traynor, The [35S]GTPgammaS binding assay: approaches and applications in pharmacology, Life Sci. 74 (2003) 489–508.
[9] W.R. Leifert, A.L. Aloia, O. Bucco, R.V. Glatz, E.J. McMurchie, G-protein-coupled receptors in drug discovery: nanosizing using cell-free technologies and molecular biology approaches, J. Biomol. Screen. 10 (2005) 765–779.
[10] G. Milligan, Principles: extending the utility of [35S]GTP gamma S binding assays, Trends Pharmacol. Sci. 24 (2003) 87–90.
[11] Y. Zheng, M.J. Hart, R.A. Cerione, Guanine nucleotide exchange catalyzed by dbl oncogene product, Methods Enzymol. 256 (1995) 77–84.
[12] A. Kikuchi, H. Nakanishi, Y. Takai, Purification and properties of Rab3A, Methods Enzymol. 257 (1995) 57–70.
[13] P. Chavrier, M. Franco, Expression, purification, and biochemical properties of EFA6, a Sec7 domain-containing guanine exchange factor for ADP-ribosylation factor 6 (ARF6), Methods Enzymol. 329 (2001) 272–279.
[14] D.P. McEwen, K.R. Gee, H.C. Kang, R.R. Neubig, Fluorescent BODIPY-GTP analogs: real-time measurement of nucleotide binding to G proteins, Anal. Biochem. 291 (2001) 109–117.
[15] E.E. Jameson, R.A. Roof, M.R. Whorton, H.I. Mosberg, R.K. Sunahara, R.R. Neubig, R.T. Kennedy, Real-time detection of basal and stimulated G protein GTPase activity using fluorescent GTP analogues, J. Biol. Chem. 280 (2005) 7712–7719.
[16] A. Gille, R. Seifert, Low-affinity interactions of BODIPY-FL-GTPgammaS and BODIPY-FL-GppNHp with G(i)- and G(s)-proteins, Naunyn Schmiedebergs Arch. Pharmacol. 368 (2003) 210–215.
[17] H. Frang, V.M. Mukkala, R. Syysto, P. Ollikka, P. Hurskainen, M. Scheinin, I. Hemmila, Nonradioactive GTP binding assay to monitor activation of g protein-coupled receptors, Assay Drug Dev. Technol. 1 (2003) 275–280.
[18] I.A. Hemmila, P. Hurskainen, Novel detection strategies for drug discovery, Drug Discov. Today 7 (2002) S150–S156.
[19] M. Engstrom, A. Narvanen, J.M. Savola, S. Wurster, Assessing activation of the human neuropeptide FF2 receptor with a non-radioactive GTP binding assay, Peptides 25 (2004) 2099–2104.
[20] M. Leopoldo, E. Lacivita, N.A. Colabufo, M. Contino, F. Berardi, R. Perrone, First structure-activity relationship study on dopamine D3 receptor agents with *N*-[4-(4-arylpiperazin-1-yl)butyl]arylcarboxamide structure, J. Med. Chem. 48 (2005) 7919–7922.
[21] H.Y. Zhang, M.L. Watson, M. Gallagher, M.M. Nicolle, Muscarinic receptor-mediated GTP-Eu binding in the hippocampus and prefrontal cortex is correlated with spatial memory impairment in aged rats, Neurobiol. Aging 28 (2007) 619–626.
[22] J. Labrecque, R.S. Wong, S.P. Fricker, A time-resolved fluorescent lanthanide (Eu)-GTP binding assay for chemokine receptors as targets in drug discovery, Methods Mol. Biol. 552 (2009) 153–169.
[23] D. Kopein, V.L. Katanaev, *Drosophila* GoLoco-protein pins is a target of Galpha(o)-mediated G protein-coupled receptor signaling, Mol. Biol. Cell 20 (2009) 3865–3877.
[24] P.C. Sternweis, J.D. Robishaw, Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain, J. Biol. Chem. 259 (1984) 13806–13813.
[25] R.W. Nipper, K.H. Siller, N.R. Smith, C.Q. Doe, K.E. Prehoda, Galphai generates multiple Pins activation states to link cortical polarity and spindle orientation in *Drosophila* neuroblasts, Proc. Natl. Acad. Sci. USA 104 (2007) 14306–14311.
[26] L.J. Sim, Q. Liu, S.R. Childers, D.E. Selley, Endomorphin-stimulated [35S]GTPgammaS binding in rat brain: evidence for partial agonist activity at mu-opioid receptors, J. Neurochem. 70 (1998) 1567–1576.
[27] N.M. Barnes, T. Sharp, A review of central 5-HT receptors and their function, Neuropharmacology 38 (1999) 1083–1152.
[28] B. Bertin, M. Freissmuth, R.M. Breyer, W. Schutz, A.D. Strosberg, S. Marullo, Functional expression of the human serotonin 5-HT1A receptor in *Escherichia coli* ligand binding properties and interaction with recombinant G protein alpha-subunits, J. Biol. Chem. 267 (1992) 8200–8206.
[29] T. Higashijima, S. Uzu, T. Nakajima, E.M. Ross, Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins), J. Biol. Chem. 263 (1988) 6491–6494.
[30] D.R. Brandt, E.M. Ross, GTPase activity of the stimulatory GTP-binding regulatory protein of adenylate cyclase, Gs. Accumulation and turnover of enzyme-nucleotide intermediates, J. Biol. Chem. 260 (1985) 266–272.
[31] T. Higashijima, K.M. Ferguson, P.C. Sternweis, M.D. Smigel, A.G. Gilman, Effects of Mg^{2+} and the beta gamma-subunit complex on the interactions of guanine nucleotides with G proteins, J. Biol. Chem. 262 (1987) 762–766.