Corrections

BIOCHEMISTRY. For the article “Spontaneous nucleotide exchange in low molecular weight GTPases by fluorescently labeled γ-phosphate-linked GTP analogs,” by Jonas Korchlach, Daniel W. Baird, Ahmed A. Heikal, Kyle R. Gee, Gregory R. Hoffman, and Watt W. Webb, which appeared in issue 9, March 2, 2004, of Proc. Natl. Acad. Sci. USA (101, 2800–2805; first published February 18, 2004; 10.1073/pnas.0308579100), due to a printer’s error in the legend for Fig. 7, the second to last sentence appeared incorrectly. The word “deleted” should read “detected” and “His6” should read “His6.” The figure and its corrected legend appear below.

![Fig. 7.](image)

- **A** BODIPY FL GT-P-γ-PA
- **B** BODIPY FL GT-P-γ-S
- MANT-GMPPNP

**Table 1. Kinetic binding parameters of ORFV NZ2 CBP to various human chemokines**

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>$k_{on} \times 10^7$, M$^{-1}$s$^{-1}$</th>
<th>$k_{off} \times 10^{-3}$, s$^{-1}$</th>
<th>$K_d$, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-chemokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>$0.56 \pm 0.02$</td>
<td>$0.05 \pm 0.003$</td>
<td>0.008</td>
</tr>
<tr>
<td>MCP-3</td>
<td>$0.71 \pm 0.14$</td>
<td>$0.29 \pm 0.08$</td>
<td>0.043</td>
</tr>
<tr>
<td>MCP-1</td>
<td>$1.02 \pm 0.23$</td>
<td>$1.86 \pm 0.13$</td>
<td>0.186</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>$0.64 \pm 0.13$</td>
<td>$2.12 \pm 0.36$</td>
<td>0.331</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>$2.02 \pm 0.39$</td>
<td>$11.99 \pm 4.03$</td>
<td>0.583</td>
</tr>
<tr>
<td>I-309</td>
<td>$0.23 \pm 0.08$</td>
<td>$20.27 \pm 5.69$</td>
<td>9.25</td>
</tr>
<tr>
<td>MDC</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>TARC</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>C-chemokine</td>
<td>$1.33 \pm 0.48$</td>
<td>$8.01 \pm 3.02$</td>
<td>0.598</td>
</tr>
</tbody>
</table>

Values represent mean ± SD and were obtained from global fitting analysis of four different concentrations, each performed in triplicate. Sensorgrams were generated by observing the association and dissociation phases of chemokines binding immobilized ORFV NZ2 CBP. Chemokines that did not bind are indicated by NB. CXC-chemokine (fractalkine) and CXC-chemokines [murine stromal cell-derived factor (SDF)-1, human SDF-1α and β, and IL-8] did not bind and are not shown. MDC, monocyte-derived chemokine; TARC, thymus- and activation-regulated chemokine.

MEDICAL SCIENCES. For the article “Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity,” by Bangyan Stiles, Ying Wang, Andreas Stahl, Sara Bassiliian, W. Paul Lee, Yoon-Jung Kim, Robert Sherwin, Sherin Devaskar, Ralf Lesche, Mark A. Magnuson, and Hong Wu, which appeared in issue 7, February 17, 2004, of Proc. Natl. Acad. Sci. USA (101, 2082–2087; first published February 9, 2004; 10.1073/pnas.0308617100), due to a printer’s error, the title appeared incorrectly and should read “Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity.” The online version has been corrected.

Genetics. For the article “Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect,” by Gloria Esposito, Bijay S. Jaiswal, Fang Xie, Magda A. M. Krajnc-Franken, Tamara J. A. A. Robben, Ankie M. Strik, Cor Kuil, Ria L. A. Philipsen, Marcel van Duin, Marco Conti, and Jan A. Gossen, which appeared in issue 25, December 9, 2003, of Proc. Natl. Acad. Sci. USA (100, 15137–15142; first published December 1, 2003; 10.1073/pnas.2336648100), the author name Byjay S. Jaiswal should have appeared as Bijay S. Jaiswal. The online version has been corrected.

Microbiology. For the article “Analysis of an orf virus chemokine-binding protein: Shifting ligand specificities among a family of poxvirus viroceptors,” by Bruce T. Seet, Catherine A. McCaughan, Tracy M. Handel, Andrew Mercer, Craig Brunetti, Grant McFadden, and Stephen B. Fleming, which appeared in issue 25, December 9, 2003, of Proc. Natl. Acad. Sci. USA (100, 15137–15142; first published December 1, 2003; 10.1073/pnas.2336648100), the authors note the following errors in Table 1. In the “Chemokine” column, MIP-1α and MIP-1β have inadvertently been switched. Also, in the “Kd, nM” column, the value for MIP-1β should read 0.331 instead of 0.032. The corrected table appears below.

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PSYCHOLOGY. For the article “Memory’s echo: Vivid remembering reactivates sensory-specific cortex,” by Mark E. Wheeler, Steven E. Petersen, and Randy L. Buckner, which appeared in issue 20, September 26, 2000, of Proc. Natl. Acad. Sci. USA (97, 11125–11129), the authors note that they inadvertently plotted the data in Fig. 3f from a retrieval condition in a different region of the brain. The corrected figure and its legend appear below. This correction does not affect the conclusions of the article.

Fig. 3. Regions in fusiform (a) and superior temporal (d) gyri (see Tables 1 and 2 for peak coordinates) associated with retrieval of pictures and sounds, respectively. Time courses in fusiform (b) and superior temporal (e) regions representing signal changes relative to fixation for Recall of pictures (open squares) and sounds (open circles). All time courses reflect an increased response, with picture > sound in fusiform gyrus and sound > picture in superior temporal gyrus. Note that a certain level of positive response in fusiform gyrus to sound trials was expected because of the presence of visually presented labels during sound trials. Time courses for regions in fusiform (c) and superior temporal (f) gyri representing signal changes relative to fixation for perception of Old (open circles) and New (open squares) items. Signal change for New items in fusiform gyrus was slightly higher than for Old items but similar in superior temporal gyrus.

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Spontaneous nucleotide exchange in low molecular weight GTPases by fluorescently labeled γ-phosphate-linked GTP analogs


*Field of Biochemistry, Molecular and Cell Biology, †School of Applied and Engineering Physics, and §Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853; and Molecular Probes, Inc., 4849 Pitchford Avenue, Eugene, OR 97402

Contributed by Watt W. Webb, December 23, 2003

Regulated guanosine nucleotide exchange and hydrolysis constitute the fundamental activities of low molecular weight GTPases. We show that three guanosine 5′-triphosphate analogs with BODIPY fluorophores coupled via the gamma phosphate bind to the GTPases Cdc42, Rac1, RhoA, and Ras and displace guanosine 5′-diphosphate with high intrinsic exchange rates in the presence of Mg2+ ions, thereby acting as synthetic, low molecular weight guanine nucleotide exchange factors. The accompanying large fluorescence enhancements (as high as 12-fold), caused by a reduction in guanine quenching of the environmentally sensitive BODIPY dye fluorescence on protein binding, allow for real-time monitoring of this spontaneous nucleotide exchange in the visible spectrum with high signal-to-noise ratios. Binding affinities increased with longer aliphatic linkers connecting the nucleotide and BODIPY fluorophore and were in the 10–100 nM range. Steady-state and time-resolved fluorescence spectroscopy showed an inverse relationship between linker length and fluorescence enhancement factors and differences in protein-bound fluorophore mobilities, providing optimization criteria for future applications of such compounds as efficient elicitors and reporters of nucleotide exchange. EDTA markedly enhanced nucleotide exchange, enabling rapid loading of GTPases with these probes. Differences in active site geometries, in the absence of Mg2+ ions, caused qualitatively different reporting of the bound state by the different analogs. The BODIPY analogs also prevented the interaction of Cdc42 with p21 activated kinase. Together, these results validate the use of these analogs as valuable tools for studying GTPase functions and for developing potent synthetic nucleotide exchange factors for this important class of signaling molecules.

Low molecular weight GTP-binding proteins play a fundamental role in a broad range of signal transduction pathways in eukaryotic cells, controlling events including cell growth, intracellular transport, cell motility, and morphology (1). Misregulation of these signaling events has been widely implicated in cancer (2, 3). Regulated GTP binding and hydrolysis underlies the function of all GTPases, which act as molecular switches that cycle between inactive (GDP-bound) and active (GTP-bound) states (4). Nucleotide exchange on the GTPase triggers signaling events by promoting the binding of GTP, which in turn induces conformational changes crucial for interactions with downstream effector proteins (5). Thus, studying the dynamics of nucleotide exchange constitutes an important prerequisite for elucidating signaling pathways mediated by GTP-binding proteins, with the eventual goal of their controlled manipulation for treatments of disease states caused by signaling dysfunction (6).

Separate from radioactively labeled nucleotides, fluorescent probes provide an important tool to measure activity and regulation of low molecular weight GTPases. The most widely used methods have involved intrinsic tryptophan residues or N-methylanthraniloyl (MANT)-labeled guanosine analogs (7). Although the exploitation of intrinsic tryptophan fluorescence does not require any label, it is limited to highly purified in vitro systems and suffers from only small changes in fluorescence intensity on binding (8). Tryptophan-based measurements also require UV optics and lack high sensitivity because of the low fluorophore quantum yield.

Labeled MANT guanine nucleotide analogs (9), with the fluorophore attached to the ribose, provide a distinctive and significant fluorescence enhancement on binding. However, its modest brightness and spectral characteristics have precluded in vivo studies (7). In addition, MANT analogs only exchange the native bound nucleotide in the absence of Mg2+ ions (10, 11).

Sensitive probing of nucleotide binding and hydrolysis by fluorophores attached via the γ-phosphate moiety has also been described (12). It is based on strong dynamical fluorescence quenching by the base while the compound is in a folded/stacked configuration in solution (13). Marked fluorescence enhancements have been observed for a more extended configuration induced by the nucleotide-binding site of a protein, due to hindrance of this chromophore-base quenching interaction. Several proteins have been studied in this way, such as RNA polymerase (14), UDP-glucose 4-epimerase (15), and a large number of dehydrogenases using NAD or etheno-NAD as probes (16). Draganescu et al. (17) have described the binding of BODIPY FL GTP-γ-S and other nonfluorescent γ-phosphate-linked analogs to Flt, a member of the histidine triad superfamily of nucleotide-binding proteins involved in tumor suppression. Subsequently, the usefulness of several fluorescent BODIPY GTP analogs for real-time detection of nucleotide binding to heterotrimeric G proteins with high affinities and signal-to-noise ratios was reported (18).

In vivo, rates of nucleotide exchange and hydrolysis in low molecular weight GTPases can be drastically modified by several regulatory factors (19). GTPase activation by exchange between bound GDP and free GTP, intrinsically very slow (5), can be accelerated by guanine nucleotide exchange factors. Their action, in combination with GTPase activating proteins (GAPs) and GDP dissociation inhibitors (GDIs), is essential for overall GTPase regulation and downstream signaling control. Defects in nucleotide exchange regulation have been implicated in oncogenic transformation, e.g., by the large family of Db1 proto-oncogenes (20). Therefore, there has been an active search for synthetic compounds that mimic the activities of these important regulatory proteins. Nonfluorescent γ-phosphate-linked GTP analogs capable of inducing hydrolysis in oncogenic Ras mutants normally deficient of GTP hydrolysis have been described (21). However, initially nucleotide-free Ras had to be used for binding these analogs. To our knowledge, synthetic compounds that can...
rapidly displace GDP from the binding sites of low molecular weight GTP-binding proteins under physiological conditions have not been reported so far.

In this article, three different γ-phosphate-linked fluorescent BODIPY GTP analogs are characterized with respect to their binding to the GTPases Cdc42, Rac1, RhoA, and Ras. We show that these analogs readily displace the native bound GDP with high intrinsic exchange rates in the presence of Mg2+, accompanied by substantial fluorescence enhancements. Subsequent analog hydrolysis appears to be prevented by the presence of the fluorophore and/or linker. The underlying photophysical properties of free and protein-bound BODIPY analogs are characterized by using steady-state and time-resolved fluorescence spectroscopy. Differences in reporting nucleotide exchange by these analogs in the presence and absence of Mg2+ ions in the nucleotide-binding site are evaluated. The interaction of the effector protein p21-activated kinase (PAK) with analog-bound Cdc42 is also studied.

Materials and Methods
BODIPY FL GTP-γ-S, BODIPY FL ATP-γ-S, MANT-GMPPNP, and MANT-GTP were purchased from Molecular Probes and used without further purification. BODIPY 515 GTP-γ-S was synthesized as described (18); the synthesis of BODIPY FL GTP-γ-P is given in Supporting Methods, which is published as supporting information on the PNAS web site. The molecular structures of the BODIPY GTP analogs are shown in Fig. 8, which is published as supporting information on the PNAS web site. Expression and purification of recombinant GTPases (22) as well as the PAK-binding assay are described in detail in Supporting Methods.

Fluorescence intensity time traces were measured at room temperature (23°C) in continuously stirred 1 × 1-cm cuvettes by using a PTI spectrofluorimeter (Photon Technologies, Monmouth Junction, NJ) with 2-nm spectral resolution. The buffer was 10 mM Hepes (pH 7.5)/5 mM MgCl2/100 mM NaCl. Traces were normalized to the baseline of BODIPY analog fluorescence levels. Excitation/emission wavelengths were 508/520 nm (BODIPY 515 GTP-γ-S), 504/520 nm (BODIPY FL GTP-γ-S and BODIPY FL ATP-γ-S), and 495/515 nm (BODIPY FL GTP-γ-P). Total concentrations of analog and GTPase are given in the figure legends. Fluorescence time traces were fit to a single exponential to determine association and dissociation rates.

For phosphodiesterase treatments, 5 μl of snake venom phosphodiesterase (1 μg/μl, United States Biochemical) was added to 2 ml of 250 mM nucleotide analog in 50 mM Tris-HCl (pH 8.0)/10 mM MgCl2/0.1 mM DTT. Kd determinations were performed on an SLM 8000C spectrofluorimeter (Perkin–Elmer) with an 8-nm spectral resolution (11). Two microliters of calf intestinal alkaline phosphatase (CIAP, Invitrogen) was added after 2 min to 125 mM BODIPY GTP analog [1 ml in 20 mM Hepes buffer (pH 7.5)/5 mM MgCl2] to hydrolyze initially bound GDP on release from the GTPase, thus removing the dissociated unlabeled nucleotide from the reaction. Different amounts of Cdc42 were added after 4 min, and the fluorescence exchange kinetics was recorded for 2 h. Saturating fluorescence yields were fit to a simple bimolecular association model to determine Kd for analog binding (23) or according to the equilibrium binding assay are described in detail in Supporting Methods.

Time-resolved fluorescence decays and anisotropies were measured by using time-correlated single photon counting techniques described in detail elsewhere (Ref. 24 and Supporting Methods). The concentrations used were 5 μM analog, 20 μM GTPase, and 40 μM PAK, as applicable. Completion of phosphodiesterase cleavage or GTPase binding was confirmed by following fluorescence intensity time courses before these measurements.

Results
Molecular Structures. The length of the linker between the γ-phosphate and BODIPY fluorophore was varied to investigate its effect on the fluorescence quenching efficiency in solution, as well as affinities, fluorescence enhancements, and nucleotide exchange rates on binding to the GTPases (Fig. 8). For BODIPY 515 GTP-γ-S and BODIPY FL GTP-γ-S, the nucleotide and fluorophore are linked via a thioether bond, whereas the coupling in BODIPY FL GTP-γ-P is attained via a phosphoramidate linkage. Both bond types are stable and essentially nonhydrolyzable.

Photophysical Properties of BODIPY GTP Analogs. Effects of guanine on BODIPY dye fluorescence were characterized by using steady-state spectroscopy and time-correlated single photon counting. Treatment with a phosphodiesterase breaks the α-β-phosphodiester bond and separates the dye from the base, thus allowing quantification of the quenching strength of guanine in the intact analog (Fig. 1). Large maximal fluorescence enhancements on such complete disruption of the guanine–label interaction were observed for all three compounds, from ~20-fold in BODIPY 515 GTP-γ-S, and decreasing with linker length to ~3-fold for the BODIPY FL GTP-γ-P analog with the longest linker (Table 1).

The absorption maximum of BODIPY 515 GTP-γ-S was ~513 nm, which is ~6 nm red-shifted with respect to the cleaved molecule. By comparison, BODIPY 515 alone absorbs at 500 nm. The emission peak of BODIPY 515 GTP is located at 520 nm, which is 3-fold for the BODIPY FL GTP-γ-P analog with the longest linker (Table 1).

The absorption maximum of BODIPY 515 GTP-γ-S was ~513 nm, which is ~6 nm red-shifted with respect to the cleaved molecule. By comparison, BODIPY 515 alone absorbs at 500 nm. The emission peak of BODIPY 515 GTP is located at 520 nm, which is 3-fold for the BODIPY FL GTP-γ-P analog with the longest linker (Table 1). The excited-state dynamics of BODIPY FL GTP-γ-S and BODIPY FL dUTP-γ-S were essentially invariant on cleavage, indicating that this quenching mechanism is specific for guanine (Fig. 9, which is published as supporting information on the PNAS web site). All three GTP analogs show multiexponential fluorescence decays, indicating several conformations with different quenching strengths (Table 2).

Molecular configurations were characterized further by using time-resolved fluorescence anisotropy, which provides compli-
shown by example for BODIPY FL GTP-/H9253 GTP-Cdc42. GTPases preloaded with MANT-GDP were not able to lysis, showed nucleotide exchange very similar to wild-type constitutively active mutant Cdc42Q61L, deficient in GTP hydrolysis and nucleotide binding (25), demonstrating that a functional active site is required for nucleotide exchange. The 

mentary insights into fluorophore mobilities and hydrodynamic volumes. Anisotropies of the free thioether-linked GTP analogs decayed as single exponentials with a rotational time that is consistent with their molecular weights (Table 1). For BODIPY FL GTP-γ-PA, the rotational time was smaller than the value predicted from the overall molecular weight, indicating a larger rotational freedom of motion of the fluorophore around its long linker in a limited angular range. The observed initial anisotropy values, r0, were slightly smaller than the theoretical value (0.4), indicating a relatively large angle between the absorbing and emitting dipoles (Table 3).

Spontaneous Nucleotide Exchange of BODIPY GTP Analogs in GTPases. All three BODIPY GTP analogs spontaneously displaced the GDP initially bound in the active sites of Cdc42, Rac1, RhoA, and Ras in buffer containing millimolar amounts of MgCl2, shown by example for BODIPY FL GTP-γ-S in Fig. 3. The accompanying substantial fluorescence enhancements on binding allow for real-time monitoring of the nucleotide exchange reaction, as was previously reported for heterotrimeric G proteins (18). The binding could be reversed by adding excess GTP, indicating that analog hydrolysis was very slow or absent under these conditions. Spontaneous nucleotide exchange was confirmed by competition assays involving GTP-binding proteins carrying radioactively labeled GDP (data not shown). BODIPY GTP analogs were specific in their association with GTPases because no fluorescence enhancement for any of the three compounds was observed with GST alone, BSA, or Cdc42T17N, a dominant-negative Cdc42 mutant deficient in Mg2+ ion coordination and nucleotide binding (25), demonstrating that a functional active site is required for nucleotide exchange. The constitutively active mutant Cdc42Q61L, deficient in GTP hydrolysis, showed nucleotide exchange very similar to wild-type Cdc42. GTPases preloaded with MANT-GDP were not able to bind BODIPY analogs (not shown), presumably because of tighter binding of MANT analogs mediated by interactions between the MANT fluorophore and the protein (11, 26). As expected, nucleotide exchange was specific for guanine analogs as demonstrated by the lack of BODIPY FL ATP-γ-S binding to the GTPases (Table 3).

Association and dissociation time traces are well described by single exponentials to obtain kinetic rates, shown by example for Cdc42 and BODIPY FL GTP-γ-S in Fig. 3. Association rates were similar for Cdc42 and Rac but 2- to 6-fold higher for Rho and Ras (Table 4, which is published as supporting information on the PNAS web site). Dissociation from Rho was particularly slow, and its fluorescence enhancement level was almost 2-fold higher, most likely reflecting different accessibilities and active-site environments for the analog in these proteins. Similar differences in fluorescence enhancements have been observed when using MANT analogs (unpublished observations).

In the experiments shown in Fig. 3, initially bound GDP competes with analog binding to the GTPase and significantly reduces overall fluorescence enhancement levels. This limitation could be

Table 1. Fluorescence properties of BODIPY GTP analogs

<table>
<thead>
<tr>
<th></th>
<th>BODIPY 515 GTP-γ-S</th>
<th>BODIPY FL GTP-γ-S</th>
<th>BODIPY FL GTP-γ-PA</th>
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</thead>
<tbody>
<tr>
<td>Linker length, carbon bonds</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Fluorescence enhancement on phosphodiesterase cleavage, fold</td>
<td>19.8</td>
<td>7.5</td>
<td>2.8</td>
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<tr>
<td>Fluorescence enhancement on Cdc42 binding*, fold</td>
<td>12.3</td>
<td>4.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Average excited-state fluorescence lifetime, ns</td>
<td>0.6</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Average excited-state fluorescence lifetime after phosphodiesterase cleavage, ns</td>
<td>4.9</td>
<td>5.6</td>
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<tr>
<td>Average excited-state fluorescence lifetime, Cdc42-bound*, ns</td>
<td>6.3</td>
<td>4.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Rotational time, ns</td>
<td>0.28</td>
<td>0.30</td>
<td>0.18</td>
</tr>
<tr>
<td>Rotational time components, Cdc42-bound*, ns, and relative contribution (%)</td>
<td>1.2 (5)</td>
<td>0.2 (59)</td>
<td>0.2 (40)</td>
</tr>
<tr>
<td></td>
<td>14.9 (95)</td>
<td>1.8 (20)</td>
<td>1.9 (25)</td>
</tr>
<tr>
<td></td>
<td>9.9 (21)</td>
<td>23.5 (35)</td>
<td></td>
</tr>
</tbody>
</table>

Kd Cdc42*, nM | 107 ± 28 | 22 ± 6 | 14 ± 5 |

*Phosphatase treated to remove GDP from equilibrium.

Fig. 2. Fluorescence decays of BODIPY FL GTP-γ-S (A) and BODIPY 515 GTP-γ-S (B) before (red) and after (green) phosphodiesterase cleavage. Lifetime decays on binding to Cdc42 (blue traces; see Fig. 3) are also shown. Lifetime components obtained from exponential fits are listed in Table 2.

Fig. 3. Spontaneous nucleotide exchange of BODIPY γ-phosphate-linked GTP analogs in low molecular weight GTPases. Shown are comparative fluorescence intensity time traces of association and dissociation of BODIPY FL GTP-γ-S for all four GTP-binding proteins. Two micromolar GTPase was added to 250 nM BODIPY FL GTP-γ-S at t = 0.5 min. At t = 30.5 min, 250 μM GTP was added to dissociate the bound analog. The dominant-negative mutant Cdc42T17N does not show any fluorescence change (gray trace). An example of single exponential fits to obtain kinetic rates of nucleotide exchange is shown for Cdc42. Association and dissociation rates and levels of fluorescence enhancements are compared in Table 2.
overcome by addition of CIAP, which selectively removes the released GDP from the reaction (27), thus ensuring complete exchange of all GTPase molecules with a BODIPY GTP analog (Fig. 4). Phosphatase degradation of these analogs is prevented because the fluorophore and linker are coupled to the γ-phosphate (28).

Efficient removal of GDP by the phosphatase allows for accurate determination of absolute enhancement levels and equilibrium dissociation constants of analog binding to the GTPases (Fig. 10, which is published as supporting information on the PNAS web site). A typical titration assay is shown in Fig. 4B for Cdc42 and BODIPY FL GTP-γ-S. Values for the three analogs range from ~10 to 100 nM for this protein, increasing with shorter linkers (Table 1). With these measured analog binding affinities, equilibrium competition assays of analog and GDP binding in the absence of CIAP allowed for determining the affinity of GDP to Cdc42 (Fig. 4B and Supporting Methods). The obtained K_d value of 60 mM is in good agreement to affinities reported for several low molecular weight GTPases (5, 27).

Fluorescence enhancement levels on complete GTPase binding increased for analogs with shorter linkers, yielding values as large as ~12-fold for BODIPY 515 GTP-γ-S binding to Cdc42 and significantly reduced to ~3-fold for BODIPY FL GTP-γ-PA (Table 1). However, the quenching interaction is weaker in the compounds with longer linkers. Therefore, similar relative enhancements were found for all three analogs, ~40–60% of maximum enhancement values obtained by complete disruption of the base-fluorophore interaction on phosphodiesterase treatment. Average fluorescence lifetimes increased on binding to Cdc42, supporting the interpretation that nonradiative decay pathways resulting from base-fluorophore stacking interactions are substantially reduced when the analog is bound to the protein (Fig. 2 and Table 2).

Time-resolved anisotropy decay measurements were performed to gain further insights into the conformational degrees of freedom of the BODIPY analogs in the Cdc42-bound configuration. The fluorophore mobilities were substantially restricted on binding to this GTPase, with overall slow rotational times (~10 ns) consistent with the combined molecular weight of Cdc42 (~21 kDa) and GTP analog (~0.8 kDa) (Fig. 5 and Tables 1 and 3). Anisotropies decayed as multieponential, with additional segmental rotational mobilities (~1.2–1.9 ns) that we assign to a wobbling motion of the analog within the protein restrictive environment. A third depolarization process slightly slower than rotational diffusion of the free dye was also observed. This component was absent in the BODIPY 515 GTP-γ-S/Cdc42 complex, demonstrating that the fluorophore of this analog is tightly bound to the protein surface. As the linker length increases, the contribution of this depolarization component in the anisotropy decay becomes more apparent, indicating a higher rotational flexibility of the label.

**Effect of Mg^{2+} on Nucleotide Exchange.** Nucleotide exchange rates were increased in the presence of excess EDTA over Mg^{2+} in the buffer. However, larger exchange rates were only directly reported as fluorescence increases for all four GTPases when BODIPY FL GTP-γ-PA was used (Fig. 11, which is published as supporting information on the PNAS web site). A more complex situation was found when using BODIPY FL GTP-γ-S and BODIPY 515 GTP-γ-S. For Rho and Ras, the behavior was identical to that described above for BODIPY FL GTP-γ-PA, whereas with Cdc42 and Rac, addition of excess EDTA caused the fluorescence to reach a constant plateau at a value close to the initial fluorescence (Fig. 6D). However, the large fluorescence jump on subsequent addition of excess Mg^{2+} indicated that the continuation of nucleotide exchange under these conditions. Fluorescence saturation levels were also reached faster compared to a trace without EDTA. Nucleotide exchange was monitored directly by these two probes only in the presence of excess Mg^{2+} over EDTA, with exchange rates increasing with the total EDTA concentration (Fig. 6B). This effect was not caused by a dependence on the Mg^{2+} concentration in the range studied (Fig. 12, which is published as supporting information on the PNAS web site). EDTA therefore acts as an additional catalyst for nucleotide exchange, even in the presence of excess Mg^{2+}, allowing for rapid loading of BODIPY analogs onto the GTPases (Fig. 13, which is published as supporting information on the PNAS web site).
on the PNAS web site). Dissociation rates obtained by GTP competition were also increased under these conditions.

**Interaction of Analog-Bound GTPase Complexes with Downstream Effector Proteins.** The ability of analog-bound Cdc42 to interact with downstream effector proteins was studied by using PAK as an example. Cdc42 preloaded with BODIPY FL GTP-γ-S was no longer able to bind the PAK-binding domain (PBD), as determined by fluorescence lifetime, anisotropy, and Western blot analysis (Fig. 7 and Tables 2 and 3).

**Discussion**

Rates of guanine nucleotide exchange and GTP hydrolysis establish the concentration of activated GTP-binding proteins in vivo, making them essential points of regulation in G protein-mediated signaling pathways. We show that GTP analogs with a BODIPY fluorophore coupled via the γ-phosphate spontaneously bind to several low molecular weight GTPases, thereby acting as small synthetic guanine nucleotide exchange factors. The accompanying fluorescence increases allowed us to study the kinetics of this nucleotide exchange in real time.

High intrinsic exchange rates found for these compounds are in contrast to MANT-labeled GTP analogs that do not exchange for bound GDP in the presence of Mg2+ (10, 11). Because intrinsic GDP dissociation rates are extremely low for these GTPases [e.g., 5 × 10−7 s−1 for Ras (27, 29)], an active mechanism by the analog to cause GDP release must be implied. Several possibilities exist that explain how this could be mediated. Both the linker and the fluorophore contain chemical groups suitable for protein surface interactions that could induce conformational changes similar to native exchange factor activities. Structural studies of a number of low molecular weight GTPases in complex with their specific guanine nucleotide-binding site even in the more open apoprotein configuration (37). Interactions of the linker and fluorophore with the guanine base lead to drastic fluorescence quenching in solution. GTPase structures determined by x-ray crystallography show the γ-phosphate exposed to the ambient medium (32, 33), with the GTPase nucleotide-binding pocket not providing enough space for the fluorophore in the closed, GTP-bound form so that the linker and fluorophore can only be accommodated on the protein periphery. The fluorophore is thereby separated from the base while the analog has to adopt a more stretched conformation, resulting in the observed increase in fluorescence brightness and decay time.

Steady-state and time-resolved excited-state decay spectroscopy was used to characterize the inhibition of these quenching pathways on GTPase binding and phosphodiesterase cleavage (Figs. 1 and 2). The integrated steady-state fluorescence signal enhancement was found to depend on the linker length, confirming the importance of guanine proximity in the fluorescence quenching mechanism (Table 1). Because the fluorescence quantum yield (Φfl) of a molecule is a function of the excited state fluorescence lifetime (τfl) and radiative (k rad) and nonradiative (k nonrad) rate constants via Φfl = k rad/(k rad + k nonrad) = k rad · τfl, the excited-state dynamics provides complimentary information about this fluorescence enhancement. The expected correlation between the average excited-state lifetime and fluorescence enhancement was only qualitative (Table 1), indicating that both radiative and nonradiative rate constants are affected. A more detailed analysis is complicated by the observed multieponential decays. It is known that guanine exhibits the lowest reduction and oxidation potentials compared to other bases (34), so that one nonradiative relaxation pathway that competes with fluorescence is likely attributable to electron transfer reactions with guanine (35, 36).

The measured exchange rates and fluorescence enhancement levels show that the analogs report differences in both accessibility and environment of the nucleotide-binding pocket for the four proteins studied. The analog with the longest linker between the fluorophore and nucleotide, BODIPY FL GTP-γ-PA, most directly monitored nucleotide exchange by exhibiting fluorescence enhancements even in the absence of the coordinating Mg2+ ion. Here, the long linker may not be accommodated in the nucleotide-binding site even in the more open apoprotein configuration (37). Interactions of the linker and fluorophore with amino acid residues outside the binding pocket may also stabilize the stretched configuration of this analog.

For the thioether analog with shorter linkers, GTPase-specific differences in the properties of the fluorescent probe were observed that may reflect interesting differences in the
protein active-site geometries. For Cdc42 and Rac, nucleotide exchange is accelerated in the absence of Mg\textsuperscript{2+}, but the fluorophore is positioned in a way that still permits efficient quenching by guanine, explaining the lack of fluorescence enhancement under these conditions. Addition of Mg\textsuperscript{2+} to the active site causes a conformational change that displaces the fluorophore from the base in the nucleotide-binding cleft, which is observed as a fluorescence increase. These probes should therefore allow a direct determination of the rates of conformational changes on coordination of the active-site Mg\textsuperscript{2+} ion, an event that is very difficult to monitor by other techniques (27). In contrast, fluorescence quenching in the absence of Mg\textsuperscript{2+} was not observed for Ras and Rho, indicating more restricted active-site geometries. Some structural evidence suggests that the switch regions of Cdc42 are more flexible than the corresponding regions of Ras [ref. 38 and G. Calero, personal communication], and it is intriguing to speculate that the differences in fluorescence quenching reported here reflect important differences in the mobility of these regions between various GTPases. These observations provide potential design strategies for more specific guanine nucleotide exchange factors, inhibitors, or imaging probes that are tailored to certain classes of GTPases. A balance between GTPase affinity and levels of fluorescence enhancement, based on the type of fluorophore and linker length, should be determined for the generation of optimized compounds for eliciting and reporting nucleotide exchange for a given application.

The presence of the BODIPY GTP analog interferes with the binding of key effector proteins such as PAK, resulting in potential inhibition of downstream signaling. It will be interesting to investigate whether spontaneous nucleotide exchange is induced by these analogs in oncogenic GTPase mutants that are defective in GTP hydrolysis, such as Ras\textsuperscript{G12V} (39). High affinity analogs could thereby inactivate these proteins by inhibiting their interaction with downstream effectors, thus constituting interesting compounds with respect to drug development efforts. Our results when using the constitutively active Cdc42\textsuperscript{G12V} mutant are promising in this respect. It is also possible that some effectors can still interact with the protein at other binding sites so that only certain pathways are selectively affected.

The nucleotide analogs reported here could complement existing approaches to the study of low molecular weight GTPase dynamics in living cells (40–42). The brightness and spectral characteristics of BODIPY fluorophores would ensure excellent signal-to-noise ratios for such measurements and allow the use of laser-based optics and potential multicolor detection. Methods for efficient loading of the compounds into cells have to be evaluated toward such studies.

BODIPY GTP analog hydrolysis appeared to be essentially absent for any of the GTPases studied, or at least much slower than intrinsic or MANT-analog GTPase activities (10, 11, 43, 44), constituting a potential limitation of using these analogs to study the coupling between nucleotide exchange and hydrolysis. Addition of GTPase activating protein (GAP) did not induce or accelerate hydrolysis rates (data not shown), possibly because of impaired GAP binding or analog interference with GAP activity. Analogs mediating GTP hydrolysis via an involvement of certain aromatic moieties coupled via the \(\gamma\)-phosphate of GTP have been reported (21, 45); however, these analogs were not capable of spontaneous nucleotide exchange so that nucleotide-free protein had to be used. It is desirable to combine the responsible chemical groups for generating more advanced visible wavelength fluorescence GTP analogs that induce and report both nucleotide exchange and GTP hydrolysis, thereby more closely mimicking the biochemistry of GTP, with intriguing potential applications for monitoring GTPase signaling activities in vivo.

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