REPORT

Real-time NMR Study of Three Small GTPases Reveals That Fluorescent 2′(3′)-O-(N-Methylanthraniloyl)-tagged Nucleotides Alter Hydrolysis and Exchange Kinetics[*][S]

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The Ras family of small GTPases control diverse signaling pathways through a conserved "switch" mechanism, which is turned on by binding of GTP and turned off by GTP hydrolysis to GDP. Full understanding of GTPase switch functions requires reliable, quantitative assays for nucleotide binding and hydrolysis. Fluorescently labeled guanine nucleotides, such as 2′(3′)-O-(N-methylanthraniloyl) (mant)-substituted GTP and GDP analogs, have been widely used to investigate the molecular properties of small GTPases, including Ras and Rho. Using a recently developed NMR method, we show that the kinetics of nucleotide hydrolysis and exchange by three small GTPases, alone and in the presence of their cognate GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors, are affected by the presence of the fluorescent mant moiety. Intrinsic hydrolysis of mantGTP by Ras homolog enriched in brain (Rheb) is ∼10 times faster than that of GTP, whereas it is 3.4 times slower with RhoA. On the other hand, the mant tag inhibits TSC2GAP-catalyzed GTP hydrolysis by Rheb but promotes p120 RasGAP-catalyzed GTP hydrolysis by H-Ras. Guanine nucleotide exchange factor-catalyzed nucleotide exchange for both H-Ras and RhoA was inhibited by mant-substituted nucleotides, and the degree of inhibition depends highly on the GTPase and whether the assay measures association of mantGTP with, or dissociation of mantGDP from the GTPase. These results indicate that the mant moiety has significant and unpredictable effects on GTPase reaction kinetics and underscore the importance of validating its use in each assay.

The Ras superfamily of small GTPases plays vital roles in the integrated network of cellular signaling. They are "turned on" by binding to GTP and adopting a conformation that allows modulation of their downstream effectors. The proteins are then "turned off" by hydrolysis of the γ-phosphate of GTP and conversion to GDP (see Fig. 1a). The relative amounts of activated GTP-bound and inactive GDP-bound forms of GTPases are tightly regulated by GAPs,[5] which catalyze nucleotide hydrolysis, and GEFs, which promote nucleotide exchange. Mutation or unregulated expression of the small GTPase proteins or their respective GAPs and GEFs can deregulate the GTPase cycle and lead to diseases such as cancer, neurodegeneration, and mental disabilities (1).

Although several methods for monitoring GTP hydrolysis and nucleotide exchange of small GTPase proteins have been developed (1–4), the assay most widely used to monitor kinetics employs the fluorescently labeled guanosine nucleotide analogs mantGTP/mantGDP, which are sensitive to the hydrophobic environment of proteins (see Fig. 1b). Because of high sensitivity and selectivity, mantGTP and mantGDP have been widely used in the field (5–7); however, the use of these nucleotide analogs is justified only if they report reaction kinetics and thermodynamics that are consistent with the natural ligands GTP and GDP. Previously, some inconsistencies between native GTP and mantGTP were observed in nucleotide hydrolysis assays of Ras and RhoA with their cognate GAPs (8, 9). However, these fluorescent probes have never been fully assessed due to the lack of appropriate methodology.

Recently we developed an NMR-based real-time assay to monitor the rate of GTP hydrolysis of Ras homolog enriched in brain (Rheb), enabling us to monitor GTPase reactions using native GTP and GDP (supplemental Fig. S2b) (10). We have demonstrated that the real-time NMR methodology can be successfully used to assay nucleotide exchange in RhoA (11). This methodology requires no chemical modification of the protein or the nucleotide, which can perturb the native structure of the protein (supplemental Fig. S1), and has the ability to sense subtle changes in the rate of catalysis in real-time fashion. In this study, we employed the NMR methodology to examine how the fluorescent adduct on mantGTP and mantGDP affects the kinetics of nucleotide hydrolysis and exchange of three small GTPases (H-Ras, Rheb, and RhoA) alone and in the presence of their GAPs or GEFs.

EXPERIMENTAL PROCEDURES

Protein Preparation—Murine Rheb (residues 1–169), human H-Ras (residues 1–171), and murine RhoA (residues 1–181) were prepared as described in the supplemental material

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according to previous protocols (10, 12, 13). Tuberous sclerosis 2 (TSC2) GAP domain (residues 1525–1742: hereafter termed TSC2GAP) and the DH-PH fragment of PDZ-RhoGEF (residues 713–1081: hereafter termed DH-PH^{PRG}) were prepared using pGEX2T and pGEX4T1 vectors, respectively (10, 13). Catalytic domain constructs of the Son of Sevenless (SOS, Cdc25 residues 566–1049: hereafter referred to as Cdc25^{SOS}) and the GTPase-activating domain of human GTPase-activating protein p120GAP (residues 715–1047: hereafter referred to as RasGAP) were prepared as His-tagged proteins from the pET15b vector. All proteins were cleaved from their tags via thrombin.

NMR-based GTPase, GAP, and GEF Assays—H-Ras and Rheb were loaded with GTP or mantGTP by incubation with a 10-fold excess of nucleotide in the presence of 10 mM EDTA. RhoA was loaded with GTP or mantGTP in the presence of 0.5 M urea and 10 mM EDTA. A heteronuclear single quantum coherence (^{1}H-^{15}N HSQC) spectrum was collected to confirm full nucleotide loading, and the mixture was then passed through a desalting column (PD MidiTrap^{TM} G-25 (GE healthcare)) equilibrated with NMR buffer to produce a 1:1 complex of GTPase and the nucleotide.

All NMR experiments were run on a Bruker AVANCE II 800-MHz spectrometer equipped with a 5-mm TCI CryoProbe. Sensitivity-enhanced ^{1}H-^{15}N HSQCs with two scans (5 min) were run in succession to monitor the intrinsic GTP hydrolysis activity of GTPases (0.1–0.3 mM) at 20 °C. The spectra were processed with NMRPipe (14), and the peak heights were analyzed with Sparky (15) via Gaussian line fitting. Residues from switches I and II, P-loop, β3 and β4, and the α3 helix that exhibit distinct well resolved peaks in each nucleotide-bound state were used as reporters of the reaction rates for each of the three GTPases, as described previously for Rheb (10).

For the intrinsic nucleotide hydrolysis, the fraction of GTPase protein in the GDP-bound state was calculated for each reporter residue using supplemental Equation 1 for Ras and Rheb relying on both GDP and GTP peaks. In the case of RhoA, the active state exhibited broadened and split peaks that complicate peak integration; hence we monitored (see supplemental Equation 2) the appearance of GDP peaks. Data fitting was done using PRISM (GraphPad software).

To assay GAP-mediated nucleotide hydrolysis, RasGAP or TSC2GAP was added to GTP-loaded H-Ras or Rheb at a GAP-to-GTPase molar ratio of 1:2,500 or 1:2.2, respectively. The hydrolysis rate was determined by fitting the data to supplemental Equation 1.

For GEF assays, DH-PH^{PRG} or Cdc25^{SOS} was added at a molar ratio of 1:30,000 GEF to RhoA or Ras in the presence of a 10-fold molar excess of GTP or mantGTP. All the experiments were performed in duplicate with 0.1 mM GTPase. The observed rates of nucleotide exchange assays performed with hydrolyzable nucleotides are affected by intrinsic hydrolysis. Thus, the observed data were fitted to equations that consider both exchange and hydrolysis (see supplemental material, Equations 4 and 6) to extract the true exchange rate. Using this rate, an exponential decay curve was generated to approximate nucleotide exchange in the absence of hydrolysis.

RESULTS

The Effect of mant on the Intrinsic Rate of Nucleotide Hydrolysis—mant-substituted nucleotides have been used extensively to investigate many aspects of G-protein signaling, including the kinetics and mechanisms of RasGAP-mediated GTP hydrolysis (9, 16–18) and Cdc25^{SOS}-mediated nucleotide exchange (6, 7). We first used NMR methodology to examine whether the addition of the mant moiety alters the intrinsic hydrolysis rate of the GTPase domain of H-Ras (1–171) (Fig. 1c) and found that it had little effect (supplemental Table S1), consistent with previous reports (19).

Next, we analyzed GTP hydrolysis by Rheb, which shares $\sim$33% sequence identity with H-Ras. Surprisingly, the hydrolysis rate of mantGTP by Rheb (3.2×10^{-3} min^{-1}) was >10 times faster than that of native GTP when both reactions were monitored by NMR (Fig. 1c). The rate constant obtained with mantGTP by NMR was identical to the value obtained using fluorescence spectroscopy (Fig. 1c and supplemental Fig. S2a), demonstrating that the results are independent of the detection method. Using mantGTP to compare the activity of different GTPases, the intrinsic GTPase activity of Rheb would appear approximately two times lower than that of H-Ras, whereas it is actually $\sim$32 times lower with native GTP, demonstrating that reliance on mantGTP would overlook this biologically important difference.

As a third case, the effect of the mant substitution on the GTPase activity of RhoA, which shares $\sim$31% sequence identity with H-Ras and Rheb, was investigated and found to be opposite to that observed with Rheb (Fig. 1c). The half-life of mantGTP bound to RhoA was $\sim$155 min, 3.5-fold longer than that of native GTP ($\sim$45 min) (supplemental Table S1). Taken together, the results show that mant-substituted nucleotides can substantially alter the kinetics of nucleotide hydrolysis by small GTPases in a manner that could not have been predicted *a priori*.

The Effect of mant on the Rate of GAP-catalyzed Nucleotide Hydrolysis—Having established that mant can have a substantial effect on intrinsic GTPase reaction rates, we investigated the effect of mantGTP on GAP-accelerated GTPase reactions. First, we used the GAP domain of the human p120-RasGAP. GTP was hydrolyzed with a rate of 2.6×10^{-2} min^{-1} by H-Ras in the presence of a 1:2,500 molar ratio of RasGAP, whereas mantGTP was turned over approximately five times faster (Fig. 2a and supplemental Table S1). Indeed, Moore *et al.* (9) previously noted that in the presence of RasGAP, p21^{N-Ras} hydrolyzes mantGTP more rapidly than native GTP, although the cleavage of the two nucleotides was not monitored by the same method.

We then examined how mantGTP might affect GAP-mediated GTP hydrolysis of a second, unrelated GAP with a distinct mechanism of action by studying Rheb and its well characterized GAP, TSC2GAP (10). At a TSC2GAP-to-Rheb ratio of 1:2.2, GTP was hydrolyzed at a rate of 1.9×10^{-2} min^{-1}, whereas mantGTP was hydrolyzed $\sim$2.5-fold more slowly (Fig. 2b and supplemental Table S1). Comparing Rheb's intrinsic rate of mantGTP hydrolysis to the GAP-catalyzed rate, TSC2GAP only produced a 2-fold enhancement (supplemental Table S1). This contrasts sharply

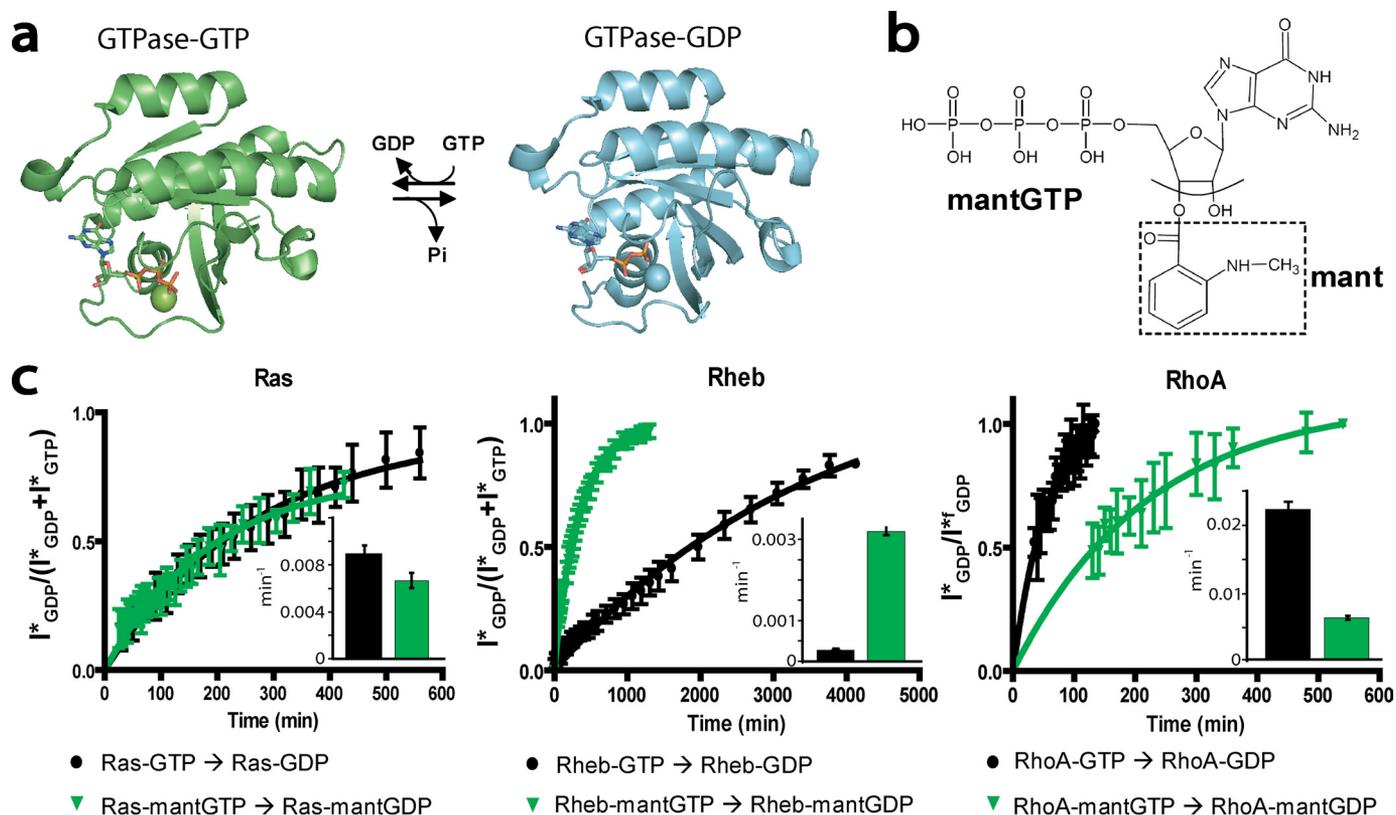**REPORT:** *Effects of mant-tagged Nucleotides*

FIGURE 1. **Effect of mant-substituted GTP on the intrinsic nucleotide hydrolysis of Ras, Rheb, and RhoA.** *a*, nucleotide-dependent conformational changes in GTPases. Nucleotide hydrolysis and exchange are correlated with conformational changes that are readily probed in two-dimensional ^{1}H-^{15}N HSQC spectra (Protein Data Bank (PDB): 1XTS (29) *green*; 1XTQ (29) *cyan*). *b*, the chemical structure of mantGTP (ChemSketch (30)) is shown with the mant moiety at the 3′ position of the ribose ring. In solution, this moiety exhibits slow chemical exchange between the 2′ and 3′ positions of the ribose ring, producing an equilibrium ratio of 4:6 (31). *c*, the time course of intrinsic GTP (*black*) and mantGTP (*green*) hydrolysis by H-Ras, Rheb, and RhoA probed via real-time solution NMR spectroscopy. All the proteins were fully loaded with GTP or mantGTP before the start of the assay, as assessed by ^{1}H-^{15}N HSQC. The rates are displayed with a histogram for each curve in inserted *panel c*. See supplemental material for descriptions of the equations.

with the ~50-fold stimulation of Rheb GTPase activity by TSC2GAP when native GTP is used in the assay. Hence, exclusive use of mantGTP would lead to a gross underestimation of the GAP activity of TSC2 toward Rheb, underscoring the utility of the NMR-based methodology.

The Effect of mant on the GEF-mediated Nucleotide Exchange— We examined how mant-substituted nucleotides affect the DH-PH^{PRG}-mediated nucleotide exchange of RhoA (Fig. 3a) using a procedure described elsewhere (11). In the NMR GEF assay with hydrolyzable nucleotides, the readout (*i.e.* GDP- and GTP-specific protein cross-peaks) is determined by both exchange and intrinsic nucleotide hydrolysis. This is evident in the case of Ras and RhoA (Fig. 3), which do not become 100% saturated with GTP or mantGTP. Hence, the observed data were fit to an equation that considers exchange and hydrolysis (see supplemental material) to derive the true exchange rate. We have shown that this derived rate agrees well with the rate determined for the non-hydrolyzable GTPγS (11). In an assay of nucleotide association with RhoA, mantGTP exhibits a 30% lower exchange rate (4.9×10^{-2} min^{-1}) when compared with native GTP (7.0×10^{-2} min^{-1}) (supplemental Table S2). In the literature, both the incorporation (13, 20, 21) and the dissociation (22, 23) of the mant-substituted nucleotide have been employed to study the function of RhoA and its interaction with GEFs. Hence, we also performed the dissociation assay, initially loading RhoA with GDP or mantGDP and monitoring

exchange to GTP. In this reaction, the rate of DH-PH^{PRG}-mediated nucleotide dissociation was approximately six times slower for mantGDP than for GDP (supplemental Fig. S3, *a* and *c*).

Previous studies have used mantGDP extensively to probe the Son of Sevenless (Cdc25^{SOS})-catalyzed nucleotide exchange of Ras GTPase. Generally, Ras is preloaded with mantGDP, and the decay in fluorescent intensity is monitored as this fluorescent nucleotide is displaced by unlabeled GTP (6, 7, 24–27). Here, using an NMR-based protocol similar to that described for RhoA, we show (Fig. 3b and supplemental Table S2) that the rate of Cdc25^{SOS}-catalyzed mantGDP dissociation (7.2×10^{-3} min^{-1}) is ~30% slower when compared with that of native GDP. GEF assays using association of mant-tagged nucleotide with Ras have also been reported (28); thus, we used NMR to compare the Cdc25^{SOS}-catalyzed association of mantGTP and GTP. We found that the rate of association of mantGTP with H-Ras is ~3-fold slower than that of GTP (supplemental Fig. S3, *b* and *d*).

DISCUSSION

Fluorescently labeled guanosine nucleotides have been used extensively to study nucleotide hydrolysis and exchange of GTPases. However, covalent modification of the nucleotide with a bulky fluorophore raises concerns about how this reporter moiety may perturb enzymatic activity. The NMR
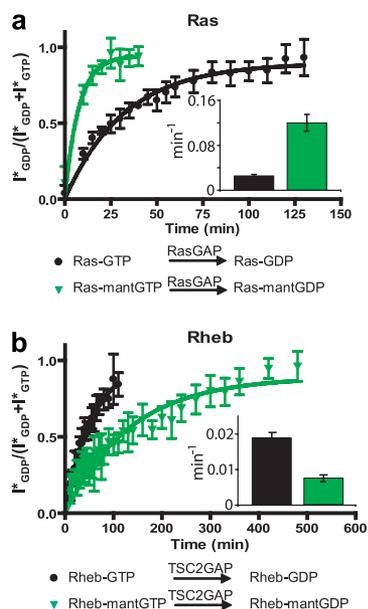
FIGURE 2. **Effects of mant-substituted GTP on GAP-mediated nucleotide hydrolysis by H-Ras and Rheb.** *a*, time course of RasGAP-mediated nucleotide hydrolysis of H-Ras with GTP (*black*) and mantGTP (*green*). A molar ratio of 1:2,500 RasGAP to H-Ras was used. *b*, time course of TSC2GAP-mediated hydrolysis of GTP (*black*) and mantGTP (*green*) bound to Rheb. A molar ratio of 1:2.2 TSC2GAP to Rheb was used for both assays. Rates extracted from each curve are displayed with an inserted histogram. Each experiment was performed in duplicate, and each curve represents a single representative experiment.

FIGURE 3. **Effects of mant-substituted nucleotides on GEF-mediated nucleotide exchange for H-Ras and RhoA.** *a*, time course of DH-PH^{PRG}-mediated nucleotide exchange of RhoA-GDP to GTP (*black*) and mantGTP (*green*). A molar ratio of 1:30,000 RhoA to DH-PH^{PRG} was used for both assays. *b*, time course of CDC25^{SOS}-mediated nucleotide exchange of H-Ras-GDP (*black*) and H-Ras-mantGDP (*green*) to GTP with 1:30,000 H-Ras to CDC25^{SOS} molar ratio. Observed data (*continuous lines*) were fitted to an equation that considers both exchange and hydrolysis to extract the true exchange rate k_{ex} in each experiment supplemental material). Using this rate, exponential decay curves (*dashed lines*) were generated to approximate nucleotide exchange in the absence of hydrolysis. The corrected rates are displayed with an inserted histogram for each curve. Each experiment was performed in duplicate, and each curve represents a single representative experiment.

methodology recently developed by our group (10) does not require any chemical modification of GTP or GDP because it makes direct observation of protein resonances that depend on nucleotide-induced changes in the chemical environment of protein (supplemental Fig. S2*b*). In this study, we employed this NMR method to compare native *versus* mant-labeled nucleotides in the kinetics of intrinsic GTPase reactions, GAP-mediated nucleotide hydrolysis, and GEF-mediated nucleotide exchange reactions of three small GTPases, H-Ras, Rheb, and RhoA. Our results clearly demonstrate that mant-labeled nucleotides had substantial effects on the kinetics of these reactions and that these effects were remarkably different and unpredictable with each GTPase, GAP, and GEF.

Intrinsic Hydrolysis of GTP and mantGTP—H-Ras exhibited a small decrease in the intrinsic hydrolysis rate of mantGTP *versus* native GTP; however, Rheb and RhoA were affected more drastically by this fluorescent tag. Remarkably, mant had opposite effects on Rheb and RhoA; Rheb hydrolyzed mantGTP ~10-fold faster than native GTP, whereas RhoA hydrolyzed this analog 3-fold more slowly than GTP. Thus, the effects of mant are specific to the structure and catalytic mechanism of each GTPase rather than the inherent lability of the nucleotide. With all three GTPases, the mant adduct perturbed proximal residues in the P-loop, switch I, and G-5 box but also induced long range perturbations in several regions including switch II (supplemental Fig. S4). The catalytic Gln of RhoA is found in switch II; thus, distortion of the structure of this loop could inhibit the hydrolysis of mantGTP. Interestingly, the analogous Gln in Rheb is in an orientation that does not contribute to catalysis (10, 29). To understand the rapid hydrolysis of mantGTP by Rheb, we asked whether the mant-induced per-
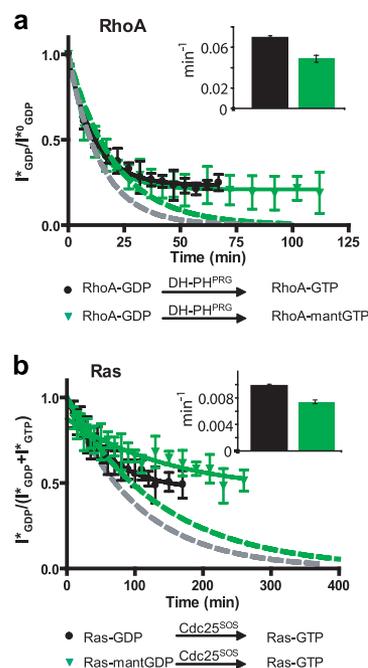
turbation of switch II might favor a catalytically competent conformation of Gln-64. However the Q64L mutation had no effect on hydrolysis of mantGTP, indicating that this reaction occurs through a more complex mechanism (supplemental Fig. S5).

GAP-catalyzed GTP Hydrolysis by H-Ras and Rheb—p120-RasGAP-catalyzed hydrolysis of mantGTP by H-Ras was ~5-fold faster than that of native GTP, whereas TSC2GAP-mediated hydrolysis of mantGTP by Rheb was slower by a factor of ~2.5 relative to unmodified GTP. Considering the rapid intrinsic GTPase activity of Rheb toward mantGTP, it is apparent that TSC2GAP activity is severely inhibited by mant. These results demonstrate the unpredictable effects of mant-tagged nucleotides on intrinsic and GAP-mediated GTPase activities and highlight the utility of the NMR approach.

Previous studies using a phosphate release assay showed that the K_m of p120 RasGAP-mediated hydrolysis of p21 N-Ras-mantGTP is lower than that of the p21 N-Ras-GTP complex (9). This result suggests that mant may increase the affinity of the H-Ras-nucleotide complex for RasGAP, thus increasing the rate of nucleotide hydrolysis. Conversely, we propose that for Rheb, the mant moiety probably hinders docking of the TSC2GAP domain to Rheb. Note that TSC2 and p120 RasGAP are not homologous, have different folds, and function through distinct catalytic mechanisms. Further structural insights are required to address the mechanistic basis for the "mant effect" on GAP-mediated nucleotide hydrolysis for both H-Ras and Rheb.

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GEF-accelerated Nucleotide Exchange of H-Ras and RhoA— Using the aforementioned NMR-based approach (11), we performed GEF assays to measure both association and dissociation of mant-tagged nucleotides as fluorescence-based experiments have been reported both ways in the literature (6, 7, 13, 20–23, 24–28). Comparing dissociation, Cdc25^{SOS}-mediated nucleotide exchange was 30% slower when starting with mantGDP-bound H-Ras (mantGDP-to-GTP) than with GDP-bound H-Ras (GDP-to-GTP). Measuring association under the same conditions, the GDP-to-mantGTP exchange was approximately three times slower than the GDP-to-GTP exchange. Similarly, DH-PH^{PRG}-mediated nucleotide exchange was 30% slower for the GDP-to-mantGTP than the GDP-to-GTP exchange in the association assay and ~6-fold slower for the mantGDP-to-GTP than the GDP-to-GTP exchange in the dissociation assay. In the crystal structure of H-Ras-mant dGppNHp (12), mant is near residue Tyr-31 in switch I, which would introduce steric clashes at the primary contact point between the GTPase and Cdc25^{SOS} and could interfere with nucleotide exchange. Assuming that the mant moiety of the nucleotide is similarly positioned on the switch I region of RhoA, it would also hinder DH-PH^{PRG} binding to RhoA (13). Furthermore, mant-induced structural perturbations of the GEF binding sites in the GTPase switch regions may inhibit interactions with GEFs. The exchange kinetics reported by mant are more reliable when the dissociation assay is used for the Ras-CDC25^{SOS} system and when the association assay is used for the RhoA-DH-PH^{PRG} system. Note that these are the conventional fluorescence methods for each protein; nevertheless, the less accurate alternative approaches are still in use. Finally, the reduced sensitivity of mantGDP-bound GTPases to the action of GEFs suggests an avenue for design of GTPase inhibitors.

In conclusion, we have demonstrated that mant-labeled nucleotides can alter the intrinsic GTPase activity of Rheb and RhoA, the GAP-catalyzed GTP hydrolysis of H-Ras-RasGAP and Rheb-TSC2GAP, as well as the DH-PH^{PRG}- and CDC25^{SOS}-mediated nucleotide exchange of RhoA and H-Ras, respectively. These results reveal that the fluorescent probes could yield biochemically inaccurate data and potentially lead to misleading conclusions. The significant and unpredictable effects of the mant tag clearly indicate that mant-tagged nucleotides should be used with caution and should be validated for each GTPase system studied. At the same time, these findings provide clues as to how one could inhibit or activate specific signaling pathways using small organic molecules, which may mimic the effects of the mant tag on small GTPases. This study also extends the utility and value of the NMR-based assays for both GTPase and GEF reactions for three small GTPases, suggesting that it will be broadly applicable to the GTPase superfamily.

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