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Specific and Effective Interaction of a Guanine Nucleotide Analogue with Small G Proteins

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SUMMARY

G proteins are molecular switches that use a cycle of GTP binding and hydrolysis to regulate a wide variety of cellular biochemical processes. Because the functional state of these proteins is allosterically determined by bound guanine nucleotides, a nucleotide analogue with protein specificity might have pharmacological or biochemical value. The binding of [α-^{32}P]GTP to four small G proteins immobilized on nitrocellulose was competed by a series of analogues with modifications at multiple sites. One analogue, N^2-(*p*-*n*-butylphenyl)guanosine 5′-(β,γ-difluoromethylene)triphosphate, had a ~40-fold higher affinity for one small G protein than for two of the others. Systematic analysis of each modification in the synthetic nucleotide revealed that specificity was conferred by the carbon substitution in the β,γ-phosphoanhydride bond. These observations were then extended to purified proteins of known sequence in solution by filtration binding studies with H-*ras* and *rab5*. *Ras* was 9-fold more discriminant between guanosine-5′-(β,γ-difluoromethylene)triphosphate and guanosine-5′-*O*-(3-thiotriphosphate) than was *rab5*, and the Q79L GTPase-defective mutant of *rab5* was 6-fold more discriminant than wild-type *rab5*. Guanosine-5′-(β,γ-difluoromethylene)triphosphate protected a 20-kDa fragment of *rab5* from tryptic proteolysis with greater efficacy than guanosine-5′-*O*-(3-thiotriphosphate) or guanosine-5′-(β,γ-imido)triphosphate despite its lower affinity, and GMP stabilized a conformation indistinguishable from apo-*rab5*. These results identify a synthetic guanine nucleotide analogue with differential affinity for closely related G proteins, determine the atomic substitution in the analogue that confers specificity, demonstrate discrimination by the analogue between wild-type and a point-mutant G protein, and establish efficacy of the analogue in inducing conformational change of a target protein disproportionate to the affinity of the interaction.

Regulatory G proteins have been likened to molecular switches that function in the control of a wide variety of cellular biochemical processes. Protein families comprising the regulatory G protein superfamily include the cytoplasmic factors involved in protein synthesis, the signal-transducing G proteins, dynamin and related proteins, signal recognition particle and its receptor, and the family of small *ras*-related G proteins (1, 2). By employing a cycle of GTP binding and hydrolysis, followed by the dissociation of GDP and the initiation of another round of GTP binding and hydrolysis, all of these proteins shuttle between two activity states which are determined by whether the di- or tri-phosphoguanine nucleoside occupies the nucleotide binding pocket. These two activity states have been shown to reflect two distinct conformations for c-H-*ras* p21, EF-Tu, and transducin (3). Overall topology of the GTP binding and hydrolyzing domains of these proteins is strikingly similar, although subtle structural differences can be found. Regions outside of the nucleotide binding pocket differ substantially.

Metabolically stable guanine nucleotide analogues have been used to great advantage in the biochemical study of many G proteins during the past 20 years (1, 2). Due to their ability to constitutively activate or block G protein-dependent cellular processes, even in the absence of other regulatory ligands such as heptahelical receptor agonists, these reagents have played an important role in elucidating the regulatory functions of many G proteins. Little thought has been given, however, to the possibility that an analogue might have differential affinities and efficacies for multiple distinct G proteins that regulate a single biochemical process

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ABBREVIATIONS: BuPGMPPCF$_2$P, N^2-(*p*-*n*-butylphenyl)guanosine-5′-(β,γ-difluoromethylene)triphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); GMPPNHP, guanosine-5′-(β,γ-imido)triphosphate; GMPPCH$_2$P, guanosine-5′-(β,γ-methylene)triphosphate; GMPPCF$_2$P, guanosine-5′-(β,γ-difluoromethylene)triphosphate; BuPGTP, *N*2-(*p*-*n*-butylphenyl)-GTP; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

(e.g., the antagonistic regulation of adenylyl cyclase by G_s and G_i). Furthermore, if such differential effects of nucleotide analogues could be demonstrated, the introduction of a metabolically stable guanine nucleotide analogue with specificity for an individual G protein would be an obvious strategy to control the activity of a targeted G protein-regulated biochemical pathway for pharmacological or investigational purposes. The present study was initiated to determine whether relatively closely related G proteins (i.e., members of the *ras*-related small G protein family) could be differentially acted on by synthetic guanine nucleotide analogues.

Materials and Methods

Preparation of bovine lung surfactant secretory vesicles (lamellar bodies). Lamellar bodies were used as the source of G proteins in our initial studies because of the focus in one of our laboratories on the role of G proteins in exocytosis from pulmonary epithelial cells (4). Lungs from freshly slaughtered calves were grossly dissected to obtain parenchyma free of large airways and blood vessels. Tissue was then suspended 1:1.5 (w/v) in lysis buffer consisting of 10 mM HEPES, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride, and 10 μM leupeptin. The tissue suspension was initially homogenized in a Waring blender for 120 sec, filtered through cheesecloth, and then further homogenized with a Polytron for 10 sec followed by three strokes with a Potter-Elvejhem motor-driven Teflon pestle. This homogenate was centrifuged at 600 × *g* for 20 min, and the supernatant was collected and pelleted onto a 0.65 M sucrose cushion at 100,000 × *g* for 60 min. The interface was collected and brought to 0.25 M sucrose, and the previous density centrifugation was repeated. The interface was again harvested; diluted with two volumes of 10 mM Tris, pH 8.0, 3 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol (buffer A); pelleted by centrifugation at 100,000 × *g* for 60 min; and frozen at −70°.

Fractionation of lamellar body G proteins. Protein was extracted from the lamellar body preparation for 1 hr at 4° using 4% cholate in buffer A at a cholate/protein ratio of 8:1 (w/w). The suspension was centrifuged at 100,000 × g for 1 hr, and the soluble supernatant was then diluted to 0.9% cholate and was brought to 25 mM with NaCl. The protein solution (50 mg from 400 g lung) was loaded onto a 20 ml DEAE-Sephacryl column (Pharmacia LKB, Piscataway, NJ), and washed with two column volumes of buffer A with 25 mM NaCl and 0.9% cholate at 1 ml/min. Elution was accomplished with three column volumes of buffer A with 0.9% cholate using a NaCl gradient from 25 mM to 400 mM, followed by a 1 M NaCl bump. Fractions (1 ml) were assayed for absorbance at 280 nm, [^{35}S]GTPγS binding activity (5), and [α-^{32}P]GTP binding to Western blot-analyzed proteins (see below). Fractions of the DEAE eluate in which [α-^{32}P]GTP binding to Western blot-analyzed proteins revealed bands of relatively equal intensity on autoradiograms were pooled and used for subsequent experiments.

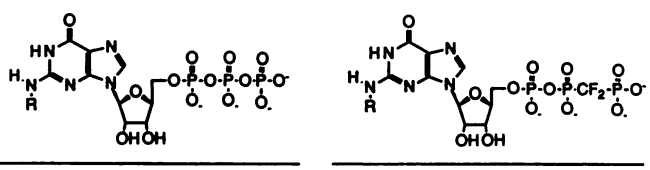
Preparation of guanine nucleotide analogues. BuPGTP was prepared according to the method of Kelleher *et al.* (6), and the corresponding BuPGMPPCF$_2$P was prepared as described by Arabshahi *et al.* (7). GMPPCF$_2$P was prepared according to the method described for the synthesis of BuPGMPPCF$_2$P (7), using ^{19}F and ^{31}P. NMR spectra were identical to those previously reported (8). The structures of GTP, BuPGTP, BuPGMPPCF$_2$P, and GMPPCF$_2$P are illustrated in Fig. 1. 2-bromoinosine-5′-triphosphate, N^2-(*p*-fluorophenyl)guanosine-5′-triphosphate, and N^2-(*p*-tolyl)guanosine-5′-triphosphate were synthesized as described (9). GMPPNHP, GMPPCH$_2$P, GTP, and GDP were purchased from Boehringer Mannheim (Indianapolis, IN). 7-Methylguanosine 5′-triphosphate was obtained from Sigma Chemical Co. (St. Louis, MO). 9-[(2-Hydroxyethoxy)methyl]guanine triphosphate (acyclovir triphosphate) was a gift from Burroughs Wellcome (Research Triangle Park, NC).

[α-^{32}P]GTP binding to Western-blotted proteins. Proteins were subjected to SDS-PAGE through 18% Laemmli gels and then transferred electrophoretically for 3500 mA/hr to BA83 0.20-μm-pore nitrocellulose (Schleicher & Schuell, Keene, NH). Blotted nitrocellulose was blocked with 5% nonfat milk and 0.05% Tween-20 in Tris-buffered saline (pH 7.5) for 1 hr, washed with two 15-min exchanges of buffer B (50 mM Tris, pH 7.5, 0.3% Tween-20, 5 mM $MgCl_2$, 1 mM EGTA), and then incubated with 0.1 nM [α-^{32}P]GTP (Amersham, Arlington Heights, IL) at 2 μCi/ml in buffer B at 22° for 1 hr (10). The blots were washed with three 20-min exchanges of buffer B and then dried and exposed to Kodak XAR5 film for 16–48 hr.

Competitive binding of guanine nucleotide analogues to Western-blotted proteins. The differential binding of guanine nucleotide analogues was initially assessed by the ability of unlabeled analogues to inhibit the binding of [α-^{32}P]GTP to Western-blotted small G proteins. An aliquot (0.5 mg) of pooled protein from the DEAE chromatography was run on a single lane preparative 18% SDS-PAGE gel and electrophoretically transferred to nitrocellulose as above. Binding of guanine nucleotides to the horizontally arrayed protein bands was then determined using a vertically arrayed slot-blot apparatus (Immunetics, Cambridge, MA). Aliquots containing increasing concentrations from 100 pM to 100 μM of unlabeled nucleotide in a solution of buffer B containing 100 pM [α-^{32}P]GTP were added to the vertical slots. After incubation at 22° for 60 min, the binding solutions were aspirated, the apparatus was disassembled, and the nitrocellulose sheets were washed as above and air dried. Autoradiograms were obtained in the linear range for film and densitometer, and relative band intensity was determined with an Ultroscan XL Laser Densitometer (Pharmacia LKB). Comparison was made to competition with GTP for each protein pool to ensure uniformity of the preparations.

Competitive binding of guanine nucleotide analogues to recombinant *ras* and *rab5* proteins. A cDNA encoding *rab5* was obtained from Dr. A. Tavitian, site-directed mutagenesis was performed to generate the Q79L point mutant, and wild-type and mutant cDNAs were cloned into the inducible vector pT7.7, as described (11). Wild-type and Q79L mutant proteins were purified by sequential DEAE-Sepharose and Sephacryl S-100 chromatographies to >95% purity as determined with SDS-PAGE (11). Recombinant H-*ras* was a gift from Dr. Jackson Gibbs (Merck Sharp and Dohme Research Laboratories, West Point, PA).

Pseudoequilibrium competition binding was carried out by adding recombinant H-*ras*, *rab5* wild-type, or *rab5* Q79L (125 ng/assay) to buffer C (20 mM Tris·HCl, pH 8.0, 0.1% 3[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol) containing 1.0 nM [^{35}S]GTPγS and increasing concentrations of GTPγS or GMPPCF$_2$P. Final reaction volume was 100 μl. Binding was allowed to progress at 30° for 60 min and was terminated by the addition of 4 ml ice-cold buffer C followed by rapid vacuum filtration through BA85 filters (Schleicher and Schuell) and two washes with 4 ml iced buffer C. Dried filters were immersed in scintillation fluid (Formula 989, DuPont-NEN, Boston, MA) and counted by β spectroscopy. Data points are the mean of triplicate determinations, and values are representative of three experiments for H-*ras*/*rab5* wild-type and two for *rab5* wild-type/*rab5* Q79L. Data



R = H, GTP

R = *p*-n-butylphenyl, BuPGTP

R = H, GMPPCF$_2$P

R = *p*-n-butylphenyl, BuPGMPPCF$_2$P

Fig. 1. Structures of GTP and its N^2-(*p*-*n*-butylphenyl)- and β,γ-difluoromethylene-substituted analogues.

were plotted and IC_{50} values were calculated with the computer program Enzfitter (Biosoft, Ferguson, MO), and statistical analysis was made by paired *t* test with the computer program Sigmastat (Jandel, San Rafael, CA). It should be noted that although this assay yields highly reproducible relative affinities, absolute affinity is not determined (12).

Nucleotide-dependent proteolysis. Proteolysis experiments were carried out as described previously (11). In brief, *rab5* proteins (2.5 μg) were preincubated in the presence or absence of 10^{-2} M nucleotides in buffer C for 2 hr at 30° and then with 0.25 μg (1:10) or 0.005 (1:50) μg trypsin for 1 hr in a total volume of 50 μl at 30°. Polypeptides were then resolved by SDS-PAGE in a Tris-Tricine buffer system and visualized using an enhanced Coomassie Brilliant blue-staining procedure.

Results

Fractionation of Western blot-analyzed G proteins. More than 90% of lamellar body protein and [^{35}S]GTPγS binding activity was solubilized by 4% cholate. The purpose of the anion exchange chromatography was 2-fold: first, to fractionate and recombine the small G proteins for binding studies and, second, to delipidate the protein because purified lamellar bodies contain lipids in a mass/protein ratio of >10:1. A representative DEAE elution profile is shown in Fig. 2. The peak of [^{35}S]GTPγS binding activity eluted at 160–180 mM NaCl, partially resolved from the protein peak (Fig. 2A). The binding of [α-^{32}P]GTP to fractions after Western blotting is shown in Fig. 2B. Four major bands of 26, 25, 24, and 23 kDa were identified, and the peak of [α-^{32}P]GTP binding to Western-blotted proteins correlated with the peak of [^{35}S]GTPγS binding activity. The intense [α-^{32}P]GTP binding activity of the 26-kDa band impaired visualization of other bands in early eluting fractions. Therefore, only later eluting fractions (e.g., 30–38, Fig. 2B) were pooled and used for subsequent experiments, yielding four bands of similar autoradiographic intensities (Fig. 3, *left lanes*).

Competitive nucleotide binding to blotted G proteins. The results of a representative competition binding experiment using unlabeled GTP are shown in Fig. 3A. There is slight preferential inhibition by GTP of [α-^{32}P]GTP binding to the 26- and 23-kDa bands compared with the 25- and 24-kDa bands, indicating a somewhat higher affinity of GTP for the 26- and 23-kDa proteins. GTPγS (Fig. 3B) and GDP (not shown) had patterns of competition similar to that of GTP, whereas GMP, ATP, and ADP did not substantially inhibit binding even at 10 μM, indicating low affinities for the blotted proteins (not shown).

We then assayed a series of synthetic GTP analogues for differential binding to lamellar body small G proteins. Among these, BuPGMPPCF$_2$P showed a pattern strikingly different from GDP, GTP, or GTPγS, with virtually total inhibition of [α-^{32}P]GTP binding to the 25-kDa band at 1 μM BuPGMPPCF$_2$P but little inhibition of binding to the other bands at this concentration (Fig. 3C). To determine whether it was the N^2-butylphenyl group or the β,γ-CF$_2$ substitution that conferred specificity, compounds containing only one of these modifications were tested. BuPGTP showed a pattern and affinity similar to that of GTP and GTPγS, and all of the specificity was found to reside in the β,γ-CF$_2$ substitution (Fig. 4). Densitometric scanning of the autoradiogram and plotting of relative band density as a function of increasing nucleotide concentration yielded 56-, 42-, and 3.4-fold greater affinities of GMPPCF$_2$P for the 25-kDa band than for the 23-, 24-, and 26-kDa bands, respectively. GMPPCH$_2$P was then assayed to determine whether the selectivity for the 25-kDa

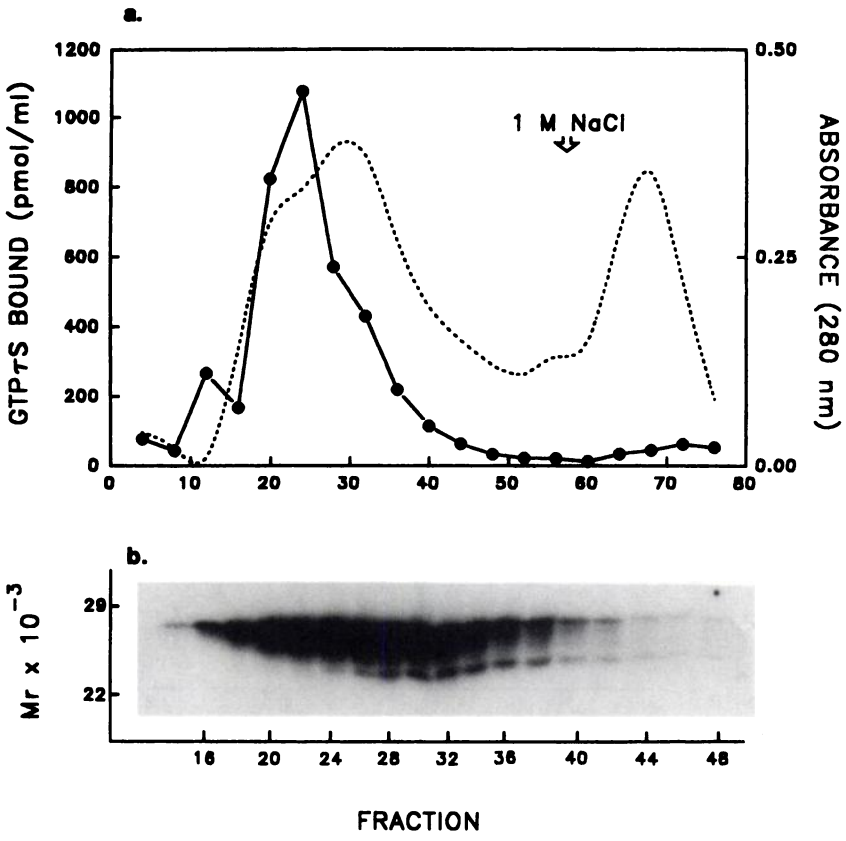


Fig. 2. DEAE fractionation of small G proteins. Crude lamellar bodies from whole bovine lung were solubilized with cholate, loaded onto a DEAE-Sephacryl column, and eluted with a linear 25–400 mM NaCl gradient and a 1 M NaCl bump. A, Absorbance at 280 nm (*dotted line*) and [^{35}S]GTPγS binding activity (*solid line*) of eluted fractions. B, Autoradiogram of [α-^{32}P]GTP bound to aliquots (100 μl) of individual DEAE fractions electrophoresed through an 18% SDS-PAGE gel and then transferred to nitrocellulose.

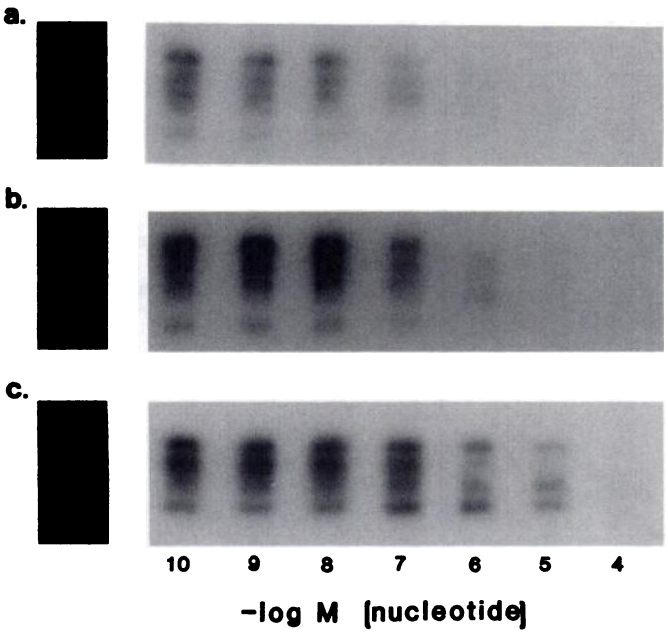


Fig. 3. Competitive binding of guanine nucleotide analogues to blotted proteins. Aliquots of pooled lamellar body protein eluted from DEAE-Sephacryl were electrophoresed on single-well 18% SDS-PAGE gels, transferred to nitrocellulose, and then incubated with 0.1 nM [α-^{32}P]GTP in the presence of varying concentrations of unlabeled guanine nucleotide analogues in a slot-blot apparatus. Autoradiograms were developed after exposure to the nitrocellulose without an intensifying screen. A, Competition by GTP. B, Competition by GTPγS. C, Competition by BuPGMPPCF$_2$P.

band was conferred by the fluorines of the β,γ-CF_2 analogue or by the phosphoanhydride substitution. The results indicate that the β,γ-CH_2 modification maintained selective binding to the 25-kDa band (Fig. 4), even though the phosphoanhydride-substituted analogue GMPPNHP showed a pattern of competitive binding similar to that of GTP and GTPγS but with a slightly reduced affinity (not shown).

Screening of other nucleotide analogues for differential affinity for the four blotted small G proteins revealed no selective inhibition of binding to a single band as observed with the β,γ-methylene series. Two N^2-substituted compounds, N^2-(*p*-fluorophenyl)guanosine-5′-triphosphate and N^2-(*p*-tolyl)guanosine-5′-triphosphate, showed a pattern of competitive inhibition similar to that of GTP but with a reduction in affinity of 10–100-fold (not shown). Taken together with our current and previous findings (6, 9), it appears that the exocyclic amine position can be extensively derivatized with modest or no loss of affinity. In contrast, 2-bromoinosine-5′-triphosphate, a GTP analogue in which the exocyclic (N^2) amino group is replaced by a bromo group, showed very little inhibition of [α-^{32}P]GTP binding to any of the Western blot-analyzed small G proteins, even at 10 μM (not shown), indicating severely reduced affinity. The presence of a methyl group on guanine N' also severely reduced binding of 7-methylguanosine 5′-triphosphate to all of the bands (not shown), similar to a recent finding with H-*ras* (13), although 7-Me-8-oxoguanosine has been previously shown to activate peritoneal exudate cells when administered *in vivo* (14). Similarly, the open ribose ring of acyclovir triphosphate severely reduced competition for all bands (not shown), even though opening the ribose ring between the 2′ and 3′ carbons previously yielded an analogue with activity for transducin (15) and the ribose hydroxyls have been extensively derivatized in the setting of an intact ring structure with only modest effects on affinity or activity (16–18).

Competitive nucleotide binding to recombinant small G proteins in solution. To confirm in a conventional binding assay the discriminant results obtained with the Western blot assay, pseudoequilibrium competitive binding of guanine nucleotide analogues to purified H-*ras* was compared with binding to *rab5* in solution by filtration assay. These two G proteins were chosen because of the extensive prior structural study of H-*ras* (3, 19) and the more modest study of *rab5* (11, 20), the ready availability of both proteins, and the fact that the significance of discriminant binding would be greatest for more closely related proteins. Amino acid identity between these two small, *ras*-related family members is 30% compared with the 15% identity between *ras* and *EF-Tu*, which are members of distinct G protein families. Truncated sequences of H-*ras* and *rab5* that show the nucleotide binding domains are compared in Fig. 5. GTPγS was used as a native guanine nucleotide for comparison to GMPPCF$_2$P in these studies because (i) a triphosphate guanine nucleoside had to be used for comparison with the triphosphate analogue GMPPCF$_2$P as some G proteins display a marked differential affinity for GDP and GTP (21) while others do not (16); (ii) like GMPPCF$_2$P, GTPγS is a slowly hydrolyzed GTP analogue, thereby avoiding the possibility that a binding assay might reflect a mixture of bound GTP and GDP if the more readily hydrolyzed GTP were used; and (iii) in preliminary studies (Figs. 3 and 4), GTPγS showed a pattern and affinity of binding to Western blot-analyzed small G proteins similar to that of GTP.

The IC_{50} values for GTPγS binding to recombinant H-*ras* and *rab5* (Fig. 6) did not significantly differ (IC_{50} for *ras*, 5.95×10^{-8} M; IC_{50} for *rab5*, 7.08×10^{-8} M; $p > 0.05$). These values are also similar to those determined for GTPγS and GTP with the Western blot-analyzed small G proteins (Table 1 and Fig. 4). GMPPCF$_2$P, in contrast, competed for [^{35}S]GTPγS binding to *ras* 7.6-fold less effectively than to *rab5* (IC_{50} for *ras*, 5.41×10^{-5} M; IC_{50} for *rab5*, 7.08×10^{-6} M; $p < 0.05$). The discrimination by *ras* between GTPγS and GMPPCF$_2$P (IC_{50} for GMPPCF$_2$P/IC_{50} for GTPγS = 909) was 9-fold greater than by *rab5* (IC_{50} for GMPPCF$_2$P/IC_{50} for GTPγS = 99.9; $p < 0.001$). These results confirm the capacity of guanine nucleotide analogues to bind differentially to relatively closely related G proteins in solution.

To determine whether GMPPCF$_2$P can discriminate between a wild-type and point-mutant G protein, competitive binding to *rab5* wild-type and *rab5* Q79L was compared (Fig. 7). The *rab5* Q79L mutant is the cognate of the H-*ras* Q61L mutant and, similar to the *ras* protein, *rab5* Q79L has been shown to have a reduced GTPase activity (11, 20). In H-*ras*, Q61 lies in loop 4, which overlies the γ-phosphate of GTP and indirectly is in contact with it via G60 (Fig. 5). The Q79L mutation in *rab5* induced a GMPPCF$_2$P-binding phenotype more similar to H-*ras* than to *rab5* wild-type. There was 6.5-fold greater discrimination between GMPPCF$_2$P and GTPγS by *rab5* Q79L (IC_{50} for GMPPCF$_2$P/IC_{50} for GTPγS, 1.96×10^{-5} M/3.27×10^{-7} M = 599) than by *rab5* wild-type (IC_{50} for GMPPCF$_2$P/IC_{50} for GTPγS, 2.61×10^{-5} M/2.83×10^{-7} M = 92.2; $p < 0.001$), and no significant difference in GTPγS binding ($p > 0.05$). These results establish that dif-

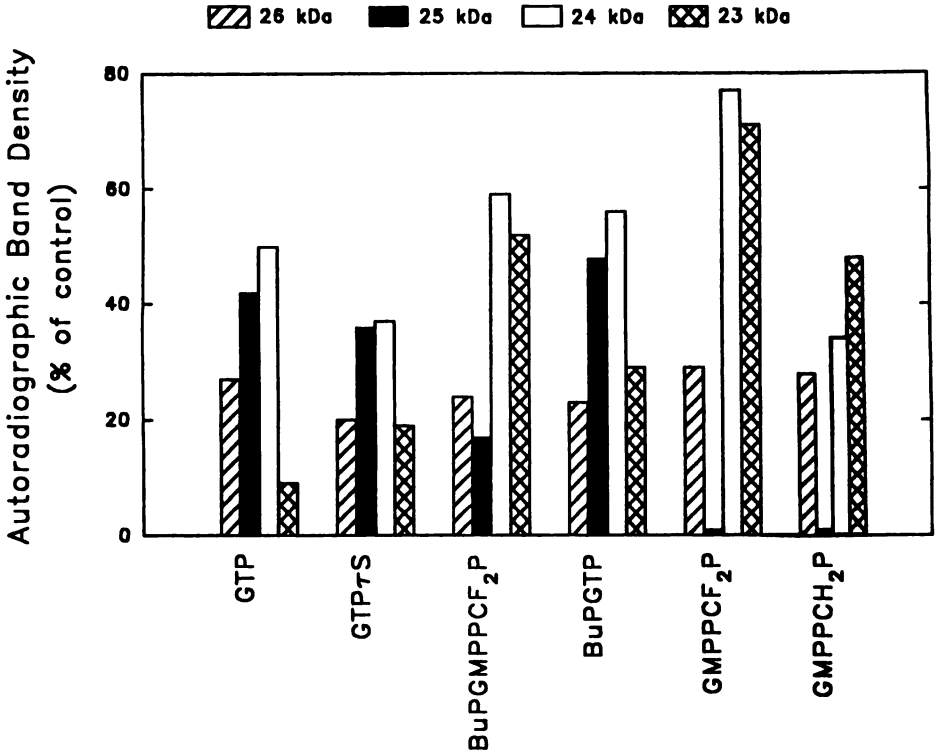


Fig. 4. Densitometric analysis of competitive nucleotide binding to blotted proteins. Densitometry was performed on representative autoradiograms generated as in Fig. 3. The reduction in autoradiographic density in the presence of competing unlabeled nucleotide is plotted as a percentage of autoradiographic density in the absence of competing unlabeled nucleotide. The concentration of competing nucleotide that is plotted was 100 nM for high affinity nucleotides (GTP, GTPγS, BuPGTP) and 1 μM for low affinity nucleotides (BuPGMPPCF$_2$P, GMPPCF$_2$P, GMPPCH$_2$P).

A.

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          5           15          25          35
H-ras  KLVVVGAGGV GKSALTIQLI QNHFVDEYDP TI-EDSYRKQV
rab5   KLVLLGESAV GKSSLVLRFV KGQFHEFQES TIGAAFLTQTV
          45          55          65          75
       VIDGETCLLD ILDTAGQEEY SAMRDPYMRT GEGFLCVFAI
       CLDDTTVKFE IWDTAGQERY HSLVPMYYRG AQAAIVVYDI
       85          95          105         115
       NNTKSFEDIH QYREQIKRVK DSDDVPMVLV GNKCDLAA-RT
       TNEESFARAK NWVKELQRQA SP-NIVIALS GNKADLANKRA
       125         135         145         155
       VESRQAQDLA RSYGIPYIET SAKTRQGVED AFYTLVREIR
       VDFQEAQSYA DDNSLLFMET SAKTSMNVNE IFMAIAKKLP
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B.

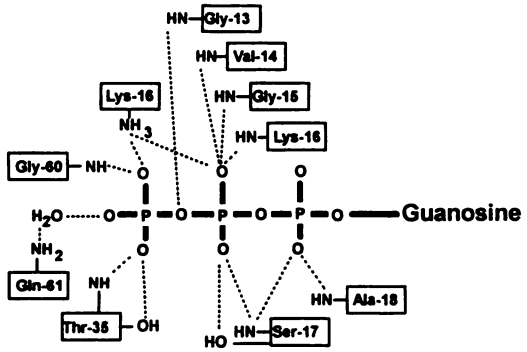

Fig. 5. A, Alignment of truncated amino acid sequences of H-*ras* and *rab5*. *Numbering*, residues in H-*ras*. Alignment is in accordance with Valencia *et al.* (32), with changes from the published sequence of *rab5* as described (11). *Bold*, G-domains. B, Schematic of the most important phosphate-region interactions between GTP and H-*ras*. All nucleotide-protein hydrogen bonds up to a length of 3.2 Å have been included. Modified from the analogue-liganded structures of Kim *et al.* (18) and Pai *et al.* (25).

ferential binding to mutant and wild-type G proteins by guanine nucleotide analogues may occur despite the absence of discriminant binding by native guanine nucleotides (i.e., GTPγS, see above).

Nucleotide-dependent proteolysis. To assess the conformations of G protein induced by guanine nucleotide analogues, nucleotide-dependent proteolysis of *rab5* was examined. We previously described the protection of a 20-kDa proteolytic doublet from further hydrolysis by GDP, GTP, and GTPγS and the appearance of 12- and 8-kDa fragments in the absence of added guanine nucleotide (11). The addition of 10 mM ATP resulted in proteolysis of Rab5 to undetectable fragments, suggesting that ATP effectively interacts with *rab5* wild-type at this concentration and induces a conformation with an increased susceptibility to trypsin compared with the absence of added nucleotides. This is consistent with a recent computer experiment in which the polypeptide backbone of H-*ras* around residue A146 (conserved in *rab5*, see Fig. 5) could not be positioned so to avoid an unfavorable interaction with the N^6-exocyclic amino group of ATP (13). The interpretation of our data as reflecting an ATP-induced, globally altered structure of *rab5* rather than activation of a contaminating ATP-dependent protease is supported by our finding that ATP competes with 1 nM [^{35}S]GTPγS for binding to Rab5 wild-type by filtration assay with an IC$_{50}$ of ~10 mM (not shown). This is comparable to the affinity of ATP relative to that of GTP for H-*ras* determined by a stopped-flow kinetic method (13). Also ruling against the possibility of activation of an ATP-dependent protease is the fact that contaminating ATP should have been sufficiently abundant in the concentrated guanine nucleotide solutions to activate an ATP-dependent protease in the absence of added ATP.

GMP neither induced protection nor promoted hydrolysis relative to the absence of added nucleotide (Fig. 8). However, competition binding revealed that GMP had an affinity for *rab5* (IC$_{50}$ of ~200 μM, not shown) considerably higher than that of ATP. Together, these data suggest that fractional receptor occupancy by 10 mM GMP is high but that GMP induces a conformation similar to that of the apoprotein.

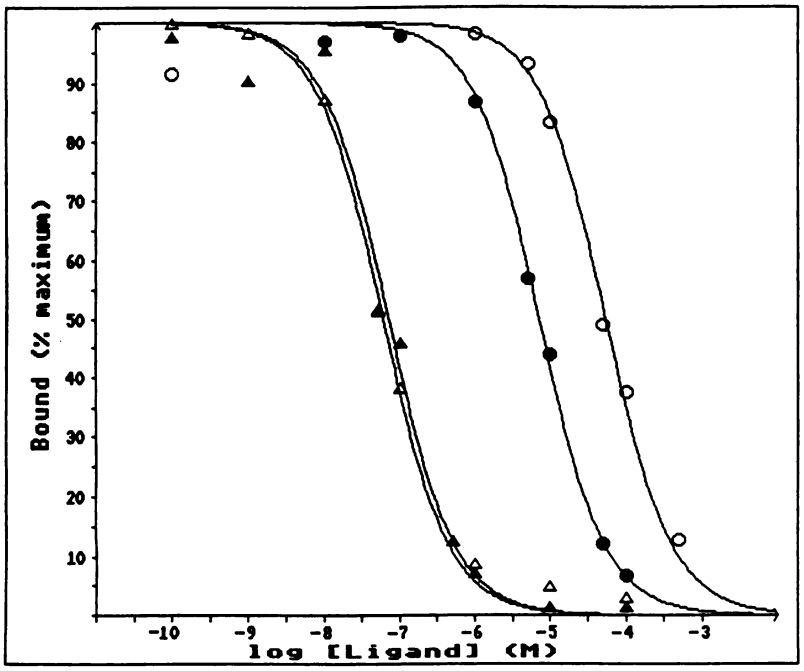

Fig. 6. Competitive binding of GTPγS and $GMPPCF_2P$ to recombinant H-*ras* and *rab5* in solution. Bacterially expressed H-*ras* and *rab5* were incubated with 1.0 nM [^{35}S]GTPγS and varying concentrations of unlabeled guanine nucleotide analogues for 60 min at 30°. Binding was terminated by rapid filtration and filters were counted. Data were plotted by the computer program Enzfitter. ▲ and △, Competition with GTPγS. ● and ○, Competition with $GMPPCF_2P$. △ and ○, H-*ras*. ▲ and ●, *rab5* wild-type.

TABLE 1

IC_{50} values of GTP and $GMPPCF_2P$ for [α-^{32}P]GTP binding to lung small G proteins

	IC_{50}	
	GTP	$GMPPCF_2P$
	nM	μM
26 kDa	27.5	2.7
25 kDa	22.9	0.8
24 kDa	43.7	33.5
23 kDa	7.2	44.0

IC_{50} values were defined as the concentrations of unlabeled nucleotides yielding 50% reductions in autoradiographic density of individual [α-^{32}P]GTP-labeled bands and were calculated with use of the computer program Enzfitter with data obtained from densitometric scanning of representative autoradiograms generated as described in Fig. 3 legend.

Among the guanine nucleoside triphosphates, only GTP induced a conformation that protected *rab5* from any apparent proteolysis at a trypsin/*rab5* ratio of 1:50 (Fig. 8B). The rank order of protection of *rab5* from proteolysis to the lower fragment in the 20-kDa doublet and to the 12- and 8-kDa fragments was as follows: GTP > $GMPPCF_2P$ > GTPγS > GDP = GMPPNHP > $GMPPCH_2P$ (Fig. 8, A and B). Thus, despite the lower affinity of $GMPPCF_2P$ compared with GTPγS, GDP, or GMPPNHP, this analogue protected *rab5* from proteolysis more effectively. Furthermore, despite the similar affinities and discriminant binding patterns of $GMPPCF_2P$ and $GMPPCH_2P$, $GMPPCF_2P$ was considerably more effective in protecting *rab5*.

Discussion

Previous work by us (6, 9) and others (17, 18, 22) demonstrated that guanine nucleotides could be substantially derivatized with retention of high affinity binding to G proteins. To test the feasibility of synthesizing protein-specific guanine nucleotide analogues, we developed a sensitive and highly reproducible screening assay for differential binding to blotted small G proteins. Using this assay, we determined that a 25-kDa lamellar body protein is preferentially bound by βγ-methylene guanine nucleotide analogues (Figs. 3 and 4). The identity of the 25-kDa protein is unknown, and it is possible that more than one molecular species is present in the 25-kDa band. In that case, the 25-kDa protein with high affinity binding must have an even higher affinity relative to the 23- and 24-kDa proteins than the ~40-fold measured because the apparent competition would be attenuated by the nondiscriminant G protein(s). The differential affinity of *rab5* and H-*ras* for the $GMPPCF_2P$ is less dramatic than that of the lamellar body proteins. However, *rab5* and H-*ras* were used only because of their extensive prior characterization and ready availability, and there was no screening of a wider group of small G proteins for differential binding. The identification of discriminant binding on two such limited searches suggests that more systematic analysis would yield substantially more specific ligands.

The fact that βγ-methylene analogues were discriminant in both assays described in this work and the finding that fluoroaluminate activates trimeric G proteins but not *ras*-related G proteins (23) are consistent with recently determined structural differences among G proteins in the β,γ-phosphate binding region (3, 24). Although certain P-loop residues, such as Lys^{16} of H-*ras* which hydrogen-bonds with both the β and γ phosphates (Fig. 5), appear to be invariant, differences in H-*ras* and *rab5* at amino acids 13 and 18, both of which hydrogen bond with the nucleotide phosphates, should generate a local configuration in *rab5* that differs from that of H-*ras*. Most substitutions at position 12 or 13 in H-*ras*, both of which are occupied by glycine, result in a reduced GTPase activity and a transforming phenotype (19), and *rab5* differs at both positions. It is also apparent that substitutions in other regions may affect nucleotide phosphate interactions because the Q79L mutation of *rab5* altered binding of $GMPPCF_2P$ compared with *rab5* wild-type (Fig. 7). Trimeric G proteins contain a structural element that hydrogen bonds with the γ-phosphate and both bridging oxygens that has no counterpart in small G proteins (3, 24).

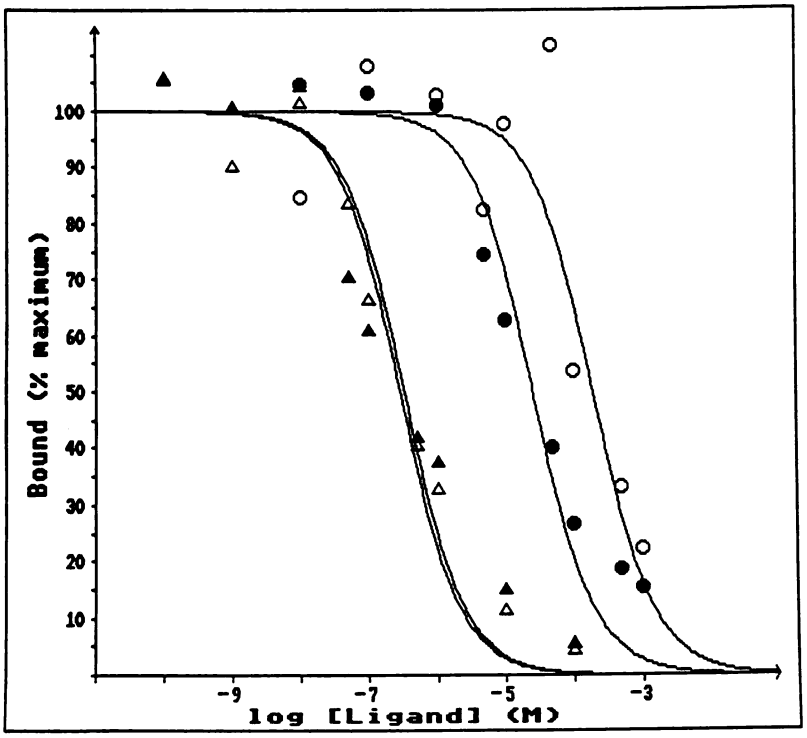

Fig. 7. Competitive binding of GTPγS and $GMPPCF_2P$ to recombinant *rab5* wild-type and *rab5* Q79L in solution. Binding was carried out exactly as in Fig. 5. ▲ and △, Competition with GTPγS. ● and ○, Competition with $GMPPCF_2P$. △ and ○, *rab5* Q79L. ▲ and ●, *rab5* wild-type.

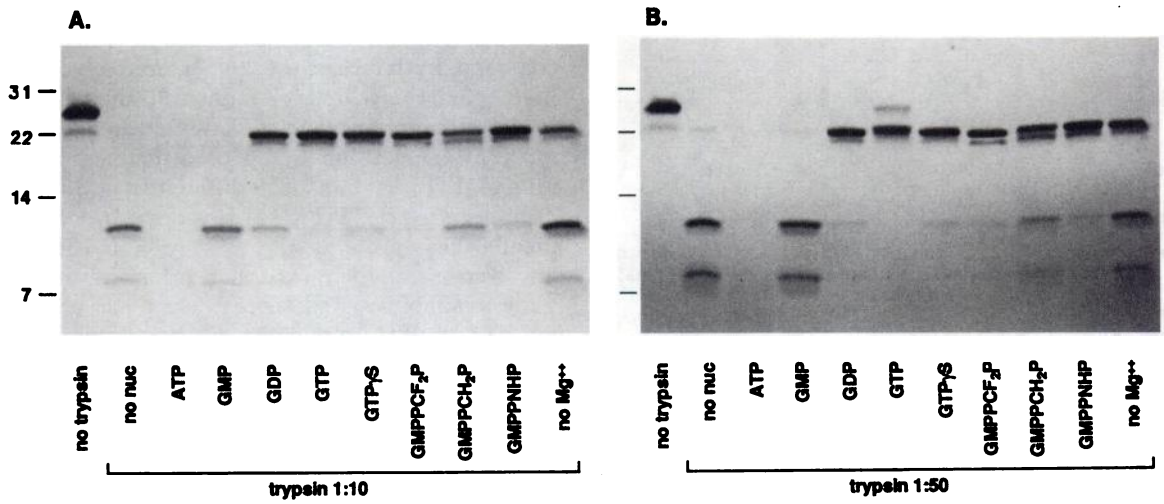

Fig. 8. Protection of *rab5* from limited proteolysis by guanine nucleotide analogues. Purified recombinant *rab5* wild-type was incubated with trypsin in the presence of the indicated nucleotide analogues (10^{-2} M), 5 mM $MgCl_2$, and 1 mM EDTA, except where indicated. *First lanes*, without trypsin; *last lanes*, in the presence of 10^{-2} M GDP without added magnesium and with 1 mM EDTA. A, Trypsin/*rab5* (w/w), 1:10. B. Trypsin/*rab5* (w/w), 1:50.

The affinity of the β,γ-methylene analogues for all of the small G proteins in our study was reduced ~100-fold compared with the affinities of native nucleotides, and the affinity of GMPPNHP was intermediate, similar to the results of others (7, 25). The rank order of affinities of these nucleotides parallels the ability of the bridging anhydride oxygen, nitrogen, or carbon to hydrogen bond with G13 of H-*ras* or the cognate amino acid in other G proteins. These findings suggest that disruption of this hydrogen bond is central to the reduced affinity of the analogues. Consistent with this idea, the hydrogen bond between G13 and the β,γ-phosphate bridging atom is 3.1 Å in the H-*ras*/GMPPNHP structure (26) but 3.3 Å in the H-*ras*/$GMPPCH_2P$ structure (19), and G13 was hydrogen-bonded to the β phosphate in 2/4 molecules in the $GMPPCH_2P$ structure. However, the selectivity displayed by the β,γ-methylene analogues is unlikely simply due to a reduced dependence of some G proteins on hydrogen bonding with the β,γ bridging atom since GMPPNHP did not display comparable selectivity. Instead, selectivity is likely to be related to changes in the geometry of the β and γ phosphates in the methylene analogues relative to the native triphosphate group. However, opposite effects on P—O bond lengths and C—C—P bond angles have been observed in crystallographic structures of a methylenephosphonate and a difluoromethylenephosphonate (27, including references), making it impossible to determine at present what conformational changes in the β,γ-methylene series of synthetic ligands are responsible for protein specificity.

In addition to binding affinity, the capacity of a ligand to influence the activity state of a target protein depends on its ability to influence the protein's conformation when bound. We assessed the nucleotide-dependent conformations of *rab5* using limited proteolysis. None of the triphosphate guanine nucleoside analogues were able to induce the distinctive GTP-liganded pattern of apparently complete protease protection at a trypsin ration of 1:50 (Fig. 8B), suggesting that they induce this conformation with less efficacy than GTP or are unable to induce it. However, another hallmark of protection induced by GTP is reduced appearance of 12- and 8-kDa bands compared with GDP, and by this criterion $GMPPCF_2P$ exceeded protection by the other analogues, including GTPγS (Fig. 8A). The β,γ-difluoromethylene substitution was found to yield isosteric and isopolar analogues of ATP and GTP with metal binding characteristics similar to those of the native nucleotides (8), and these properties probably account for the ability of $GMPPCF_2P$ to induce the GTP conformation of *rab5* disproportionate to its relative affinity. This analogue might be thought of as a *rab5* agonist of low potency but high efficacy. In contrast, $GMPPCH_2P$ protected *rab5* poorly, consistent with NMR analyses that identified multiple differences in structure between H-*ras*/$GMPPCH_2P$ and the structures of H-*ras* liganded with GTP, GTPγS, or GMPPNHP (28, 29) and also consistent with the substantial structural and metal-binding differences noted between β,γ-methylene analogues and native nucleotides (8, 30). GMP failed to induce a trypsin-susceptible conformation distinct from that of apo-*rab5* (Fig. 8). This is similar to the failure of GMP or guanosine to induce the isomerization of H-*ras*, which is characteristic of the second slow step of GDP binding, even though they undergo the weak initial step of binding and stabilize H-*ras* against denaturation during storage (15). It is also consistent with the inhibition by GMP of adenylyl cyclase activation by GMPPNHP (31). Taken together, GMP may be viewed as a neutral antagonist of *rab5*, H-*ras*, and G_s, and it may prove to be useful in stabilizing an "empty" G protein structure for crystallization.

In summary, on a very limited search, we empirically identified a guanine nucleotide analogue with relatively high protein specificity. This result indicates that use of guanine nucleotide analogues in biochemical assays should take into account the possibility of differential protein binding or efficacy, as was recently noted for a photoreactive GTP analogue (32). This result also suggests that systematic modification of guanine nucleotides, nucleosides, and bases, based on empirical and structural data and taking advantage of nonconserved regions, might yield useful discriminant analogues. Some G proteins appear to shuttle between two activity states and can be locked in either an "off" or "on" state by metabolically stable nucleotide analogues, bacterial toxins, or mutations grossly affecting nucleotide exchange or hydrolysis (e.g., *ras*, trimeric G proteins, *rho*), whereas other G proteins apparently must cycle in a precisely timed fashion and can be locked only in an "off" state (e.g., *EF-Tu*, *rab*, signal recognition particle). The options for influencing the activity of an individual G proteins will therefore be determined by the manner in which the GTPase cycle is used by that G protein.

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References

1. Bourne, H. R., D. A. Sanders, and F. McCormick. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.)* **349:**117–127 (1991).
2. Dickey, B. F., and L. Birnbaumer, eds. *GTPases in Biology*. Springer-Verlag, Heidelberg (1993).
3. Wittinghofer, A. The structure of $G_{\alpha t}$: more to view than just ras. *Cell* **76:**201–204 (1994).
4. Joyce-Brady, M., J. B. Rubins, M. P. Panchenko, J. Bernardo, M. P. Steele, L. Kolm, E. R. Simons, and B. F. Dickey. Mechanisms of mastoparan-stimulated surfactant secretion from isolated pulmonary alveolar type 2 cells. *J. Biol. Chem.* **266:**6859–6865 (1991).
5. Northup, J. K., M. D. Smigel, and A. G. Gilman. The guanine nucleotide activating site of the regulatory component of adenylate cyclase: identification by ligand binding. *J. Biol. Chem.* **257:**11416–11423 (1982).
6. Kelleher, D. J., L. W. Dudycz, G. E. Wright, and G. L. Johnson. Ability of guanine nucleotide derivatives to bind and activate bovine transducin. *Mol. Pharmacol.* **30:**603–607 (1986).
7. Arabshahi, L., N. Khan, M. Butler, T. Noonan, N. Brown, and G. Wright. (Difluoromethylene)phosphates of guanine nucleotides as probes of DNA polymerases and G proteins. *Biochemistry* **29:**6820–6826 (1990).
8. Blackburn, G. M., D. E. Kent, and F. J. Kolkmann. The synthesis and metal binding characteristics of novel, isopolar phosphate analogues of nucleotides. *J. Chem. Soc. Perkins Trans. I.* 1119–1125 (1984).
9. Noonan, T., N. Brown, L. Dudycz, and G. E. Wright. Interaction of GTP derivatives with cellular and oncogenic ras-p21 proteins. *J. Med. Chem.* **34:**1302–1307 (1991).
10. Dexter, D., J. B. Rubins, E. C. Manning, L. Khachatrian, and B. F. Dickey. Compartmentalization of low molecular mass GTP-binding proteins among neutrophil secretory granules. *J. Immunol.* **145:**1845–1850 (1990).
11. Hoffenberg, S., J. S. Sanford, S. Liu, D. S. Daniel, M. Tuvin, B. J. Knoll, M. Wessling-Resnick, and B. F. Dickey. Biochemical and functional characterization of a recombinant GTPase, Rab5, and two of its mutants. *J. Biol. Chem.* **270:**5048–5056 (1995).
12. Goody, R. S., M. Frech, and A. Wittinghofer. Affinity of guanine nucleotide binding proteins for their ligands: facts and artifacts. *Trends Biochem. Sci.* **16:**327–328 (1991).
13. Rensland, H., J. John, R. Linke, I. Simon, I. Schlichting, A. Wittinghofer, and R. S. Goody. Substrate and product structural requirements for binding of nucleotide to H-ras p21: the mechanism of discrimination between guanosine and adenosine nucleotides. *Biochemistry* **34:**593–599 (1995).
14. Ojo-Amaize, E. A., B. Rubalcava, T. L. Avery, H. B. Cottam, S. S. Matsumoto, W. B. Jolley, and R. K. Robins. Activation of the respiratory burst in murine phagocytes by certain guanine ribonucleosides modified at the 7 and 8 positions: possible involvement of a pertussis toxin-sensitive G-protein. *Immunol. Lett.* **23:**173–178 (1990).
15. Hingorani, V. T., L.-F. H. Chang, and Y.-K. Ho. Chemical modification of bovine transducin: probing the GTP-binding site with affinity analogues. *Biochemistry* **28:**7424–7432 (1989).
16. John, J., R. Sohmen, J. Feuerstein, R. Linke, A. Wittinghofer, and R. S. Goody. Kinetics of interaction of nucleotides with nucleotide-free H-ras p21. *Biochemistry* **29:**6058–6065 (1990).
17. Neal, S., J. F. Eccleston, and M. R. Webb. Hydrolysis of GTP by $p21^{NRAS}$, the *NRAS* protooncogene product, is accompanied by a conformational change in the wild-type protein: use of a single fluorescent probe at the catalytic site. *Proc. Natl. Acad. Sci. USA* **87:**3562–3565 (1990).
18. Hoyer, P. B., P. Fletcher, and B. E. Haley. Synthesis of 2′,3′-O-(2,4,6-trinitrocycloheadienylidine) guanosine 5′-triphosphate and a study of its inhibitory properties with adenylate cyclase. *Arch. Biochem. Biophys.* **245:**369–378 (1986).
19. Kim, S.-H, G. G. Privé, and M. V. Milburn. Conformational switch and structural basis for oncogenic mutations of Ras proteins, in *GTPases in Biology* (B. F. Dickey and L. Birnbaumer, eds.). Springer-Verlag, Heidelberg, 177–194 (1993).
20. Stenmark, H., A. Valencia, O. Martinez, O. Ullrich, B. Goud., and M. Zerial. Distinct structural elements of rab5 define its functional specificity. *EMBO J.* **13:**575–583 (1994).
21. Kabcenell, A. K., B. Boud, J. Northup, and P. J. Novick. Binding and hydrolysis of guanine nucleotides by Sec4p, a yeast protein involved in the regulation of vesicular traffic. *J. Biol. Chem.* **265:**9366–9372 (1990).
22. Hiratsuka, T. A chromophoric and fluorescent analogue of GTP, 2′,3′-O-(2,4,6-trinitrocycloheadienylidene)-GTP, as a spectroscopic probe for the GTP inhibitory site of liver glutamate dehydrogenase. *J. Biol. Chem.* **260:**4784–4790 (1985).
23. Kahn, R. Fluoride is not an activator of the smaller (20–25 kDa) GTP-binding proteins. *J. Biol. Chem.* **266:**15595–15597 (1991).
24. Coleman, D. E., A. M. Berghuis, E. Lee, M. E. Linder, A. E. Gilman, and S. R. Sprang. Structures of active conformations of $G_{i\alpha 1}$ and the mechanism of GTP hydrolysis. *Science (Washington D. C.)* **265:**1405–1412 (1994).

25. John, J., I. Schlichting, E. Schiltz, P. Rosch, and A. Wittinghofer. C-terminal truncation of $p21^{H}$ preserves crucial kinetic and structural properties. *J. Biol. Chem.* **264**:13086–13092 (1989).
26. Pai, E. F., U. Krengel, G. A. Petsko, R. S. Goody, W. Kabsch, and A. Wittinghofer. Refined crystal structure of the triphosphate conformation of H-*ras* p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. *EMBO J.* **9**:2351–2359 (1990).
27. Chambers, R. D., D. O'Hagan, R. B. Lamont, and S. C. Jain. The difluoromethylenephosphonate moiety as a phosphate mimic: X-ray structure of 2-amino-1,1-difluoromethylphosphonic acid. *J. Chem. Soc. Chem. Commun.* **15**:1053–1054 (1990).
28. Miller, A. F., C. J. Halkides, and A. G. Redfield. An NMR comparison of the changes produced by different guanosine 5′-triphosphate analogues in wild-type and oncogenic mutant $p21^{ras}$. *Biochemistry* **32**:7367–7376 (1993).
29. Campbell-Burk, S. L., and T. E. Van Aken. Analysis of ras structure and dynamics by nuclear magnetic resonance, in *GTPases in Biology* (B. F. Dickey and L. Birnbaumer, eds.). Springer-Verlag, Heidelberg, 213–234 (1993).
30. Yount, R. G., D. Babcock, W. Ballantyne, and D. Ojala. Adenylyl imidodiphosphate, an adenosine triphosphate analogue containing a P-N-P linkage. *Biochemistry* **10**:2484–2489 (1971).
31. Lad, P. M., T. B. Nielsen, M. S. Preston, and M. M. Rodbell. The role of the guanine nucleotide exchange reaction in the regulation of the β-adrenergic receptor and in the actions of catecholamines and cholera toxin on adenylate cyclase in turkey erythrocytes. *J. Biol. Chem.* **255**:988–995 (1980).
32. Fields, T. A., M. E. Linder, and P. J. Casey. Subtype-specific binding of azidoanilido-GTP by purified G protein α subunits. *Biochemistry* **33**:6877–6883 (1994).
33. Valencia, A., P. Chardin, A. Wittinghofer, and C. Sander. The *ras* protein family: evolutionary tree and role of conserved amino acids. *Biochemistry* **30**:4637–4648 (1991).

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