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FUNCTIONAL AND STRUCTURAL INTERACTIONS OF THE RAB5 D136N MUTANT WITH XANTHINE NUCLEOTIDES

Simon Hoffenberg*, Lydia Nikolova*, Julie Y. Pan#, D. Sundarsingh Daniel*, Marianne Wessling-Resnick#, Brian J. Knoll*@, and Burton F. Dickey*@[1]

*Baylor College of Medicine, Departments of Medicine and Cell Biology, Houston, TX 77030

@Houston VA Medical Center, Houston, TX 77030

#Harvard School of Public Health, Department of Nutrition, Boston, MA 02115

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Rab5 is a Ras-related GTPase which regulates endosomal fusion. The D136N mutant of Rab5, which was predicted to switch specificity from guanine to xanthine nucleotides, was expressed in *E. coli*, extracted with urea, purified by column chromatography, and refolded by stepwise dialysis against buffer containing XDP. The purified protein bound xanthine nucleotides with considerably higher affinity than guanine nucleotides. *In vitro* prenylation of the mutant protein was highly dependent on xanthosine diphosphate. In contrast, both the wild type and mutant proteins were protected from proteolysis equally well by non-cognate and cognate triphosphate nucleosides at high concentration. The D136N Rab5 mutant appears to be a valuable reagent in conjunction with xanthine nucleotides for the study of protein-nucleotide interactions in systems in which multiple GTPases are active, although interactions with non-cognate nucleotides should be evaluated if they are present at high concentration.

Broken cell assays which reconstitute specific steps of intercompartmental transport have been powerful tools in the identification of molecular components mediating transport and elucidation of their interactions (1-3). A requirement for guanine nucleotides has been demonstrated in many systems, and several distinct families of GTPases have been shown to regulate vesicle traffic including the Ras-related Rab proteins, the ARF proteins, and the heterotrimeric G-proteins. Because multiple GTPases are active in a single step of vesicle transport, analysis of nucleotide-protein interactions is complicated. In addition, because of the ubiquity of GTPases as intracellular regulatory proteins, it is possible that putative guanine nucleotide-dependent activities of known GTPases could be regulated by currently unknown GTPases.

A mutant of EF-Tu in which ligand specificity was switched from guanine to xanthine nucleotides was previously used to determine the stoichiometry of nucleotide hydrolysis by EF-Tu in protein synthesis (4). This strategy was needed because multiple GTPases participate in nascent polypeptide elongation. The point mutation responsible for the switch in nucleotide specificity of EF-Tu was

[1]Corresponding author. FAX: 713-794-7853.

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present in one of the G-domains which are highly conserved among members of the GTPase superfamily. This strategy should therefore be suitable for other GTPase family members.

Rab5 is a Ras-related GTPase which regulates uncoated vesicle-early endosome and homotypic early endosome fusion (5,6). We have generated a mutant of Rab5 with predicted specificity for xanthine nucleotides, and confirm that the differential binding of guanine and xanthine nucleotides is reversed from that of wild type Rab5. We find that prenylation of the mutant protein is highly specific for its cognate diphosphate nucleoside. In contrast, non-cognate nucleotides protect from tryptic proteolysis with efficacies equal to or greater than those of cognate nucleotides.

METHODS

Site-specific mutagenesis. The cDNA for human Rab5 had been previously cloned into M13, and the D136N mutant was constructed using the oligonucleotide 5'-TATTTGCTAGGTTGGCCTTGTTT-3' as described (5). The mutant cDNA was then inserted by directional subcloning into pT7.7, an expression vector for protein synthesis in *E. coli* (5), and into pAGA, a T7 polymerase-based transcription vector for *in vitro* transcription and translation (7). The DNA sequence of Rab5 D136N in both vectors was confirmed using the dideoxy chain termination method.

Expression, purification and refolding of Rab5 D136N. Induction of Rab5 D136N expression in *E. coli* and cell harvesting and lysis were essentially as described previously for Rab5 wild type (5). Since almost all of the mutant protein was found in the insoluble pellet after cell lysis, the supernatant was discarded and the pellet from a 2 l culture was resuspended in 160 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, and 2 mM β-mercaptoethanol) containing 1% Triton X-100 and homogenized using an Ultra-Turrax T25 (IKA-Labortechnik, Germany) at maximal setting for two pulses of 30 sec. This suspension was centrifuged at 20,000 g for 30 min, and the pellet was washed with 0.1% CHAPS to remove residual Triton X-100. The pellet was then extracted with 100 ml 8 M urea, filtered through a glass fiber filter, and applied to a 500 ml DEAE-Sepharose Fast Flow column equilibrated with lysis buffer containing 6 M urea. The unbound fraction (100 ml) containing Rab5 D136N was split in two, with half stored at 4°C for processing at a later time and half processed immediately. Each 50 ml aliquot was dialyzed against 4 M urea in lysis buffer for 6 h at 4°C, then against 2 M urea containing 10 μM XDP and 0.1% CHAPS. The urea concentration was then reduced by 0.5 M for each subsequent step of dialysis and the time increased to 8 h. The final sample was concentrated on an Amicon YM10 membrane and applied to a Sephacryl S-100 column equilibrated with lysis buffer containing 10 μM XDP, 0.1% CHAPS and 150 mM NaCl. Homogeneous fractions based on SDS-PAGE were pooled for subsequent experiments.

Competitive nucleotide binding. Purified Rab5 wild type (5) and D136N proteins (both 50 nM) were incubated in triplicate with 2 nM [^{35}S]GTPγS (255 Ci/mmol, New England Nuclear, Boston, MA) or 66 nM [^{3}H]XTP (6.8 Ci/mmol, Sigma Radiochemicals, St. Louis, MO) for 4 h at 30°C in 100 μl binding buffer containing various concentrations of unlabelled nucleotides. Binding buffer consisted of 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% CHAPS. Binding was terminated by dilution with 4 ml iced washing buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 25 mM $MgCl_2$) and rapid filtration through BA85 filters (Schleicher & Schuell, Keene, NH) , followed by two washings of 4 ml each. Filters were then counted by scintillation spectroscopy.

***In vitro* transcription, translation and prenylation.** *In vitro* transcription, translation and prenylation of Rab5 proteins using rabbit reticulocyte lysate were as described (7), except that various concentrations of XDP or GTP were added to the reactions. Prenylation of Rab5 D136N expressed in *E. coli* and purified as above was performed by incubation of 1 μg protein at 37°C for 4 h with 100 nM [^{3}H]geranylgeranyl pyrophosphate (3.33 x 10^5 dpm/pmol, American Radiolabelled Chemicals), 8 μl reticulocyte lysate, and varying concentrations of XDP in a volume of 10 μl. Both prenylation

reactions were stopped by the addition of Laemmli sample buffer, and aliquots were electrophoresed through urea/acrylamide gradient SDS gels (7). Incorporation of geranylgeranyl pyrophosphate was assessed for *in vitro* translated, [^{35}S]methionine-labeled protein by densitometric comparison of the processed and unprocessed proteins with differing mobilities on fluorographs, and for protein purified from *E. coli* by scintillation counting of excised gel slices containing protein labeled with [^{3}H]geranylgeranyl pyrophosphate.

RESULTS

Expression, purification and refolding of Rab5 D136N. Four hours after induction, a heavy 24 kDa band consistent with Rab5 D136N expression was seen in Coomassie Blue-stained SDS-PAGE gels of *E. coli* lysates. All attempts to obtain soluble protein by growing cells at lower temperature and/or under hyperosmotic conditions failed (8). Extraction of the pellet with urea following washing with Triton X-100 solubilized most of the 24 kDa band and provided several-fold purification from total lysate. Our refolding procedure differed from one recently described for H-Ras extracted with guanidine hydrochloride in that we used stepwise dialysis, a higher protein concentration, and detergent in the dialysate (9). Yield was maximized by collecting protein which precipitated at each step after the urea concentration was lowered below 2M, resolubilizing it in 4 M urea, and then dialyzing it as described in Methods. Refolding also provided some purification. Following molecular sieve chromatography, the protein was >95% pure as estimated from SDS-PAGE and Coomassie Blue staining, and the protein was stable in aqueous solution at 4°C for weeks or frozen at -80°C for months.

Nucleotide binding. Binding of non-cognate nucleotides was not directly measurable by filtration assay, and therefore all binding studies were performed by competition against radiolabeled cognate nucleotide. The differential affinities of GTP and XTP for Rab5 were essentially reversed for the D136N mutant (Fig. 1, Table 1 and Table 2). The affinities of diphosphate nucleosides for wild type and mutant proteins were similarly reversed (Table 1), although Rab5 wild type was considerably more discriminant than Rab5 D136N against non-cognate nucleotides (Table 2). The binding of ITP to both proteins was intermediate in affinity between XTP and GTP (Table 1), as might be expected since the interactions of N_1 should be preserved with both proteins even though there is no N_2 exocyclic substituent in ITP for hydrogen bonding with amino acid 136.

***In vitro* prenylation.** We have previously demonstrated that prenylation of Rab5 is supported by GDP but not GTP (7), so only diphosphate nucleosides were examined. Concentrations of GDP up to 1 mM did not support prenylation of *in vitro* translated Rab5 D136N above background level, whereas 1mM XDP resulted in prenylation of nearly 90% of protein after 1 h (Fig. 2). The EC_{50} of XDP for this reaction was approximately 50 μM.

As previously described, *in vitro* prenylation of bacterially expressed Rab proteins is inefficient (5), and only ~1% of purified Rab5 D136N present in the prenylation reaction incorporated

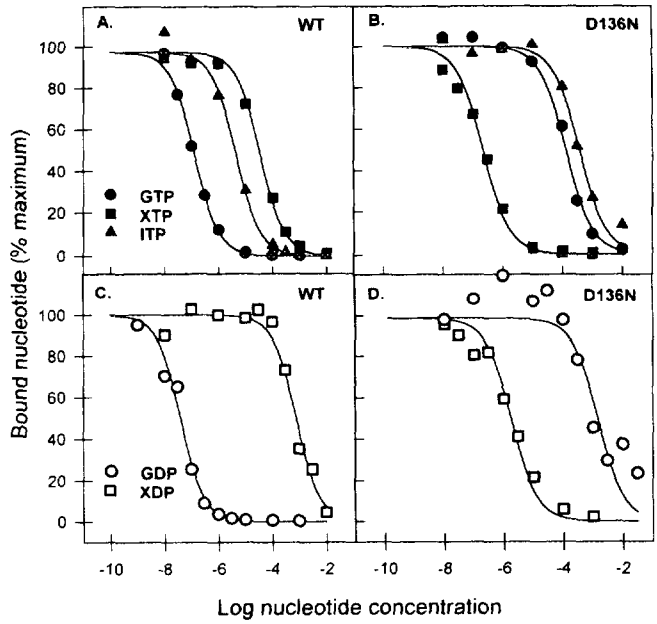

Figure 1. Competitive nucleotide binding. The binding of 2 nM [^{35}S]GTPγS to Rab5 wild type or 66 nM [^{3}H]XTP to Rab5 D136N was competed with unlabeled nucleotides as described in Methods. Shown are representative experiments which were repeated as indicated in Table 1. **(A)** Binding to Rab5 wild type competed with GTP, XTP and ITP. **(B)** Binding to Rab5 D136N competed with GTP, XTP and ITP. **(C)** Binding to Rab5 wild type competed with GDP and XDP. **(D)** Binding to Rab5 D136N competed with GDP and XDP.

[^{3}H]geranylgeranyl pyrophosphate in the presence of 1 mM XDP (data not shown). This is similar to previous results with Rab5 wild type and GDP (5). The EC_{50} of XDP for prenylation of purified Rab5 D136N was between 10 and 100 μM (Fig. 3), consistent with the findings for *in vitro* translated protein (Fig. 2).

Table 1. Competitve binding constants of purine nucleotides

	Rab5 wild type	Rab5 D136N
GTPγS	$1.26 \pm 0.25 \times 10^{-7}$ (3)	--
GTP	$1.19 \pm 0.15 \times 10^{-7}$ (8)	$3.65 \pm 2.29 \times 10^{-4}$ (5)
XTP	$2.81 \pm 0.85 \times 10^{-5}$ (7)	$4.20 \pm 1.02 \times 10^{-7}$ (6)
ITP	$7.89 \pm 1.59 \times 10^{-6}$ (4)	$3.79 \pm 0.97 \times 10^{-4}$ (3)
GDP	$3.49 \pm 1.20 \times 10^{-8}$ (4)	$9.22 \pm 3.02 \times 10^{-4}$ (3)
XDP	$7.86 \pm 3.55 \times 10^{-4}$ (4)	$1.93 \pm 0.77 \times 10^{-6}$ (3)

IC_{50} values were calculated from competitive binding experiments performed as in Fig. 1. Shown are the mean ± SEM of the number of separate experiments indicated in parentheses.

Table 2. Relative affinities of nucleotide binding

Rab5 wild type		Rab5 D136N	
XTP/GTP	236	GTP/XTP	869
ITP/GTP	66	ITP/XTP	902
XDP/GDP	22,521	GDP/XDP	478
GTP/GDP	3.4	XTP/XDP	0.2
XTP/XDP	0.04	GTP/GDP	0.4

IC_{50} values from Table 1 were used to calculate ratios of nucleotide binding affinities for Rab5 proteins.

Nucleotide-dependent proteolysis. We have previously demonstrated the successive degradation of Rab5 to a 20 kDa and then to 12 and 8 kDa fragments by trypsin, and stabilization of this process by guanine nucleotides (5). Only triphosphate nucleosides were used here, and only the disappearance of holoproteins and the sequential appearance and disappearance of 20 kDa polypeptides were evaluated as criteria for protection by nucleotides.

Purified Rab5 wild type protein was contaminated by a minor 20 kDa product of carboxyl-terminal degradation (Fig. 4., first lane), as previously observed (5). The smaller amount of this contaminant with the purified mutant protein may reflect protection within inclusion bodies prior to bacterial lysis or by urea during purification. Even the lowest amount of trypsin (1.25 ng) resulted in

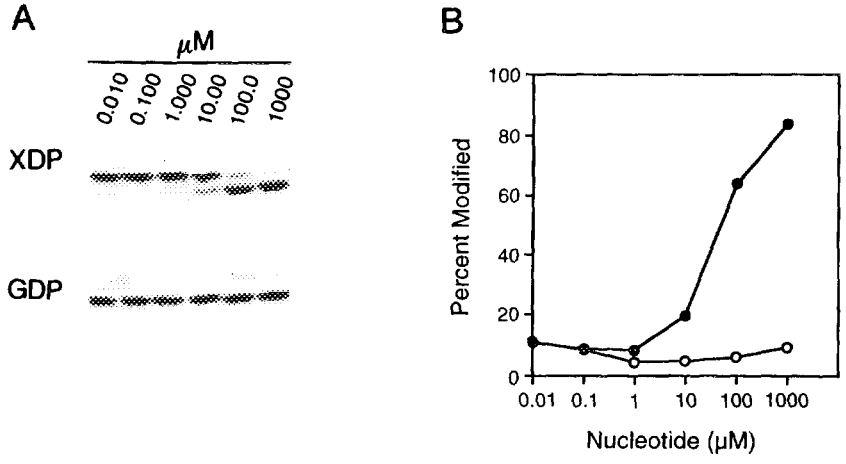

Figure 2. Prenylation of Rab5 D136N is dependent on XDP. (A) [^{35}S]methionine-labeled Rab5 D136N was synthesized and prenylated *in vitro*, then electrophoresed on a urea (4-8 M)/acrylamide (10-15%) gradient SDS gel and processed for fluorography. Varying concentrations of either XDP or GDP were included in the prenylation reaction as indicated. **(B)** The amount of prenylated (lower band) and unprenylated (upper band) polypeptide was quantified by densitometry and plotted as a function of added nucleotide concentration. GDP, open cirles; XDP, closed circles.

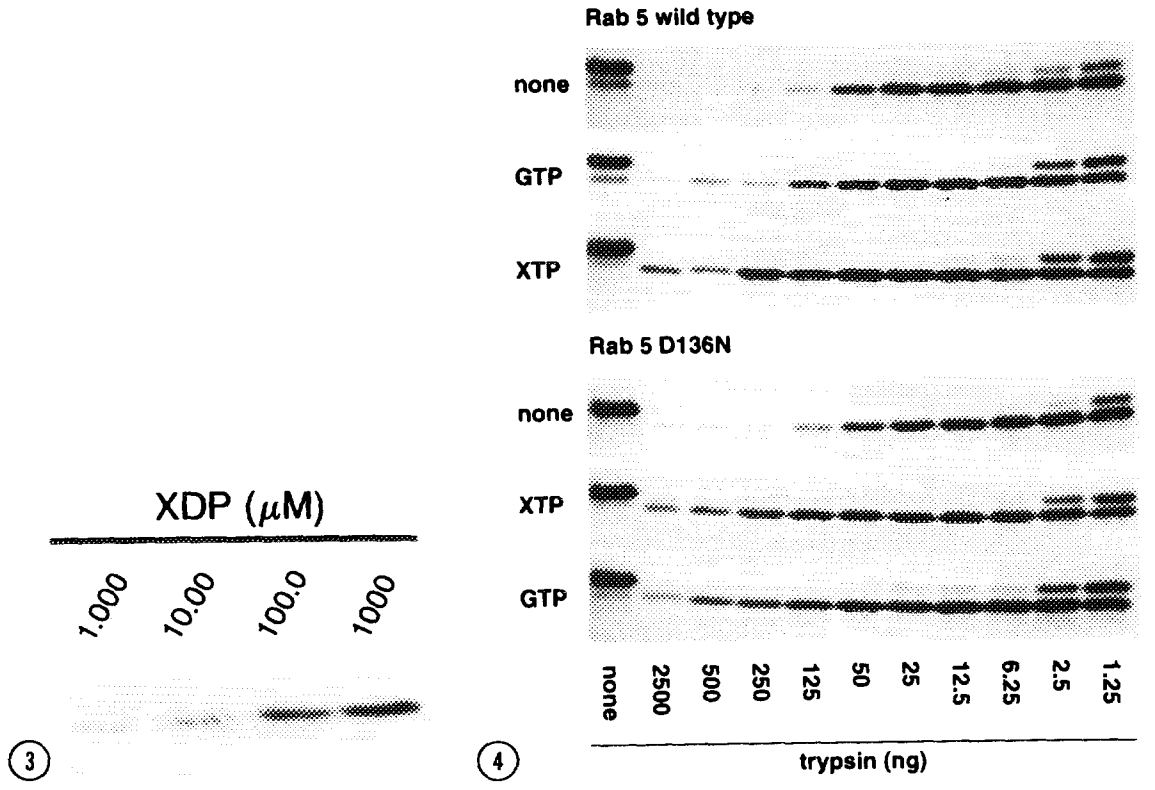

Figure 3. Purified recombinant Rab5 D136N is prenylated in an XDP-dependent manner. Bacterially expressed and chromatographically purified Rab5 D136N was prenylated *in vitro* with [^{3}H]geranylgeranyl pyrophosphate in the presence of increasing concentrations of XDP. Protein was then subjected to SDS-PAGE as for Fig. 4 and the fluorograph is shown.

Figure 4. Nulceotide-dependent proteolysis. Rab5 proteins (2.5 μg) were preincubated in the presence or absence of 10^{-2} M nucleotides in 50 μl binding buffer for 2 h at 30°C, then with various amounts of trypsin for 15 min at 30°C. The reaction was stopped by boiling in Laemmli sample buffer for 5 min, and polypeptides were resolved by SDS-PAGE in a Tris-Tricine buffer system and visualized using an enhanced Coomassie Brilliant Blue staining procedure, as described (5).

marked loss of holoproteins and increases in the abundance of 20 kDa polypeptides. Increasing amounts of trypsin progressively reduced the holoproteins to undetectable levels, and at the highest concentrations also reduced the abundance of 20 kDa polypeptides. These effects of trypsin were attenuated by the addition of 10 mM triphosphate nucleosides to the 3 μM XDP or 0.3 μM GDP which were carried through from the purification of the mutant and wild type proteins, respectively. Despite the lower affinities of non-cognate nucleotides (Table 1), their efficacies were equal to or greater than those of cognate nucleotides in protecting both Rab5 proteins (Fig. 4).

DISCUSSION

A xanthine nucleotide-specific mutant of EF-Tu was created several years ago to test the importance of amino acid-nucleotide interactions predicted from early tertiary structure models (4).

It was postulated that the hydrogen bond between the carboxyl group of D138 in EF-Tu wild type and the 2-amino group of guanine would be substituted by a hydrogen bond between the amide group of EF-Tu D138N and the 2-carbonyl group of xanthine. Not only did EF-Tu D138N bind xanthine nucleotides with high affinity and specificity, but it was also functional *in vitro* (4,10). Since the topology of all regulatory GTPases crystallized to date is similar, and D138 of EF-Tu is part of the NKXD motif which is highly conserved among GTPases, it may be possible to switch the nucleotide specificity of any member of the GTPase superfamily. This experimental strategy has been used to demonstrate that nucleotide specificity of the distantly related GTPase adenylosuccinate synthetase is similarly determined by an (N/T)KXD sequence (11), and that the stoichiometry of nucleotide hydrolysis by EF-Tu is two molecules hydrolyzed for each step of polypeptide elongation (10). Switching nucleotide specificity should be particularly useful for elucidating the role of specific GTPases in particular steps of vesicle transport since multiple GTPases have been shown to regulate these processes, and guanine nucleotide analogs could have multiple targets. Further, such mutants should be valuable for analysis of the mechanism of coupling of the GTP binding and hydrolyzing cycle of an individual GTPase to its regulatory role in vesicle traffic when used in concert with fluorescent xanthine nucleotide analogs, phosphorylation-resistant XDP analogs and hydrolysis-resistant XTP analogs.

The Rab5 D136N mutant has reversed affinity for xanthine and guanine nucleotides (Table 1), similar to affinities of cognate mutants inferred from enzymatic activities of EF-Tu (4) and adenylosuccinate synthetase (11) and directly measured for H-Ras (12). The finding that *in vitro* prenylation of Rab5 D136N is supported by XDP is significant for two reasons. First, this finding supports the functionality of the mutant protein. This was expected since D→N mutants of EF-Tu (4,10), adenylosuccinate synthetase (11), and H-Ras (12) function as wild type protein in the presence of comparable concentrations of xanthine nucleotides. Second, it establishes that the specificity for diphosphate nucleotides in prenylation is due to the requirements of the Rab protein itself and not to some other component of the prenylation reaction. For comparison, it was initially unclear whether the nucleotide requirements for cholera toxin-catalyzed ADP-ribosylation of Gs were those of Gs itself or of the ADP-ribosylation (ARF) cofactor which is also a GTPase (13).

Somewhat surprisingly, the efficacies of xanthine and guanine nucleotides in protecting Rab5 wild type and D136N from proteolysis by trypsin did not parallel the relative affinities of these nucleotides or their efficacies in supporting prenylation. Whereas the substrate for prenylation is diphosphate nucleoside-liganded Rab5, protease protection was induced by triphosphate nucleosides, and there is precedent for induction of the active triphosphate-liganded conformation of GTPases by non-cognate nucleotides. XTP and ITP activate adenylyl cyclase to a similar or greater extent than GTP, although the concentration of GTP for half-maximal activation is approximately 100-fold lower

(14,15). Similarly, ITP and XTP induce degranulation of permeabilized mast cells to a greater extent than does GTP (16), and XTP promotes exocytosis in adrenal chromaffin cells to a greater extent than does a hydrolysis-resistant GTP analog (17). It was also noted that non-cognate nucleotides promote interactions of wild type and mutant H-Ras with Raf *in vitro* to an extent similar to cognate nucleotides (4), that H-Ras D119N is transforming *in vivo* in a milieu high in GTP (18, 19), and that EF-Tu D138N functioned *in vitro* in the presence of a high concentrations of GDP (4). Taken together, these results indicate that even though the affinity of wild type and D→N mutant GTPases is low for non-cognate purine nucleotides, at high concentrations non-cognate nucleotides may have efficacies similar to or even greater than those of cognate nucleotides. This suggests that even though both the phosphate and base binding regions of GTPases contribute substantially to nucleotide binding affinity (20), protein interactions with the nucleotide γ-phosphate drive a conformational shift associated with protein activation whereas interactions with the base may impede the activating conformational shift.

In summary, we have demonstrated that the D136N mutant of Rab5 has reversed affinity for xanthine and guanine nucleotides, and that it is biochemically active in a prenylation reaction. However, based on our results and those of others, GTPases with switched nucleotide affinity should be used with some caution since nucleotide efficacy does not necessarily follow affinity, and nucleotide-specific effects seem to be predictable only at low nucleotide concentration. It is possible that further study of the relation between nucleotide base determinants of affinity and conformational or functional efficacy could be of pharmacologic value in designing nucleotide analogs with high protein specificity and efficacy.

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