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Flow Cytometry for Real-Time Measurement of Guanine Nucleotide Binding and Exchange by Ras-like GTPases

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Abstract

Ras-like small GTPases cycle between GTP-bound active and GDP-bound inactive conformational states to regulate diverse cellular processes. Despite their importance, detailed kinetic or comparative studies of family members are rarely undertaken due to the lack of real-time assays measuring nucleotide binding or exchange. Here, we report a bead-based, flow cytometric assay that quantitatively measures the nucleotide binding properties of GST-chimeras for prototypical Ras-family members Rab7 and Rho. Measurements are possible in the presence or absence of Mg²⁺, with magnesium cations principally increasing affinity and slowing nucleotide dissociation rate 8- to 10-fold. GST-Rab7 exhibited a 3-fold higher affinity for GDP relative to GTP that is consistent with a 3-fold slower dissociation rate of GDP. Strikingly, GST-Rab7 had a marked preference for GTP with ribose ring-conjugated BODIPY FL. The more commonly used γ -NH-conjugated BODIPY FL GTP analogue failed to bind to GST-Rab7. In contrast, both BODIPY analogues bound equally well to GST-RhoA and GST-RhoC. Comparisons of the GST-Rab7 and GST-RhoA GTP-binding pockets provide a structural basis for the observed binding differences. In sum, the flow cytometric assay can be used to measure nucleotide binding properties of GTPases in real-time and quantitatively assess differences between GTPases.

Keywords

Rab and Rho GTPases; membrane trafficking; actin remodeling; nucleotide binding and exchange; fluorescent GTP analogues

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Introduction

Small molecular weight, Ras-like GTPases regulate a variety of cellular functions ranging from signal transduction (Ras), cytoskeletal rearrangements (Rho), membrane dynamics and protein synthesis (Arf/Sar) to vesicular (Rab) and nuclear transport (Ran) [1–4]. Abnormal regulation of GTPases has been implicated in wide range of diseases including cancer, pigmentation and immune disorders, as well as neurological disease [5–8]. The structural conformation and hence the activity of GTPases is mainly regulated by the nature of the bound guanine nucleotides [9]. Binding to GTP activates the GTPase and induces a conformational change that regulates interactions with downstream effector proteins. The hydrolysis of GTP to GDP due to the intrinsic GTPase activity of the protein inactivates the GTPase, though often the intrinsic hydrolysis rates are slow [10–12]. In vivo, activation and inactivation is further regulated by the binding of numerous accessory proteins that serve to enhance nucleotide exchange and hydrolysis [1,3,13]. Developing a convenient method that accurately measures the kinetics of nucleotide binding of GTPases is crucial for monitoring pathways controlled by these pivotal regulatory proteins. Although most GTPases share similar structural features such as switch regions and nucleotide binding pockets, the variations within these regions enable them to play unique roles in cellular function. Discerning the differences between these GTPases, as well as what conditions affect nucleotide binding, is crucial to understanding disease pathways and developing drug targets. Detailed analyses of the kinetic parameters of nucleotide binding and release are also expected to provide key mechanistic insight and suggest rate-limiting events in pathway regulation.

A variety of methods have been utilized to measure nucleotide binding to GTPases, some of which are non-quantitative and none of which allow rapid, real-time measurement as required for high through-put screening and analyses of complex mixtures or cell lysates. In the cell biology community, nucleotide binding is most often confirmed by ligand overlay blotting, TLC analysis of bound nucleotide following protein immunoprecipitation or in vivo FRAP assays [14–17]. Such assays assess nucleotide binding qualitatively but cannot readily measure affinity for different nucleotides or interaction kinetics. Quantitative analyses have relied on the use of radiolabeled nucleotides and measurement via filter binding and liquid scintillation counting [17,18] or the use of reverse-hase HPLC [11]. A highly quantitative fluorescence approach was first pioneered for the Rab GTPases and entails monitoring intrinsic tryptophan fluorescence changes upon nucleotide binding or tryptophan FRET to 2'(3')-O-(N-Methylanthraniloyl)-(MANT)-substituted fluorescent nucleotides [11,12,19]. However, this approach is not broadly applicable to GTPases that lack the requisite tryptophan residues, including important small molecular weight GTPases such as Ha-Ras p21 and heterotrimeric G proteins among others. In general, the need for large quantities of purified protein, multi-step procedures for preparing nucleotide-free protein, specialized instrumentation, artifacts induced by filter-binding and radiolabeled analogues may explain why these techniques have not been more broadly employed in the characterization of the large number of small GTPase family members.

Recent efforts to broaden the applicability of fluorescent strategies for measuring nucleotide binding to heterotrimeric and small GTPases led to the development of a series of fluorescent BODIPY-conjugated GTP analogues and quantification of the binding and dissociation of fluorescent nucleotide analogues by spectrofluorometry [20–22]. Here we describe a rapid bead-based, flow cytometry assay using BODIPY FL nucleotide analogues to measure the kinetics of nucleotide binding and dissociation from Rab7, a small GTPase and member of the Ras superfamily involved in endosome maturation and lysosome biogenesis [23–25]. Rab7 was selected for these studies because its kinetic and structural properties are well described, thereby enabling comparative evaluation with established methods [11,12,26–28]. Real time measurements with fluorescence detection by flow cytometry discriminate free GTP analogues

from those bound to GTPases on a bead, and can be used for a wide range of binding conditions. We exploit this homogeneous discrimination to investigate the difference in affinity for different nucleotides, as well as to reveal structurally confirmed differences in nucleotide binding pocket structure between Rab7 and the RhoA and RhoC GTPases that are important in actin dynamics, cell-cell adhesion and trafficking [29]. The assay approach has already been shown to have further utility for protein-protein interaction studies, comparative evaluation of multiple GTPase family members and drug screens (PubChem AID # 757-61, and AID 764; <http://nmmlsc.health.unm.edu/assays.shtml>).

Materials and Methods

Reagents

All reagents were of analytical quality and unless otherwise noted were from Sigma (www.sigmaaldrich.com). Plasticware was from VWR (www.vwr.com). Sephadex G-25, glutathione Sepharose 4B, and Superdex peptide beads were from Amersham Biosciences (www.amershambiosciences.com); the Superdex peptide beads were 13 μm with an exclusion limit of 7,000 Da, and were extruded from a column. Purification and filter concentrators were from Millipore. Fluorescein and BODIPY® (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene or dipyrromethene-boron-difluoride) nucleotide analogues (BODIPY FL GTP 2'-(or-3')-O-(N-(2-aminoethyl)urethane, G-12411; BODIPY FL GTP- γ -NH amide, G-35778; BODIPY FL GDP 2'-(or-3')-O-(N-(2-aminoethyl)urethane), G22360) were from Invitrogen Molecular Probes (<http://probes.invitrogen.com>). Concentrations for BODIPY nucleotides were determined from absorbance measurements using an extinction coefficient value of 80,000 $\text{M}^{-1}\text{cm}^{-1}$ (Invitrogen Molecular probes). GST-RhoA purified from *E. coli* was purchased from Cytoskeleton (<http://www.cytoskeleton.com/>). GST-RhoC in vitro translated in a wheat germ system was purchased from Abnova Corp. (H00000389/P01) and glutathione (GSH) was removed by spin concentration in the presence of 0.1% bovine serum albumin.

Expression and purification of GST-tagged proteins

Recombinant DNA expression was performed in accordance with NIH guidelines and with approval by the institutional biosafety office. A fusion protein between glutathione S-alkyl transferase and green fluorescent protein (GST-GFP) was constructed by inserting an EcoRI-NotI fragment from pEGFP-N2 (Becton-Dickinson Biosciences) into pGEX-6P-1 (Amersham Biosciences) as described [30]. A vector encoding GST-Rab7 was constructed by inserting PCR-amplified, wild-type, canine Rab7 into pGEX-5X-2 (Amersham Biosciences, Piscataway, NJ) using standard recombinant DNA techniques [31]. BL21 (DE3) *E. coli* were individually transformed with the expression vectors and grown overnight at 37°C on Luria-Bertani broth (LB)-agar plates with 100 $\mu\text{g}/\text{ml}$ ampicillin. About 20 colonies were transferred into 100 ml LB broth with 100 $\mu\text{g}/\text{ml}$ ampicillin and grown at 37°C to a bacterial density of 0.6 absorbance units at 595 nm. For GST-Rab7 expression, the culture was transferred to room temperature, 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to induce expression of the properly folded, active fusion protein and further incubated at room temperature for 16–18 h. For GST-GFP, the incubation temperature was reduced to 30°C, 0.25 mM IPTG was added and culture continued at 30°C for 4–5 h. Bacterial cells expressing the fusion proteins were then collected by centrifugation and quick frozen directly as pellets at –20°C. Pellets were then resuspended in PBS to a final volume of 10 ml and bacteria were lysed using a Branson Sonicator 250 at setting '3' with 6 bursts of 10 s, followed by a 30 min incubation with the addition of 0.5 ml 10% (v/v) Triton X-100. The lysates were cleared by centrifugation at 8,000 g for 10 min. The GST-fusion proteins were subsequently affinity purified by sequential passage over two separate 0.5 ml columns of glutathione Sepharose 4B. The affinity columns were rinsed two times with 10 ml of buffer and the bound GST-fusion proteins were eluted with 10 mM GSH, incubated with the resin for 10 min at 4°C. The eluates

were again passed through gel filtration columns to remove free GSH or simultaneously purified and concentrated using Millipore Amicon Ultra Tubes MW cut-off 30,000 (Catalog# UFC903024). Following the second gel filtration, the GST-fusion proteins were concentrated using separate Microcon YM-30 filters, the retentates were washed three times with fresh buffer, and the final filtrates and retentates were collected. The purity of the retentate was verified by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining (Fig. 1A). The concentrations of the purified fusion proteins were quantified by the method of Bradford [32] using bovine serum albumin as a standard, ethylene glycol was added to 50% by volume and aliquots were stored at -20°C until needed.

Synthesis of GSH loaded beads (bGSH)

Flow cytometry suited, high glutathione density beads (bGSH) were prepared by loading Superdex peptide beads with GSH as previously described [30]. Briefly, Superdex peptide beads of 13 μm diameter and 7,000 Da exclusion limit were extruded from a column, activated with a water-soluble bis-epoxide, and then coupled to glutathione. One ml of a 50% slurry Superdex peptide beads in 20% ethanol was reduced to a wet cake using a 15 ml coarse sintered glass filter, and washed three times with 15 ml water to remove the ethanol. The wet cake was transferred to a small screw-cap tube, and the filter was rinsed with 0.3 ml of water. The beads were suspended by vortexing, then 60 μl of 10 M NaOH and 300 μl of 1,4-butanediyl diglycidyl ether were added, and the suspension was rocked gently at 40°C for 4 h. The epoxy-activated beads were rinsed four times on the filter and 600 μl of 100 mM GSH in 100 mM sodium phosphate, pH re-adjusted to 7.5, 1 mM EDTA, was added. The beads were kept in suspension for 16 h at 40°C , and then rinsed twice with 0.01% dodecyl maltoside. The remaining active sites were blocked with 1% 2-mercaptoethanol for 2 h. The beads were rinsed twice and stored in HPS (30 mM HEPES, pH 7.5, 100 mM KCl, 20 mM NaCl, 0.01% dodecyl maltoside) containing 1 mM MgCl_2 and 0.02% NaN_3 at 4°C as a 50% slurry, which corresponds to $\sim 2.5 \times 10^5$ high GSH density beads/ μl , or 25 assays of 10^4 beads/ μl .

Flow cytometry measurements and molecular assembly of GST-Rab7 and GST-RhoA proteins on GSH beads

All binding and measurements were performed in the presence of HPS buffer containing 1 mM EDTA or 1 mM MgCl_2 as noted. A Becton-Dickinson FACScan flow cytometer (Sunnyvale, CA, USA) with a 488 nm excitation laser and standard detection optics was used for all assays as previously described [33]. GST-proteins (1 μM , GST-Rab7 or GST-RhoA), were incubated in 96-well plates at 4°C , overnight with 10^3 glutathione beads/ μl in a total volume of 10 μl HPS buffer with 1 mM DTT (dithiothreitol/ Cleland's reagent) added fresh and 1 mM EDTA or 1 mM MgCl_2 as noted. Unbound protein was removed by centrifugation and beads were resuspended in fresh buffer, the wash step repeated once before storage at 4°C until needed. We have previously shown that GST-protein assemblies on GSH beads are very stable [30] and disassemble from the glutathione beads, at very slow rates $1.3 \times 10^{-4} \text{ s}^{-1}$ (or $t_{1/2}=1.7 \text{ hr}$) only when highly diluted to limit rebinding. Thus, the bead bound protein is known to be sufficiently stable to enable the study of secondary interactions between GST-proteins and fluorescently tagged guanine nucleotides with minimal loss of the "foundation" assembly.

Dose dependence binding assays

For all dose dependence assays, 10^4 beads loaded with GST-protein were incubated for 2 h at 4°C with varying concentrations of fluorescent guanine nucleotide (0–2 μM) in a total volume of 10 μl on a 96-well plate. For measurement, samples were transferred to a tube suitable for flow cytometry and diluted at least 10-fold in the appropriate HPS buffer. This dilution step is necessary to optimize the discrimination of bead-associated fluorescence and background fluorescence of soluble proteins, and also ensures sufficient sample volume for measurement.

Competitive binding assays

For the competition assays, 10^4 beads loaded with GST-protein were incubated for 2 h at 4°C with 100 nM fluorescent nucleotide and the appropriate concentration of non-fluorescent nucleotide competitor in a 96-well plate with a total volume of 10 μ l. Measurements were performed as described above.

Kinetic assays

Association measurements were performed by incubation of beads loaded with GST-protein (10^3 beads/ μ l) at 4°C with the appropriate concentration of fluorescent nucleotide in a 1.5 ml tube. At the desired time points 10 μ l aliquots were removed and measured on the flow cytometer. Dissociation was measured by removing a 10 μ l aliquot of equilibrated, fluorescent nucleotide-bound protein on beads and diluting it 10-fold in the presence of 1 mM non-fluorescent GTP. In addition, real time measurements of changes in fluorescence were taken using the kinetic mode of the cytometer.

Standardization of flow cytometry data with fluorescence calibration beads

We used standard fluorescence calibration beads to account for day-to-day variation in bead fluorescence, changes in detector settings and use of different flow cytometers [34–36]. The standard calibration beads used here were commercial standards from Bangs Labs (www.bangslabs.com) Quantum™ FITC MESF beads. These beads are optimized for fluorescein but can be used for any fluorophore being analyzed under the same excitation and detection optics as long as a suitable correction factor is applied to account for the spectroscopic differences between the fluorescein and target fluorophore (e.g. BODIPY FL) [37]. The calibration scheme has been recently described in detail [37]. Given that the measured fluorescence intensity of any given molecule is proportional to, $I_0 \epsilon_{ex} \phi \%T$; where I_0 is the intensity of the light source, ϵ_{ex} is the absorption coefficient of the fluorophore at the excitation wavelength and ϕ is the quantum yield of the fluorophore and $\%T$ is the percent fraction of fluorescence light transmitted by the 530 nm, 30 nm wide bandpass filter (upto 80% maximum transmittance) and is used to account for the spectral mismatch between the sample fluorophore and the fluorescein standard. Therefore, to apply Quantum FITC MESF beads to BODIPY labeled assemblies we derived a correction factor based on the ratio using values for unquenched fluorophores [37]:

$$(\epsilon_{ex} \phi \%T)_{\text{BODIPY}} / (\epsilon_{ex} \phi \%T)_{\text{fluorescein}} = 44,000 \times 0.90 \times 20 / 85,000 \times 0.93 \times 28 = 0.36 \quad (1)$$

Thus, the measured BODIPY intensity is attenuated by a factor of 0.36. The parameter that most significantly affects the measured output of the BODIPY fluorophore is the off-resonance excitation (55% of its λ_{max}) of the fluorophore compared to fluorescein, which is excited at its absorption maximum.

Data Analysis

Multiple trials were used to generate means and standard errors. For detailed fitting parameters see Table 1 and Table 2. Curve fitting and analyses utilized GraphPad Prism software (www.graphpad.com).

Results

Purified GTPases can be immobilized on GSH beads and GTP-binding can be detected by flow cytometry

We previously demonstrated the utility of flow cytometry for accurately measuring the dynamics of interaction between GST-GFP fusion proteins and glutathione beads [30]. Here we extend the utility of glutathione beads to the characterization of the equilibrium binding and association and dissociation kinetics between GST-Rab7 and BODIPY FL tagged guanine nucleotides. The molecular assembly schemes are shown in Equation 2 and Equation 3. The first step entails binding of purified GST-Rab7 (Fig. 1A) to the GSH beads (bGSH) (step 1 in Fig. 1B), and is expected to have characteristics similar to the previously described GST-GFP [30]. The second step is of biological interest and involves binding of the Rab7 protein to the fluorescently labeled nucleotide (BODIPY FL GTP) (Step2 in Fig. 1B).

The strong affinity interaction between glutathione beads and GST-proteins was documented in the chromatography literature over three decades ago [38]. Here, we examined the binding between glutathione beads and GST-Rab7 solely to establish quantitative limits on the number of binding sites per bead under our assay conditions. GST-Rab7 pre-incubated with saturating concentrations (10 μM , see Fig. 2C) of BODIPY FL GTP was used to determine the maximum number of GST-Rab7 occupied sites. Fig. 1C shows a parabolic plot of GTP sites/bead versus concentration of GST-Rab7 (bound to BODIPY FL GTP). The sites/bead were determined using calibration beads as described in the methods section. At the maximal concentration of protein, a proximately 5×10^6 GST-Rab7 molecules bound to each bead. This represents a concentration of 2 nM bead-bound protein and translates to ~ 20 femtomoles of protein per 10 μl reaction. The dose dependence of GST-Rab-BODIPY FL GTP binding to the GSH beads revealed an apparent dissociation constant of $K_d = 190$ nM (Fig. 1C). An alternative analysis of the apparent affinity of GST-Rab7 for the GSH beads was based on competing the non-fluorescent protein with fluorescent GST-GFP protein. The inhibition constant $K_i = 107$ nM for GST-Rab7 was comparable to the K_d value determined in Fig. 1C. It should be noted that the protein-bead binding constants were not obtained under true equilibrium conditions, which requires long term incubation at 4°C . Therefore, the K_d values are principally useful as quantitative parameters for setting up the assay platform rather than being thermodynamically meaningful. [39]. On the other hand, protein saturated beads can be generated in a relatively short time when micromolar concentrations of GST-proteins are used.

Effect of magnesium on the equilibrium exchange of endogenous for exogenous GDP in the nucleotide binding pocket

Mg^{2+} ions are believed to play a significant role in the stabilization of nucleotide binding, to Ras, Rho and Rab GTPases in addition to acting as cofactors in nucleotide hydrolysis [18, 40–42]. The nucleotide-binding pocket of purified GTPases is usually occupied with endogenous GDP. Therefore, the binding of fluorescently tagged nucleotides to Rab7 GTPase is expected to involve an exchange of the endogenous GDP. To enhance the probability of nucleotide-exchange we examined the effects of incubating exogenous nucleotides with Rab7 GTPase loaded beads in the presence of EDTA. Fig. 2A shows the differences in exchange of

endogenous GDP for fluorescently labeled GTP and affinity for BODIPY FL GTP [18,41] in Mg^{2+} -containing or -depleted buffers. The stabilizing influence of Mg^{2+} on the interaction of endogenous GDP with the Rab7 nucleotide binding pocket is a parent in the parabolic plot of exchangeable sites of BODIPY FL GTP versus concentration of the titrated exogenous nucleotide. In the presence of Mg^{2+} , BODIPY FL GTP binds with higher affinity $K_d = 20$ nM to relatively fewer exchangeable sites, whereas depletion of Mg^{2+} results in a higher number of exchangeable sites of diminished affinity ($K_d = 150$). The reduced affinity is consistent with an increase in the dissociation rate of the nucleotide in the absence of Mg^{2+} as expected. An analysis of the early kinetics of dissociation of BODIPY FL GTP from Rab7 when competed by a large excess of unlabeled GDP showed the dissociation rate to be 7 times faster in the absence of Mg^{2+} relative to when Mg^{2+} was present (Fig. 2B). The latter result is consistent with the 8-fold decrease in affinity of the nucleotide interaction with Rab7 in the absence of Mg^{2+} (Fig. 2A), demonstrating that accurate kinetic measurements of Rab7 nucleotide binding are possible in both the presence and absence of magnesium cations.

To convincingly demonstrate that under our assay conditions the binding of BODIPY FL GTP to the Rab7 nucleotide-binding pocket largely involves an exchange of endogenous GDP [11,18], we examined the association kinetics of BODIPY FL GTP with Rab7 beads by varying the concentration of ligand over a 10-fold range in Mg^{2+} -depleted buffer. The association data are shown in Fig. 2C. Because the ligand concentration remains constant throughout the experiment, the data were analyzed in terms of pseudo-first order kinetics. The on-rates ($t_{1/2}$) as a function of concentration were then plotted as shown in Fig. 2D. Because the association-rates are constant irrespective of a 10-fold change in ligand concentration, we conclude that the on-rates are indeed governed by the rate of nucleotide exchange. Therefore, in situations where the intrinsic association rate is of interest care must be taken to remove all endogenous nucleotide as previously described [11,41,42].

BODIPY FL GDP binds to the nucleotide binding pocket more tightly than GTP analogues

We used 3 different experimental measures of the binding affinity of Rab7 pocket for the fluorescently tagged GDP and GTP analogues. Fig. 3A shows a parabolic plot of bound nucleotide analogues versus concentration. The data show that BODIPY FL GDP binds to the nucleotide binding pocket with a 3-fold higher affinity ($K_d = 54$ nM) compared to BODIPY FL GTP ($K_d = 150$ nM). This difference in affinity is well correlated to a 3-fold faster dissociation of GTP ($k_{off} = 7.0 \times 10^{-3} s^{-1}$) from the Rab7 compared to the GDP ($k_{off} = 2.3 \times 10^{-3} s^{-1}$) shown in Fig. 3B. We also analyzed the competitive displacement of BODIPY FL GTP from Rab7 using GDP versus GTP γ S, a nonhydrolyzable analogue of GTP (Fig. 3C). The resulting inhibition measured for GDP, ($K_i = 13$ nM) and GTP γ S, ($K_i = 40$ nM) further corroborated the 3-fold higher affinity of Rab7 for GDP relative to GTP.

Fluorescent nucleotide analogues reveal structure/function differences between GTPases

Because BODIPY FL GTP is subject to hydrolysis, the non-hydrolyzable, BODIPY FL GTP- γ -NH analog is frequently used. BODIPY FL GTP- γ -NH differs from BODIPY FL GTP in that the fluorophore is conjugated to the nucleotide via the γ -phosphate as opposed to the 2' or 3' oxygen of the ribose ring (Fig. 4A) [20]. Remarkably, GST-Rab7 showed 90% lower binding affinity for the BODIPY FL GTP- γ -NH relative to the BODIPY FL GTP (Fig. 4B). In the presence of Mg^{2+} , GST-Rab7 yielded a maximal binding $B_{max} = 60 \times 10^3$ fluorophores/bead for BODIPY FL GTP- γ -NH compared with a maximal binding $B_{max} = 650 \times 10^3$ fluorophores/bead for BODIPY FL GTP.

To determine whether other small molecular weight GTPases were equally sensitive to the structural modification of nucleotides we also carried out similar binding experiments using GST-RhoA. In contrast to GST-Rab7, GST-RhoA, showed no difference in nucleotide

analogue binding (Fig. 4B), signifying that the nucleotide analogue preference may be GTPase specific. The dose response curve for GST-RhoA binding to BODIPY FL GTP yielded a maximal binding $B_{\max} = 700 \times 10^3$ fluorophores/bead and a $K_d = 80$ nM (Fig. 4B).

The unique inability of GST-Rab7 to bind the BODIPY FL GTP- γ -NH nucleotide prompted a structural comparison. The crystal structures for both GTP-bound Rab7 and RhoA reveal how placement of the fluorophore group could lead to such a drastic change in binding affinity (Fig. 4C). The ribose and phosphate groups make distinct contacts within the Rab7 and RhoA nucleotide binding pockets. Furthermore, the Rab nucleotide binding pocket leaves the ribose ring of the nucleotide solvent exposed, whereas the γ -phosphate is buried within the protein. A bulky BODIPY FL group on the γ -phosphate may not allow the nucleotide to be accommodated within the binding pocket of Rab7. In RhoA the binding pocket leaves both the ribose and the γ -phosphate solvent exposed so either BODIPY FL or BODIPY FL GTP- γ -NH can fit into the pocket. Analogous results were found for RhoC (not shown).

Discussion

We report a new bead based flow cytometry assay to measure in real time, the affinity and kinetics of nucleotide binding to small GTPases, using GST-fusions of Rab7 and RhoA as test cases. The particular features of the assay that assure broad utility are the facts that: (1) it circumvents the need for radiolabeled analogues; (2) only femtomoles of purified protein are needed per assay; (3) the assay may be run on a conventional flow cytometer; (4) the assay is quick and measures binding in real time without large numbers of wash steps; and (5) it may be run with or without magnesium.

The assay enables the sensitive identification of comparative binding differences between GTPases and their nucleotides, though as executed here does not establish the absolute kinetic and affinity constants for the GTPase. The nucleotide binding pocket of GTPases is nearly always occupied by GDP except in association with molecular chaperones that promote exchange and stabilize the nucleotide free form [19,43,44]. Consequently, considerable effort must be taken to purify small GTPases free of nucleotide and magnesium as described by Simon et al. [11]. Since the procedure of stripping nucleotide is laborious and accompanied by loss of activity, it is advisable only in situations where it is critical to determine absolute constants. The dissociation constant and kinetic rates determined by flow cytometry are not directly comparable to those determined in nucleotide free systems where a $K_d = 6$ nM for Rab7 binding to methylanthraniloyl (mant) GTP was reported [11]. Instead, our values agree well with those measured under conditions allowing nucleotide exchange for BODIPY FL analogues and using fluorescence spectroscopy [21]; in which case the nucleotide binding affinities (K_d) for Cdc42, Rac1, RhoA, and Ras ranged between 10–100 nM. The use of a reduced Mg^{2+} system diverges from the normal in vivo environment that GTPases reside in. Mg^{2+} and Mn^{2+} are important for securing the nucleotide into the binding pocket and Mg^{2+} drastically slows nucleotide off rates [41]. Because the flow cytometry based measurement reflects exchange of endogenous nucleotide for fluorescently labeled BODIPY FL GTP, we found it useful to remove Mg^{2+} ions, which tend to limit this exchange (c.f. Fig. 2). Nevertheless, as shown here, the applicability of our assays is independent of Mg^{2+} , but can be used when desired or necessary.

The GST-Rab7 binding affinities for several nucleotides and nucleotide analogues yielded three interesting results. First, although Rab7 has a 3-fold higher affinity for GDP $K_d = 54$ nM than for GTP $K_d = 150$ nM, however, this is a relatively small difference considering that the cell and tissue concentration of GTP is in the micromolar range (19–276 μ M with erythrocytes having the most variable levels) and 2- to 9-fold higher than GDP [45–49]. Thus, in the cellular context it makes sense that the nucleotide bound state must be regulated by accessory proteins to preclude purely concentration dependent binding. Second, recent work by Korlach et al.

[21] suggests that BODIPY FL nucleotides may act analogous to guanine nucleotide exchange factors (GEFs) and aid displacement of endogenous, unlabeled nucleotide. However, because the bound BODIPY FL nucleotides were completely competed off with unlabeled nucleotides we suggest that the BODIPY moiety is not a major factor in nucleotide exchange or binding. Third, Rab7 is sensitive to substitution at the γ -phosphate position. A recent paper by Zhang et al. suggests that during nucleotide exchange on Ras the incoming nucleotide (GTP) attacks the nucleotide binding pocket of Ras through its β - and γ -phosphate groups to displace GDP [43]. The observed sensitivity of GST-Rab7 to changes in the γ -phosphate region of the nucleotide may imply a conserved mechanism for nucleotide exchange among the Ras-related GTPases.

Unlike many other GTPases, GST-Rab7 exhibited a marked inability to bind BODIPY FL GTP when the fluorophore was attached to the γ -phosphate as in BODIPY FL GTP- γ -NH. Relative to RhoA, we find that GST-Rab7 exhibits a 90% decrease in binding affinity to BODIPY FL GTP- γ -NH, while GST-RhoA has no preference. This result is nicely explained by structural differences in the nucleotide binding pockets of the two GTPases with Rab7.

GTPases play a crucial role in regulation of a multitude of cellular functions. An understanding of the specific differences between GTPases, and their nucleotide binding will become increasingly important as we try to gain insight into mechanisms of disease. We have presented a rapid and sensitive assay for detection of nucleotide binding dynamics. With only femtomoles of protein required for individual measurements, and without complicated purification steps this assay approach can be used to quickly investigate and compare nucleotide binding of individual mutant GTPases, make comparisons of GTPases involved on the same pathway, and using modified beads for high throughput screening, identify effector molecules and or small molecule inhibitors and activators (PubChem AID # 757–61, and AID 764; <http://nmmlsc.health.unm.edu/assays.shtml>).

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References

1. Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. *Physiol. Rev* 2001;81:153–208. [PubMed: 11152757]
2. Wennerberg K, Rossman KL, Der CJ. The Ras superfamily at a glance. *J. Cell Sci* 2005;118:843–846. [PubMed: 15731001]
3. Schwartz SL, Cao C, Pylypenko O, Rak A, Wandinger-Ness A. Rab GTPases at a glance. *J. Cell Sci* 2007;120:3905–3910. [PubMed: 17989088]
4. Myers KR, Casanova JE. Regulation of actin cytoskeleton dynamics by Arf-family GTPases. *Trends Cell Biol* 2008;18:184–192. [PubMed: 18328709]
5. Ménasché G, Pastural E, Feldmann J, Certain S, Ersoy F, Dupuis S, Wulffraat N, Bianchi D, Fischer A, Le Deist F, de Saint Basile G. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat. Genet* 2000;25:173–176. [PubMed: 10835631]
6. Shy ME. Charcot-Marie-Tooth disease: an update. *Curr. Opin. Neurol* 2004;17:579–585. [PubMed: 15367862]
7. Sahai E, Marshall CJ. RHO-GTPases and cancer. *Nat. Rev. Cancer* 2002;2:133–142. [PubMed: 12635176]

8. Stein MP, Dong J, Wandering-Ness A. Rab proteins and endocytic trafficking: potential targets for therapeutic intervention. *Adv. Drug Deliv. Rev* 2003;55:1421–1437. [PubMed: 14597139]
9. Vetter IR, Wittinghofer A. The guanine nucleotide-binding switch in three dimensions. *Science* 2001;294:1299–1304. [PubMed: 11701921]
10. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 1991;349:117–127. [PubMed: 1898771]
11. Simon I, Zerial M, Goody RS. Kinetics of interaction of Rab5 and Rab7 with nucleotides and magnesium ions. *J. Biol. Chem* 1996;271:20470–20478. [PubMed: 8702787]
12. Neu M, Brachvogel V, Oschkinat H, Zerial M, Metcalf P. Rab7: NMR and kinetics analysis of intact and C-terminal truncated constructs. *Proteins* 1997;27:204–209. [PubMed: 9061784]
13. Goldfinger LE. Choose your own path: specificity in Ras GTPase signaling. *Mol. Biosyst* 2008;4:293–299. [PubMed: 18354782]
14. Xu X, Press B, Wagle NM, Cho H, Wandering-Ness A, Pierce SK. B cell antigen receptor signaling links biochemical changes in the class II peptide-loading compartment to enhanced processing. *Int. Immunol* 1996;8:1867–1876. [PubMed: 8982771]
15. Jordens I, Fernandez-Borja M, Marsman M, Dusseljee S, Janssen L, Calafat J, Janssen H, Wubbolts R, Neeffjes J. The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr. Biol* 2001;11:1680–1685. [PubMed: 11696325]
16. Handley MT, Haynes LP, Burgoyne RD. Differential dynamics of Rab3A and Rab27A on secretory granules. *J. Cell Sci* 2007;120:973–984. [PubMed: 17311845]
17. Spinosa MR, Progida C, De Luca A, Colucci AM, Alifano P, Bucci C. Functional characterization of Rab7 mutant proteins associated with Charcot-Marie-Tooth type 2B disease. *J. Neurosci* 2008;28:1640–1648. [PubMed: 18272684]
18. Kabcenell AK, Goud B, Northup JK, Novick PJ. Binding and hydrolysis of guanine nucleotides by Sec4p, a yeast protein involved in the regulation of vesicular traffic. *J. Biol. Chem* 1990;265:9366–9372. [PubMed: 2111819]
19. Itzen A, Pylypenko O, Goody RS, Alexandrov K, Rak A. Nucleotide exchange via local protein unfolding--structure of Rab8 in complex with MSS4. *EMBO J* 2006;25:1445–1455. [PubMed: 16541104]
20. McEwen DP, Gee KR, Kang HC, Neubig RR. Fluorescent BODIPY-GTP analogs: real-time measurement of nucleotide binding to G proteins. *Anal. Biochem* 2001;291:109–117. [PubMed: 11262163]
21. Korlach J, Baird DW, Heikal AA, Gee KR, Hoffman GR, Webb WW. Spontaneous nucleotide exchange in low molecular weight GTPases by fluorescently labeled gamma-phosphate-linked GTP analogs. *Proc. Natl. Acad. Sci. USA* 2004;101:2800–2805. [PubMed: 14973186]
22. Hemsath L, Ahmadian MR. Fluorescence approaches for monitoring interactions of Rho GTPases with nucleotides, regulators, and effectors. *Methods* 2005;37:173–182. [PubMed: 16289968]
23. Feng Y, Press B, Wandering-Ness A. Rab 7: an important regulator of late endocytic membrane traffic. *J. Cell Biol* 1995;131:1435–1452. [PubMed: 8522602]
24. Press B, Feng Y, Hoflack B, Wandering-Ness A. Mutant Rab7 causes the accumulation of cathepsin D and cation-independent mannose 6-phosphate receptor in an early endocytic compartment. *J. Cell Biol* 1998;140:1075–1089. [PubMed: 9490721]
25. Progida C, Malerod L, Stuffers S, Brech A, Bucci C, Stenmark H. RILP is required for the proper morphology and function of late endosomes. *J. Cell Sci* 2007;120:3729–3737. [PubMed: 17959629]
26. Brachvogel V, Neu M, Metcalf P. Rab7: crystallization of intact and C-terminal truncated constructs complexed with GDP and GppNHp. *Proteins* 1997;27:210–212. [PubMed: 9061785]
27. Alexandrov K, Scheidig AJ, Goody RS. Fluorescence methods for monitoring interactions of Rab proteins with nucleotides, Rab escort protein, and geranylgeranyltransferase. *Methods Enzymol* 2001;329:14–31. [PubMed: 11210530]
28. Rak A, Pylypenko O, Niculae A, Goody RS, Alexandrov K. Crystallization and preliminary X-ray diffraction analysis of monoprenylated Rab7 GTPase in complex with Rab escort protein 1. *J. Struct. Biol* 2003;141:93–95. [PubMed: 12576024]
29. Ridley AJ. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol* 2006;16:522–529. [PubMed: 16949823]

30. Tessema M, Simons PC, Cimino DF, Sanchez L, Waller A, Posner RG, Wandinger-Ness A, Prossnitz ER, Sklar LA. Glutathione-S-transferase-green fluorescent protein fusion protein reveals slow dissociation from high site density beads and measures free GSH. *Cytometry A* 2006;69:326–334. [PubMed: 16604533]
31. Stein MP, Cao C, Tessema M, Feng Y, Romero E, Welford A, Wandinger-Ness A. Interaction and functional analyses of human VPS34/p150 phosphatidylinositol 3-kinase complex with Rab7. *Methods Enzymol* 2005;403:628–649. [PubMed: 16473626]
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 1976;72:248–254. [PubMed: 942051]
33. Buranda T, Jones GM, Nolan JP, Keij J, Lopez GP, Sklar LA. Ligand receptor dynamics at streptavidin-coated particle surfaces: A flow cytometric and spectrofluorimetric study. *J. Phys. Chem. B* 1999;103:3399–3410.
34. Schwartz A, Wang A, Early E, Gaigalas AK, Zhang Y, Marti G, Vogt R. Quantitating fluorescence intensity from fluorophore: The definition of MESF assignment. *J. Res. Natl. Inst. Stand. Technol* 2002;107:83–91.
35. Wang L, Gaigalas AK, Abbasi F, Marti G, Vogt R. Quantitating fluorescence intensity from fluorophores: Practical use of MESF values. *J. Res. Natl. Inst. Stand. Technol* 2002;107:339–353.
36. Nolan JP, Sklar LA. The emergence of flow cytometry for sensitive, real-time measurements of molecular interactions. *Nat. Biotechnol* 1998;16:633–638. [PubMed: 9661195]
37. Wu Y, Campos SK, Lopez GP, Ozbun MA, Sklar LA, Buranda T. The development of quantum dot calibration beads and quantitative multicolor bioassays in flow cytometry and microscopy. *Anal. Biochem* 2007;364:180–192. [PubMed: 17397793]
38. Simons PC, Vander Jagt DL. Purification of glutathione S-transferases from human liver by glutathione-affinity chromatography. *Anal Biochem* 1977;82:334–341. [PubMed: 907137]
39. Motulsky HJ, Mahan LC. The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol Pharmacol* 1984;25:1–9. [PubMed: 6708928]
40. Feuerstein J, Kalbitzer HR, John J, Goody RS, Wittinghofer A. Characterisation of the metal-ion-GDP complex at the active sites of transforming and nontransforming p21 proteins by observation of the 17O-Mn superhyperfine coupling and by kinetic methods. *Eur. J. Biochem* 1987;162:49–55. [PubMed: 3028791]
41. Burstein ES, Macara IG. Interactions of the ras-like protein p25rab3A with Mg²⁺ and guanine nucleotides. *Biochem. J* 1992;282:387–392. [PubMed: 1312327]
42. Zhang B, Zhang Y, Wang Z, Zheng Y. The role of Mg²⁺ cofactor in the guanine nucleotide exchange and GTP hydrolysis reactions of Rho family GTP-binding proteins. *J. Biol. Chem* 2000;275:25299–25307. [PubMed: 10843989]
43. Zhang B, Zhang Y, Shacter E, Zheng Y. Mechanism of the guanine nucleotide exchange reaction of Ras GTPase--evidence for a GTP/GDP displacement model. *Biochemistry* 2005;44:2566–2576. [PubMed: 15709769]
44. Delprato A, Lambright DG. Structural basis for Rab GTPase activation by VPS9 domain exchange factors. *Nat. Struct. Mol. Biol* 2007;14:406–412. [PubMed: 17450153]
45. Sklar LA, Mueller H, Omann G, Oades Z. Three states for the formyl peptide receptor on intact cells. *J Biol Chem* 1989;264:8483–8486. [PubMed: 2722782]
46. Yonekura S, Iwasaki M, Kai M, Ohkura Y. Determination of guanine and its nucleosides and nucleotides in human erythrocytes by high-performance liquid chromatography with postcolumn fluorescence derivatization using phenylglyoxal reagent. *J. Chromatogr. B. Biomed. Appl* 1994;654:19–24. [PubMed: 8004240]
47. Wiebe GJ, Fung E, Biddle FG, Snyder FF. A twenty strain survey and backcross localization of the erythrocytic GTP concentration determining locus *Gtpc* on mouse chromosome 9. *Genome* 1999;42:447–452. [PubMed: 10382292]
48. Tomiya N, Ailor E, Lawrence SM, Betenbaugh MJ, Lee YC. Determination of nucleotides and sugar nucleotides involved in protein glycosylation by high-performance anion-exchange chromatography: sugar nucleotide contents in cultured insect cells and mammalian cells. *Anal. Biochem* 2001;293:129–137. [PubMed: 11373089]

49. Klawitter J, Schmitz V, Klawitter J, Leibfritz D, Christians U. Development and validation of an assay for the quantification of 11 nucleotides using LC/LC-electrospray ionization-MS. *Anal. Biochem* 2007;365:230–239. [PubMed: 17475198]

Fig. 1. Purified GST-Rab7 can be immobilized on GSH beads and GTP-binding can be detected by flow cytometry using femtomoles of protein

(A) GST-Rab7 expressed in *E. coli* cells was isolated using glutathione beads and purity confirmed by SDS-PAGE and Coomassie staining. (B) Both binding of protein to the beads (Step 1) and nucleotide binding to the GTPase (Step 2) can be assayed by flow cytometry. (C) The dose dependence of GST-Rab7 binding to GSH beads was measured in the presence of saturating concentrations ($10 \mu\text{M}$) of BODIPY FL GTP (GTP^*) and variable concentrations of bead bound GST-Rab7 protein. Samples were incubated for 2 h. Maximal binding of $6 \pm 0.1 \times 10^6$ active GST-Rab7 bound per bead with an affinity $K_d = 190 \text{ nM}$. (D) The affinity of GST-Rab7 for GSH-beads was measured via competitive inhibition of GST-GFP binding. GST-GFP (100 nM) was incubated for 2 h in the presence of varying concentrations on non-fluorescent GST-Rab7. The inhibition constant was $K_i = 107 \text{ nM}$. (C,D) The number of GFP and BODIPY FL GTP molecules was calculated as described in the Materials and Methods. (B–D) Fluorescent nucleotide is designated in the schematics as (GTP^*).

Fig. 2. Kinetic measurement of nucleotide affinity and Mg^{2+} dependence

(A) The dose dependence of BODIPY FL GTP binding to Rab7 was measured in the presence or absence of $MgCl_2$. Increasing concentrations of BODIPY FL GTP were incubated to equilibrium for 2 h with bead bound GST-Rab7 in the presence of either 1 mM $MgCl_2$ or 1 mM EDTA and fluorescence was measured via flow cytometry. BODIPY FL GTP bound with a $K_d = 20$ nM in the presence of $MgCl_2$ with a maximal binding of 650×10^3 fluorophores/ bead compared with a $K_d = 150$ nM in the presence of EDTA and a maximal binding of 1600×10^3 fluorophores/ bead. (B) The dissociation rate of BODIPY FL GTP in the presence of Mg^{2+} was measured by incubating the bead bound Rab7 with 500 nM BODIPY FL GTP. After binding to equilibrium, samples were diluted 10-fold in buffer containing either 1mM EDTA or 1mM $MgCl_2$ and 1mM non fluorescent GTP (addition indicated by arrow). Dissociation was measured over 150 seconds. The dissociation rate of BODIPY FL GTP in the presence of 1mM $MgCl_2$ was $k_{off} = 1.3 \times 10^{-3} s^{-1}$ compared with $k_{off} = 6.6 \times 10^{-3} s^{-1}$ in the presence of 1 mM EDTA. (C) Kinetic rates for GTP binding to bead bound Rab7 were measured using 50 nM, 100 nM and 500 nM BODIPY FL GTP. An observed binding rate was measured during

approach to equilibrium over time. After 120 min samples were diluted in presence of 1mM GTP competitor (arrow) and dissociation measured over 35 min. The dissociation rate for all three concentrations was $k_{\text{off}} = 7 \times 10^{-3} \text{ s}^{-1}$. (D) Half-time ($t_{1/2}$) measured in (C) was plotted as a function of GTP concentration. The observed half-time remained constant at 11.1 min over a 10-fold range of nucleotide concentration. (A–D) The number of BODIPY FL GTP molecules was estimated as described in the Materials and Methods. Fluorescent nucleotide is designated in the schematics as (GTP*).

Fig. 3. GST-Rab7 has different affinities for BODIPY FL GTP and BODIPY FL GDP

(A) The dose dependence of BODIPY FL GTP and BODIPY FL GDP binding to Rab7 was measured by incubating increasing concentrations of either nucleotide to equilibrium for 2 h with bead bound Rab7. Fluorescence measured via flow cytometry. BODIPY FL GTP bound with a $K_d = 150$ nM and BODIPY FL GDP bound with a $K_d = 54$ nM. (B) The dissociation rates for BODIPY FL GTP and BODIPY FL GDP were measured by incubating bead bound GST-Rab7 with 500 nM of either nucleotide to equilibrium for 2 h. Samples were diluted 10-fold in the presence of 1 mM non-fluorescent GTP and the dissociation of BODIPY FL nucleotide measured. BODIPY FL GTP dissociated at a rate of $6.6 \times 10^{-3} \text{ s}^{-1}$ and BODIPY FL GDP at a rate of $1.3 \times 10^{-3} \text{ s}^{-1}$. (C) The affinities of unlabeled nucleotides for GST-Rab7

were measured by competitive inhibition of BODIPY FL GTP binding to GST-Rab7. Bead bound GST-Rab7 was incubated to equilibrium with 100 nM BODIPY FL GTP in the presence of increasing concentrations of non-fluorescent GDP or GTP- γ -S. K_i values for all three respectively are GDP (13 nM) and GTP- γ -S (40 nM). (A–C) The number of BODIPY FL GTP molecules was calculated as described in the Materials and Methods. Fluorescent nucleotide is designated in the schematics as (GTP* or GDP*).

Fig. 4. Rab7 discriminates BODIPY FL GTP nucleotide analogues conjugated via theribose or at the γ -phosphate position

(A) The BODIPY FL GTP analogues that are commonly used have the BODIPY FL moiety conjugated at the 2' or 3' oxygen on the ribose ring (BODIPY FL GTP) or at the γ -phosphate position (BODIPY FL GTP- γ -NH), as illustrated. (B) The dose dependencies of BODIPY FL GTP or BODIPY FL GTP- γ -NH binding to GST-Rab7 or RhoA were comparatively evaluated. Both GST-Rab7 bound beads or GST-RhoA bound beads were incubated to equilibrium with increasing concentrations of either the BODIPY FL GTP or BODIPY FL GTP- γ -NH in the presence of 1 mM MgCl_2 . The affinity of GST-Rab7 (left) for the BODIPY FL GTP was $K_d = 20$ nM with a maximal binding of 650×10^3 fluorophores per bead whereas for BODIPY FL

GTP- γ -NH there was a maximal binding of 60×10^3 fluorophores per bead. The affinity of GST-RhoA (right) for BODIPY FL GTP was $K_d = 80$ nM with a maximal binding of 700×10^3 fluorophores per bead and for BODIPY FL GTP- γ -NH was $K_d = 95$ nM with a maximal binding of 710×10^3 fluorophores per bead. (C) Comparisons of the crystal structures of GTP-bound RhoA and Rab7 are shown as space filling models. An opening in side of the RhoA crystal structure (arrow) can be seen that causes the phosphate groups of the nucleotide to be solvent exposed. In the Rab7 crystal structure the γ -phosphate is solvent protected.

Table 1**Summary of GST-Rab7 binding to BODIPY nucleotide analogs**

Experiments carried out in 1 mM EDTA buffer unless specified

Nucleotide	BODIPY FL GDP	BODIPY FL GTP
¹ K _d (nM)	54 ± 15	150 ± 62
² B _{max} (10 ³ BODIPY/bead)	2000 ± 400	1600 ± 400
³ t _{1/2} (mins)		11.1 ± 0.6
⁴ k _{off} (sec ⁻¹)	2.3 ± 1.5 × 10 ⁻³	7.0 ± 3.0 × 10 ⁻³
⁴ k _{off} (sec ⁻¹) Mg ²⁺		1.3 × 10 ⁻³
Competitors	GDP	GTP-γ-S
⁵ K _i (nM)	13 ± 5	40 ± 10

^{1,2} Maximal number of bound BODIPY nucleotide molecules per bead ($B_{\max}$) and dissociation constants were calculated simultaneously using the GraphPad Prism software to fit dose dependence to $B = B_{\max} \frac{[\text{Ligand}]}{K_d + [\text{Ligand}]}$ where B represents the number of bound receptors. Cytometry detector output was converted to BODIPY/bead using standard fluorescein beads from Invitrogen.

³ Observed on-rate was calculated using $B = B_{\max}(1 - e^{-k_{\text{obs}}(\text{time})})$ to fit the kinetic data.

⁴ Off rates were calculated by fitting $B = \text{Span}(e^{-k_{\text{off}}(\text{time})}) + \text{Plateau}$ to fit kinetic data.

⁵ Competition of non-fluorescent nucleotide and BODIPY FL GTP. Experimental data was fit to $B = B_{\min} + \frac{\text{Span}}{1 + 10^{([\text{Ligand}] - \log EC_{50})}}$. Inhibition

constants were calculated by converting the fit EC_{50} values to $K_i = \frac{EC_{50}}{1 + \frac{100\text{nM}}{K_d}}$.

Table 2**Comparison of GST-Rab7 and GST-RhoA binding to BODIPY-GTP analogs**Experiments carried out in 1 mM MgCl₂ buffer

GTPase	GST-Rab7		GST-RhoA	
	BODIPY FL	BODIPY FL	BODIPY FL	BODIPY FL
Nucleotide	GTP	GTP- γ -NH	GTP	GTP- γ -NH
$I_{B_{\max}}$ (10^3 BODIPY/bead)	650 ± 10	60 ± 5	700 ± 20	710 ± 15
^2K_d (nM)	20 ± 5	70 ± 20	80 ± 10	95 ± 10

^{1,2}Calculated as described in Table 1