0026-895X/97/010047-05$3.00/0

MOLECULAR PHARMACOLOGY, **51:**47–51 (1997).

Synthesis and Effect of Nonhydrolyzable Xanthosine Triphosphate Derivatives on Prenylation of Rab5^{D136N}

IVAN YANACHKOV, JULIE Y. PAN, MARIANNE WESSLING-RESNICK, and GEORGE E. WRIGHT

Department of Pharmacology and Molecular Toxicology, University of Massachusetts Medical School, Worcester, Massachusetts 01655 (I.Y., G.E.W.), and Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115 (J.Y.P., M.W.-R.)

Received July 25, 1996; Accepted September 25, 1996

SUMMARY

A novel and convenient method for nucleoside triphosphate synthesis was applied to the preparation of potentially nonhydrolyzable xanthosine triphosphate derivatives. The *N*-methylimidazolide of xanthosine 5′-monophosphate reacted rapidly with methylenediphosphonic acid and imidodiphosphonic acid to give xanthosine 5′-(β,γ-methylene)triphosphate and xanthosine 5′-(β,γ-imido)triphosphate, respectively, in good yields. Both compounds inhibited the xanthosine-diphosphate-dependent prenylation of a mutant of Rab5, Rab5^{D136N}, the nucleotide specificity of which had been converted from GTP to xanthosine triphosphate. The results indicate that xanthosine 5′-(β,γ-methylene)triphosphate and xanthosine 5′-(β,γ-imido)-triphosphate bound to the mutant protein with similar affinities and were not hydrolyzed under the assay conditions. These novel derivatives may be useful tools for the study of the role of individual GTPases mutated to xanthosine triphosphate specificity in the background of other GTP-binding proteins.

Despite the fact that small-molecular-weight GTPases regulate a diverse set of biological functions, the proteins share highly conserved guanine nucleotide binding domains. X-ray crystallographic studies of the elongation factor EF-Tu showed that Asp138 interacts with the guanine base of GDP by accepting hydrogen bonds from N1—H and the 2-NH_2 group (1, 2). In support of this model, point mutation of Asp138 to Asn138 greatly reduced the affinity of the protein for GDP but dramatically increased affinity for XDP (3). It is likely that Asn138 in mutant EF-Tu interacts with the base by donating a hydrogen bond to the 2-oxo group and accepting a hydrogen bond from the N1—H of xanthine (Fig. 1). Because Asp138 of EF-Tu is highly conserved, the aspartate-to-asparagine mutation in other GTPases is predicted to alter the nucleotide binding specificities of such mutants as well. Indeed, several GTPases with the aspartate-to-asparagine mutation have been reported to have high affinity and selectivity for binding xanthosine nucleotides (4–10).

Rab proteins are a family of Ras-like small-molecular-weight G proteins that are localized to distinct subcellular compartments and are implicated in intracellular membrane trafficking. One member of this family, Rab5, is localized on the plasma membrane, clathrin-coated vesicles, and early endosomes (11). Overexpression of Rab5 stimulates the rate of endocytosis and early endosome fusion (12–14). Rab5 is post-translationally modified with two geranylgeranyl (prenyl) groups on the two cysteines closest to its carboxyl terminus (15). This modification by prenylation is required for the membrane attachment and function of the protein (12, 16, 17).

Previous studies have shown that *in vitro* Rab5 prenylation in reticulocyte lysate was inhibited by nonhydrolyzable GTP analogs and that two point mutants of Rab5, which either preferentially bound GTP or had reduced GTPase activity, exhibited reduced rates of prenylation (18). These results indicate that only the GDP-bound form of Rab5 is the substrate for geranylgeranylation. Furthermore, when a cognate mutant of EF-Tu^{D138N}, Rab5^{D136N}, was constructed, its prenylation was dependent on the concentration of XDP instead of GDP, indicating that the nucleotide binding specificity was switched from guanosine to xanthosine nucleotides (9).

Given the possibility that nonhydrolyzable XTP analogs might inhibit prenylation of Rab5^{D136N} and allow studies of Rab5-dependent processes independently of other GTPases, we undertook the synthesis of XTP derivatives that would be expected to be insensitive to the XTPase activities of the mutant Rab5. We report a simple and superior method to

This work was supported by American Cancer Society Research Grant CB-15 to M.W.-R., who is an established investigator of the American Heart Association.

I. Y. and J. Y. P. contributed equally to this article.

ABBREVIATIONS: XDP, xanthosine diphosphate; XMPPCH$_2$P, xanthosine 5′-(β,γ-methylene)triphosphate; XMPPNHP, xanthosine 5′-(β,γ-imido)triphosphate; XMP, xanthosine monophosphate; XTP, xanthosine triphosphate; TEAB, triethylammonium bicarbonate buffer; DMF, *N,N*-dimethylformamide; HR, high-resolution; FAB, fast-atom bombardment.

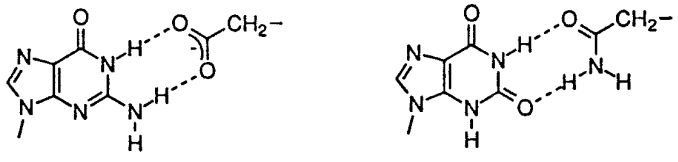

Fig. 1. Hydrogen-bonding schemes for purine ring binding of wild-type Rab5 and Rab5^{D136N}. *Left*, hydrogen bonding between guanine and wild-type Rab5. *Right*, hydrogen bonding between xanthine and Rab5^{D136N}.

prepare nucleoside triphosphate derivatives and its application to the synthesis of the xanthosine derivatives XMPPCH$_2$P and XMPPNHP. As anticipated, these compounds are potent inhibitors of XDP-dependent Rab5^{D136N} prenylation in reticulocyte lysate.

Experimental Procedures

Materials. Xanthosine phosphates (XMP, XDP, and XTP) were purchased from Sigma Chemical (St. Louis, MO), and geranylgeranyl pyrophosphate was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were from Aldrich Chemical (Milwaukee, WI); DMF and *N*-methylimidazole were Sure-seal. TEAB was prepared by bubbling CO_2 gas through a mixture of water and freshly distilled triethylamine to pH 7.7. Glass double-distilled water was used throughout.

^{1}H and ^{31}P NMR spectra were recorded on a Varian Unity 300 instrument (Palo Alto, CA). Chemical shifts (δ, ppm) are referenced from internal 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (^{1}H) and external 85% phosphoric acid (^{31}P). Nucleoside triphosphate samples for NMR were freed from traces of paramagnetic metal impurities by passing through a Chelex 100 column (BioRad, Hercules, CA) in the sodium form (0.5 × 2 cm), elution with 2 ml of water, and lyophilization. HR FAB mass spectra were obtained in the negative ion mode by the Washington University Resource for Biomedical and Bioorganic Mass Spectrometry (St. Louis, MO).

XMPPCH$_2$P. A solution of XMP sodium salt trihydrate (50 mg, 0.1 mmol) in water (1 ml) was passed through a 20- × 1-cm column of Dowex 50WX8 in the pyridinium form, and eluted with 2 column volumes of water. Tri(*n*-butyl)amine (48 μl, 0.2 mmol) was added to the eluate, followed by *iso*propyl alcohol until the mixture became homogeneous. The solution was concentrated under vacuum and lyophilized. The residue was dissolved in dry DMF (0.5 ml) in a 15-ml screw-capped centrifuge tube under N_2. A solution of triphenylphosphine (79 mg, 0.3 mmol), 2,2′-dipyridyl disulfide (69 mg, 0.3 mmol), and *N*-methylimidazole [41 mg (40 μl), 0.5 mmol] in dry DMF (0.3 ml) was added. After 6 min, the *N*-methylimidazolide of XMP was precipitated by addition of cold dry diethyl ether (10 ml), and the mixture was centrifuged. The ether was decanted, and the solid was resuspended in 10 ml of ether, centrifuged and decanted again, and the residue was dried briefly in a gentle stream of N_2.

Tri(*n*-butyl)ammonium methylenediphosphonate [prepared by neutralization of a solution of 88 mg (0.5 mmol) of methylenediphosphonic acid in water (2 ml) with tri(*n*-butyl)amine (0.283 ml, 1 mmol) followed by lyophilization] was dissolved in dry DMF (1 ml), and the solution was added to the activated XMP. After vortexing for 5 min, the mixture was homogeneous. After 30 min, the reaction mixture was diluted with 20 ml of cold 0.1 M TEAB, loaded on a DEAE-Sephadex column (2 × 20 cm), and eluted with a linear gradient of 0.1–1.0 M TEAB during 16 hr at a flow rate of 2.67 ml/min. Fractions 83–91 (16 ml each) were combined and evaporated under vacuum, and the residue was redissolved in a small volume of water and lyophilized to give 47.3 mg (51%) of XMPPCH$_2$P as the triethylammonium salt. Side fractions were combined and evaporated, and a second chromatography of the residue on DEAE-Sephadex afforded an additional 13.0 mg (14%) of product. The triethylammonium salt was converted to the sodium salt by passing through a column of Dowex 8X50 in the Na^+ form. [^{1}H NMR (D_2O) δ 7.94 (s, 1H, 8-H), 5.78 (d, 1 H, H-1′; $J_{1'2'}$ = 6.0 Hz), 4.52 (t, 1 H, H-2′, $J_{1'2'} \approx J_{2'3'} \approx$ 5.6 Hz), 4.40 (dd, 1 H, H-3′, $J_{2'3'}$ = 5.2 Hz, $J_{3'4'}$ = 3.6 Hz), 4.20 (m, 1 H, H-4′), 4.09 (ddd, 1 H, H-5′, $J_{4'5'}$ = 2.9 Hz, $J_{5'5''}$ = 11.6 Hz, $J_{5'P1}$ = 6.2 Hz), 4.03 (ddd, 1 H, H-5″, $J_{4'5''}$ = 3.3 Hz, $J_{5'5''}$ = 11.6 Hz, $J_{5''P1}$ = 4.7 Hz), 2.04 (dd, 2H, PC**H**$_2$P, J_{HP2} = 21.2 Hz, J_{HP3} = 19.0 Hz); ^{31}P NMR (D_2O) δ 14.15 (ddt, ^{1}H dec. dd, P^2, J_{P2P3} = 7.4 Hz, J_{P2P1} = 27.0 Hz, J_{P2H} = 21.2 Hz), 12.19 (dt, ^{1}H dec., d, P^3, J_{P2P3} = 7.4 Hz, J_{P3H} = 18.9 Hz), −10.12 (m, ^{1}H dec., d, P^1, J_{P1P2} = 27.0 Hz).] HR FAB mass spectra calculated for $[C_{11}H_{13}N_4O_{14}P_3Na_4+H\text{-}2Na]^-$, 546.9515; found, 546.9517.

XMPPNHP. A solution of tetrasodium imidodiphosphate (9.5 hydrate; 218 mg, 0.5 mmol) in ice cold water (2 ml) was passed through a column of Dowex 50WX8 in the H^+ form and eluted with ice cold water until the eluate was neutral. Tri(*n*-butyl)amine (0.476 ml, 2.0 mmol) was added immediately to the ice cold eluate, and *i*-PrOH was added until the mixture became homogeneous. The solution was concentrated under high vacuum at room temperature and lyophilized. This tri(*n*-butyl)ammonium salt was dissolved in dry DMF (1 ml), and the solution was added to the *N*-methylimidazolide of XMP, prepared as described above (0.1 mmol). The mixture was vortexed until it became homogeneous (about 3 min). After 30 min, the reaction mixture was diluted with 20 ml of ice cold 0.1 M TEAB, loaded on a DEAE-Sephadex column (2 × 20 cm), and eluted at 4° with a linear gradient of 0.1–1.0 M TEAB during 16 hr at a flow rate of 2.67 ml/min. Fractions 74–86 (16 ml each) were combined and evaporated under high vacuum at room temperature. The residue was dissolved in a small amount of water and lyophilized to give XMPPNHP as the triethylammonium salt (21.6 mg, 35% yield). This product was converted to the sodium salt by passing an aqueous solution through a column of Dowex 50WX8 in the Na^+ form. [^{1}H NMR (D_2O) δ 7.92 (s, 1 H, 8-H), 5.75 (d, 1 H, H-1′; $J_{1'2'}$ = 5.9 Hz), 4.49 (t, 1 H, H-2′, $J_{1'2'} \approx J_{2'3'} \approx$ 5.6 Hz), 4.41 (dd, 1 H, H-3′, $J_{2'3'}$ = 5.3 Hz, $J_{3'4'}$ = 3.6 Hz), 4.16 (m, 1 H, H-4′), 4.09 (ddd, 1 H, H-5′, $J_{4'5'}$ = 3.1 Hz, $J_{5'5''}$ = 11.7 Hz, $J_{5'P1}$ = 6.7 Hz), 4.03 (ddd, 1 H, H-5″, $J_{4'5''}$ = 3.3 Hz, $J_{5'5''}$ = 11.7 Hz, $J_{5''P1}$ = 4.7 Hz); ^{31}P NMR (D_2O) δ −0.08 (d, P^3, J_{P2P3} = 4.4 Hz), −6.95 (dd, P^2, J_{P1P2} = 20.8 Hz, J_{P2P3} = 4.6 Hz), −9.94 (m, ^{1}H dec., d, P^1, J_{P1P2} = 20.9 Hz). HR FAB mass spectra calculated for $[C_{10}H_{12}N_5O_{14}P_3Na_4+H\text{-}2Na]^-$, 565.9455; found, 565.9467.

***In vitro* prenylation of Rab5^{D136N}.** A point mutant of Rab5, Rab5^{D136N}, was constructed in the transcription competent vector, pAGA, as described previously (9). Rab5^{D136N} was synthesized *in vitro* using rabbit reticulocyte lysate programmed with the Rab5^{D136N} transcripts in the presence of [^{35}S]methionine (9.7×10^{17} cpm/pmol) according to the method described in Ref. 19. [^{35}S]-labeled Rab5^{D136N} (1 nM) was prenylated in 10 μl of geranylgeranylation mixture (12 mM Tris, pH 7.5, 0.6 mM dithiothreitol, 3 mM $MgCl_2$, 45% rabbit reticulocyte lysate, 20 μM geranylgeranyl pyrophosphate) containing various amounts of xanthine nucleotides (XDP, XTP, XMPPCH$_2$P, or XMPPNHP). Reactions were conducted at 37° for 1 hr; parallel reactions on ice were used as controls for nonmodified protein. The reactions were stopped by addition of Laemmli sample buffer, and the entire reaction mixtures were electrophoresed through a urea/acrylamide gradient sodium dodecyl sulfate gel (19). The gel was processed for fluorography, dried, and exposed to film. For quantitative analysis of the extent of protein prenylation, gel slabs corresponding to each of the ^{35}S-labeled protein bands were sliced off using the fluorograph as a guide. The gel slices were solubilized in 30% H_2O_2 by incubation at 65° overnight, and 20 ml of Scintiverse II (Fisher Scientific, Fair Lawn, NJ) was added for counting. The percentage of prenylated Rab5^{D136N} in each reaction was calculated as the ratio of radioactivity in the prenylated (lower) band to the sum of radioactivity in the prenylated and unprenylated (upper) bands after background was subtracted from each band. Apparent K_D values were calculated from IC_{50} values as described (20).

Results

***N*-Methylimidazolides in nucleoside triphosphate synthesis.** The most widely cited procedure for synthesis of nucleoside triphosphates and their modified derivatives involves activation of the monophosphates to the corresponding imidazolides with carbonyldiimidazole, followed by condensation of the activated intermediate with pyrophosphate or modified pyrophosphates (21). This procedure is lengthy (2–4 hr for activation and 12–24 hr for the condensation step) and results in partial modification of the 2′- and 3′-hydroxyl groups of ribonucleotides to 2′,3′-cyclic carbonates (22). These disadvantages prompted a search for a new method applicable to nucleoside triphosphate synthesis. It is known that *N*-methylimidazolides of carboxylic acids are more active acylating agents than the imidazolides (23), and it was reported recently that *N*-methylimidazolides of nucleoside monophosphates and oligonucleotides reacted readily with amines to give phosphoroamidates in high yields (24). A test reaction to apply this activated intermediate to nucleoside triphosphate synthesis was performed with N^2(*p*-butylphenyl)-deoxyGMP (25). Reaction of the monophosphate with triphenylphosphine, 2,2′-dipyridyl disulfide, and *N*-methylimidazole in DMF gave, after precipitation with diethyl ether, an product identified by ^{1}H and ^{31}P NMR as the *N*-methylimidazolide. Stirring of the solid with a solution of tetrabutylammonium pyrophosphate in DMF for 5 min gave, after ion exchange chromatography, a 65% yield of N^2(*p*-butylphenyl)-deoxyGTP, identical with a sample prepared by the classical imidazolide method (25) (results not shown).

To test the utility of this method to prepare β,γ-modified ribonucleoside triphosphates, we generated the *N*-methylimidazolide of xanthosine monophosphate and treated it with both methylenediphosphonic acid and imidodiphosphonic acid (in separate reactions) as the tetrabutylammonium salts. In both cases, condensation was completed within 30 min, as observed by ^{31}P NMR of the reaction mixtures. After workup, ion exchange chromatography on DEAE-Sephadex yielded XMPPCH$_2$P in 65% overall yield and XMPPNHP in 35% overall yield. Both products were converted to their sodium salts and identified by ^{1}H and ^{31}P NMR spectroscopy and FAB mass spectrometry (see Experimental Procedures).

Effect of XTP Derivatives on Prenylation of Rab5^{D136N}. To examine the properties of the XTP derivatives, their ability to inhibit prenylation of Rab^{D136N} was tested. Previous studies (18) have demonstrated that Rab geranylgeranyl transferase preferentially recognizes Rab proteins in the GDP-bound state and that nonhydrolyzable GTP derivatives block prenylation of Rab5 *in vitro*. Using an *in vitro* prenylation assay, we have also shown that modification of Rab5^{D136N} is dependent on XDP rather than GDP, demonstrating the conversion of nucleotide-binding specificity in this mutant (9). This XDP-dependent modification of Rab5^{D136N} is confirmed as illustrated in Fig. 2A. The post-translational modification of Rab5 is demonstrated by the shift to a higher mobility isoform on urea-gradient sodium dodecyl sulfate/polyacrylamide gels (19). As shown in Fig. 2A, Rab5^{D136N} is not prenylated in the absence of XDP, but it becomes completely modified when incubated at 37° for 1 hr in the presence of 100 μM XDP.

Using this *in vitro* prenylation assay, XDP-dependent modification of Rab5^{D136N} was monitored in the presence of XTP, XMPPCH$_2$P, and XMPPNHP. As shown in Fig. 2B, the presence of XTP had little effect on prenylation. This result is expected because this nucleotide can be hydrolyzed to XDP and can support post-translational modification by Rab geranylgeranyl transferase. However, both XMPPCH$_2$P and XMPPNHP inhibited this process in a concentration-dependent manner (Fig. 2B). The inhibitory effects of XMPPCH$_2$P and XMPPNHP show that both derivatives can interact functionally with Rab5^{D136N} but are resistant to the XTPase activity of the protein. For quantitative analysis of the extent of protein prenylation, gel slabs corresponding to each of the [^{35}S]-labeled protein bands were sliced off and solubilized to measure radioactivity. The extent of prenylation was calculated as the ratio between the cpm in the prenylated (lower) band and the sum of the cpm in the prenylated and unprenylated (upper) bands. The extent of prenylation of Rab5^{D136N} as a function of xanthine nucleotide concentration is shown in Fig. 3. From a series of similar experiments, IC$_{50}$ values for XMPPCH$_2$P and XMPPNHP were determined, and

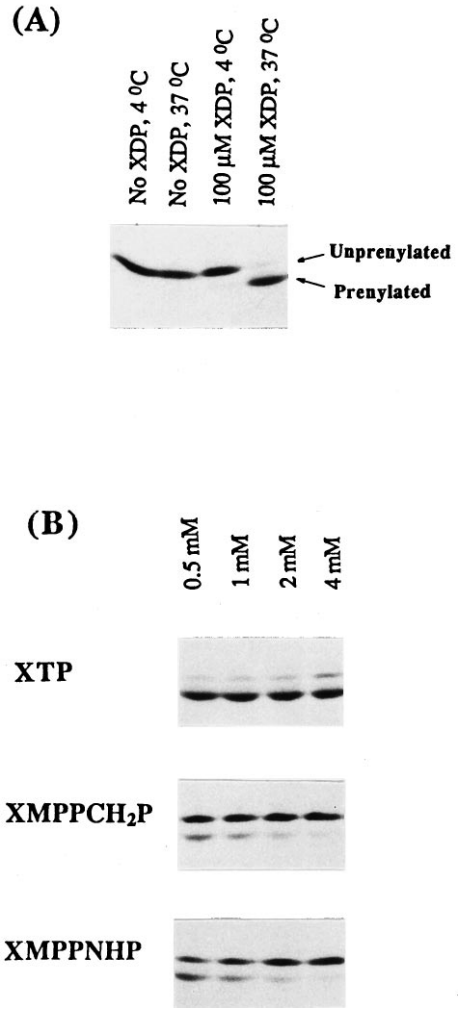

Fig. 2. Effects of xanthine nucleotides on prenylation of Rab5^{D136N}. ^{35}S-Labeled Rab5^{D136N} was synthesized as described in Experimental Procedures. The protein (1 nM final concentration) was incubated in reticulocyte lysate for 1 hr on ice or at 37° in the absence or presence of 100 μM XDP (A) or in the presence of 100 μM XDP and increasing concentrations of XTP, XMPPCH$_2$P, or XMPPNHP (B). Reactions were stopped and electrophoresed as described (19).

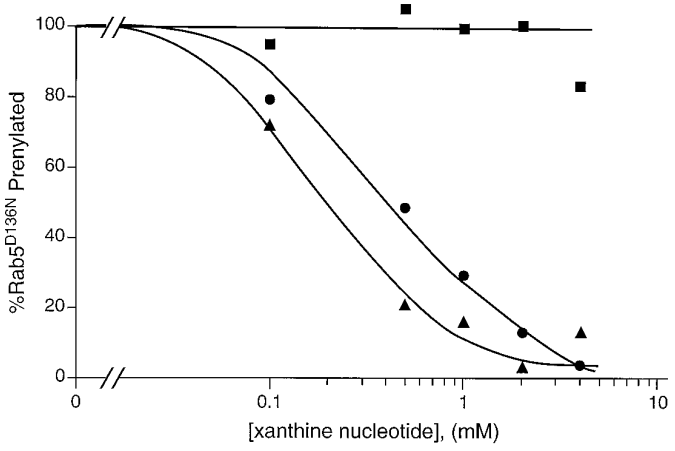

Fig. 3. Inhibition of $Rab5^{D136N}$ prenylation as a function of xanthine nucleotide concentration. ■, XTP; ▲, $XMPPCH_2P$; ●, XMPPNHP. Percentage of $Rab5^{D136N}$ prenylation, determined as described in Materials and Methods, is plotted against xanthine nucleotide concentration. A representative experiment from four repeats is shown.

their respective apparent K_D values for protein binding were calculated (Table 1). Assuming that true affinities of the nucleotides are directly related to the apparent K_D values, $XMPPCH_2P$ and XMPPNHP seem to bind to $Rab5^{D136N}$ with similar affinities.

Discussion

A rapid and convenient method to prepare nucleoside triphosphates and β,γ-modified derivatives has been described. Preparation by a reported method (24) of *N*-methylimidazolides of nucleoside monophosphates and their isolation by diethyl ether precipitation avoids both side reactions and imprecise control of reaction conditions. Coupling of the intermediate with pyrophosphate or pyrophosphate analogs gives the triphosphate derivatives rapidly and in good yields.

The xanthosine 5′-triphosphate derivatives $XMPPCH_2P$ and XMPPNHP were synthesized by the new method and characterized with respect to standard spectroscopic properties (see Experimental Procedures). Both compounds blocked the XDP-dependent prenylation of $Rab5^{D136N}$ in a concentration-dependent manner. The apparent K_D values were surprisingly similar, considering that analogous GTP derivatives (guanosine 5′-(β,γ-methylene)triphosphate and guanosine 5′-(β,γ-imido)triphosphate) typically differ significantly in their affinity to GTP-binding proteins; the imido compound has higher affinity than the methylene compound (26). Our observation that both modified triphosphates displayed persistent inhibition of $Rab5^{D136N}$ prenylation but that XTP, which is hydrolyzed by $Rab5^{D136N}$, did not (Fig. 2), strongly suggests that $XMPPCH_2P$ and XMPPNHP are not subject to hydrolysis by $Rab5^{D136N}$.

GTPases play pivotal roles in a diverse set of important cellular functions. For many of these biological pathways, such as signal transduction, intracellular membrane trafficking, and protein synthesis, multiple GTPases are involved. Thus, it is difficult to dissect the role of GTP hydrolysis by an individual GTPase in the background of many GTP-binding activities. One approach has been to selectively mutate one GTP-binding protein to XTP-binding specificity by conversion of aspartate to asparagine in the conserved NKXD guanine nucleotide-binding motif. This point mutation has been shown to alter nucleotide-binding specificity of several GTPases, including EF-Tu (3), Ras (10), Rab5 (9), Ypt1 (8), HypB (6), FstY (7), and adenylosuccinate synthetase (5). All of these mutants display altered nucleotide-binding specificity and possess XTPase activity. Furthermore, as long as xanthine nucleotide is present, the interaction with regulatory factors and the biological function of the aspartate-to-asparagine mutants remain unaffected (3, 6–8, 10). Such mutants have proven to be powerful tools for studying the mechanism of wild-type protein function (4, 6–8, 27). Such nonhydrolyzable derivatives as $XMPPCH_2P$ and XMPPNHP should prove to be valuable reagents for future studies of other XTP-binding mutants, at least in *in vitro* assay systems, with particular importance in discriminating the function of nucleotide hydrolysis in various biological pathways.

TABLE 1

IC_{50} and apparent K_D values for inhibition of XDP-dependent prenylation and xanthine nucleotide binding to $Rab5^{D136N}$

Experiments as shown in Fig. 2 and 3 were performed to determine the IC_{50} value. The apparent K_D value for each nucleotide was calculated from $IC_{50} = K_D (1 + [XDP]/EC_{50(XDP)})$ (20), where [XDP] = 100 μM and the estimated $EC_{50(XDP)}$ = 50 μM (9). Results are averages of four experiments.

	$XMPPCH_2P$	XMPPNHP
	μM	
IC_{50}	132 ± 34	202 ± 85
Apparent K_D	44 ± 11	67 ± 28

References

1. La Cour, T. F. M., J. Nyborg, S. Thirup, and B. F. C. Clark. Structural details of the binding of guanosine diphosphate to elongation factor Tu from *E. coli* as studied by x-ray crystallography. *EMBO J.* **4:**2385–2388 (1985).
2. Jurnak, F. Structure of the GDP domain of EF-Tu and location of the amino acids homologous to *ras* oncogene Proteins. *Science (Washington D. C.)* **230:**32–36 (1985).
3. Hwang, Y.-W., and D. L. Miller. A mutation that alters the nucleotide specificity of elongation factor Tu, a GTP regulatory protein. *J. Biol. Chem.* **262:**13081–13085 (1987).
4. Weijland, A., and A. Parmeggiani. Toward a model for the interaction between elongation factor Tu and the ribosome. *Science (Washington D. C.)* **259:**1311–1314 (1993).
5. Kang, C., N. Sun, R. B. Honzatko, and H. J. Fromm. Replacement of Asp333 with Asn by site-directed mutagenesis changes the substrate specificity of *Escherichia coli* adenylosuccinate synthetase from guanosine 5′-triphosphate to xanthosine 5′-triphosphate. *J. Biol. Chem.* **269:**24046–24049 (1994).
6. Maier, T., F. Lottspeich, and A. Böck. GTP hydrolysis by HypB is essential for nickel insertion into hydrogenases of *Escherichia coli*. *Eur. J. Biochem.* **230:**133–138 (1995).
7. Powers, T., and P. Walter. Reciprocal stimulation of GTP hydrolysis by two directly interacting GTPases. *Science (Washington D. C.)* **269:**1422–1424 (1995).
8. Jones, S., R. J. Litt, C. J. Richardson, and N. Segev. Requirement of nucleotide exchange factor for Ypt1 GTPase mediated protein transport. *J. Cell Biol.* **130:**1051–1061 (1995).
9. Hoffenberg, S., L. Nikolova, J. Y. Pan, D. S. Daniel, M. Wessling-Resnick, B. J. Knoll, and B. F. Dickey. Functional and structural interactions of the $Rab5^{D136N}$ mutant with xanthine nucleotides. *Biochem. Biophys. Res. Commun.* **215:**241–249 (1995).
10. Zhong, J.-M., M.-C. Chen-Hwang, and Y.-W. Hwang. Switching nucleotide specificity of Ha-Ras p21 by a single amino acid substitution at aspartate 119. *J. Biol. Chem.* **270:**10002–10007 (1995).
11. Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. Localization of low molecular weight GTP-binding proteins to exocytic and endocytic compartments. *Cell* **62:**317–329 (1990).
12. Gorvel, J. P., P. Chavrier, M. Zerial, and J. Gruenberg. Rab5 controls early endosome fusion *in vitro*. *Cell* **64:**915–925 (1991).
13. Bucci, C., R. G. Parton, I. H. Mather, H. Stunnenberg, K. Simons, B. Hoflack, and M. Zerial. The small GTPase Rab5 functions as a regulatory factor in the early endocytic pathway. *Cell* **70:**715–728 (1992).
14. Li, G., and P. D. Stahl. Structure-function relationship of the small GTPase Rab5. *J. Biol. Chem.* **268:**24475–24480 (1993).
15. Kinsella, B. T., and W. A. Maltese. Rab GTP-binding proteins with three

different carboxyl-terminal cysteine motifs are modified *in vivo* by 20-carbon isoprenoids. *J. Biol. Chem.* **267:**3940–3945 (1992).
16. Hoffenberg, S., J. C. 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).
17. Peter, M., P. Chavrier, E. A. Nigg, and M. Zerial. Isoprenylation of Rab proteins on structurally distinct cysteine motifs. *J. Cell Sci.* **102:**857–865 (1992).
18. Sanford, J. C., Y. Pan, and M. Wessling-Resnick. Prenylation of Rab5 is dependent on guanine nucleotide binding. *J. Biol. Chem.* **268:**23773–23776 (1993).
19. Sanford, J. C., L. Foster, Z. Kapadia, and M. Wessling-Resnick. Analysis of the stoichiometry of Rab protein prenylation. *Anal. Biochem.* **224:**547–556 (1995).
20. Cheng, Y., and W. H. Prusoff. Relationship between the inhibition constant (K_I) and the concentration of an inhibitor that causes a 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22:**3099–3108 (1973).
21. Hoard, D. E., and D. G. Ott. Conversion of mono- and oligodeoxyribonucleotides to 5′-triphosphates. *J. Am. Chem. Soc.* **87:**1785–1788 (1965).
22. Maeda, M., A. D. Patel, and A. Hampton. Formation of ribonucleotide 2′,3′-cyclic carbonates during conversion of ribonucleoside 5′-phosphates to diphosphates and triphosphates by the phosphoroimidazolidate procedure. *Nucleic Acids Res.* **4:**2843–2853 (1977).
23. Watkins, B. E., and H. Rapaport. Synthesis of benzyl and benzyloxycarbonyl base-blocked 2′-deoxyribonucleosides. *J. Org. Chem.* **47:**4471–4477 (1982).
24. Zaritova, V. F., T. S. Godovicova, I. V. Kutiavin, and L. M. Khalimskaya. Reactive oligonucleotide derivatives as affinity reagents and probes in molecular biology, in *Biophosphates and Their Analogues: Synthesis, Structure, Metabolism, and Activity*. (K. S. Bruzik and W. J. Stec, eds.). Elsevier, Amsterdam, 149–164 (1987).
25. Wright G. E., and L. W. Dudycz. Synthesis and characterization of N^2-(*p*-n-butylphenyl)-2′-deoxyguanosine 5′-triphosphate and its inhibition of HeLa DNA polymerase alpha. *J. Med. Chem.* **27:**175–181 (1984).
26. Yamanaka, G., F. Eckstein, and L. Stryer. Interaction of retinal transducin with guanosine triphosphate analogues: specificity of the γ-phosphate binding region. *Biochemistry* **25:**6149–6153 (1986).
27. Weijland, A., G. Parlato, and A. Parmeggiani. Elongation factor Tu D138N, a mutant with modified substrate specificity, as a tool to study energy consumption in protein biosynthesis. *Biochemistry* **33:**10711–10717 (1994).

Send reprint requests to: George E. Wright, Ph.D., Department of Pharmacology and Molecular Toxicology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655. E-mail: wright@icarus.ummed.edu