Conformational resolution of nucleotide cycling and effector interactions for multiple small GTPases determined in parallel

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Small GTPases alternatively bind GDP/GTP guanine nucleotides to gate signaling pathways that direct most cellular processes. Numerous GTPases are implicated in oncogenesis, particularly the three RAS isoforms HRAS, KRAS, and NRAS and the RHO family GTPase RAC. Signaling networks comprising small GTPases are highly connected, and there is some evidence of direct biochemical cross-talk between their functional G-domains. The activation potential of a given GTPase is contingent on a codependent interaction with the nucleotide and a Mg2+ ion, which bind to individual variants with distinct affinities coordinated by residues in the GTPase nucleotide-binding pocket. Here, we utilized a selective-labeling strategy coupled with real-time NMR spectroscopy to monitor nucleotide exchange, GTP hydrolysis, and effector interactions of multiple small GTPases in a single complex system. We provide insight into nucleotide preference and the role of Mg2+ in activating both WT and oncogenic mutant enzymes. Multiplexing revealed guanine nucleotide exchange factor (GEF), GTPase-activating protein (GAP), and effector-binding specificities in mixtures of GTPases and resolved that the three related RAS isoforms are biochemically equivalent. This work establishes that direct quantitation of the activation potential for any given GTPase, as small GTPases are highly impacted by Mg2+ abundance.

Small GTPases are a class of critical hub proteins, responsible for controlling both the direction and intensity of cell signals by acting as “molecular switches” (1–3). The RAS and RHO subfamilies constitute about one-third of all RAS superfamily GTPases (4) and are key regulators of both normal and oncogenic cellular processes, including proliferation and migration. Archetypally, small GTPases interconvert between active GTP-bound and inactive GDP-bound states. Nucleotide exchange and GTP hydrolysis occur intrinsically; however, exchange and hydrolysis rates are catalyzed by guanine nucleotide exchange factors (GEFs)3 or GTPase-activating proteins (GAPs), respectively. Contributions from intrinsic versus catalyzed exchange in vivo are not well-understood. In the active state, GTPases bind directly to downstream effector proteins via specialized recognition domains, predominantly RAS-binding domains (RBDs) for RAS GTPases and CRIB motifs for RHO GTPases (5, 6).

Within this enzyme superfamily, the most heavily studied are three highly related RAS isoforms: HRAS, KRAS, and NRAS. These small GTPases are critical mediators of signaling networks that stimulate cell growth and proliferation, including the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. Oncogenic mutations at codons 12, 13, and 61 of the HRAS, KRAS, and NRAS genes are among the most frequent genetic mutations in human cancers (7). The three RAS proteins share 80% sequence identity and are coexpressed in most cell types. Structurally, they share a nearly identical tertiary fold. As such, these proteins were initially considered functionally redundant, but multiple lines of evidence support functional specificity: RAS genes exhibit different transforming potential (8–10), are distinctly mutated in cancers (11, 12), exhibit unique sensitivities to GEFs (13), and are localized to discrete subcellular locales (14). Whether genuine biochemical variations in the core G-domains of these proteins contribute to these observed biological differences remains an open question.

Existing approaches to measure small GTPase kinetics include time-course HPLC, release of 32P, from [32P]GTP, and, most commonly, release or uptake of fluorescent nucleotide analogs. These are crucial assays used to decipher how much

3 The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; RALA, RAS-like proto-oncogene A; RBD, RAS-binding domain; RT-NMR, real-time NMR; HSQC, heteronuclear single-quantum coherence; GMPPNP, guanosine 5′-O-[β,γ-imido]triphosphate; GTP·γS, guanosine 5′-O-[γ-thio]triphosphate; REM, RAS exchanger motif; DH, Db↓ homology; PH, pleckstrin homology; GRF, guanine nucleotide–release factor; GRP, guanine nucleotide–releasing protein; GST, glutathione S-transferase; CRIB, Cdc42- and Rac-interacting binding.
activated GTPase might subsist in vivo. Unfortunately, several important drawbacks exist with these methods, foremost that fluorescently tagged analogs can impact reaction kinetics (15). Indeed, the indirect nature of these approaches are a shortcoming that has led to improper conclusions concerning the rate of WT and oncogenic RAS mutant nucleotide exchange and its overall on/off state (16, 17). To accurately quantify the activation state of GTPases requires consideration of relative nucleotide affinity (i.e., preference of a given GTPase to bind GDP or GTP), the availability of Mg$^{2+}$ cofactor, and the potential impact of multimer formation or membrane interactions. This would take into consideration the growing evidence that RAS GTPases dimerize (18, 19), which would be intensified at high protein concentrations such as those in membrane nanoclusters.

Recently, real-time NMR (RT-NMR) experiments have been adapted to quantitate small GTPase activity (20, 21). As GTPases undergo major conformational change upon binding to GDP or GTP, successive collection of $^3$H-$^{15}$N heteronuclear single quantum coherence (HSQC) spectra allows for kinetic analyses of exchange or hydrolysis. Importantly, these assays do not require fluorescent nucleotide analogs or any chemical modification of the GTPase. Furthermore, as NMR assays are functional over a wide range of protein concentrations and even on membrane-tethered GTPase (22), they can be used to probe the functional impact of proposed RAS GTPase oligomerization. To strengthen the RT-NMR approach, it is now possible to multiplex these assays (23), allowing quantification of activation states for several GTPases monitored simultaneously in real time. This approach could therefore resolve whether GTPases behave autonomously in mixtures or whether they are highly interactive with each other or at concentrations that promote oligomerization and whether this impact nucleotide exchange or GTP hydrolysis kinetics and/or effector binding.

We employed here a multiplexed RT-NMR approach to study the full GTPase nucleotide exchange and hydrolysis cycle as well as specificity of effector binding. Using a selective-labeling strategy, we employed RT-NMR to concurrently measure kinetics and effector binding specificity of the three related RAS isoforms, across RAS and RHO subfamily members, and between cancer-associated mutations of RAS and RAC1 and WT counterparts. The data improve our understanding of the complexity and interconnectedness of small GTPases in the context of cell signaling, particularly the impact of relative nucleotide affinity, Mg$^{2+}$ availability, and cross-talk mechanisms.

Results

Selective isotopic GTPase labeling and intrinsic nucleotide cycling

To juxtapose structure–function data for multiple small GTPases in parallel, we selected seven enzymes from the RAS and RHO subfamilies (Fig. 1A). Structures of these proteins demonstrate the exceptional similarity of their tertiary folds (Fig. 1B) (24–28). Identical sizes, shapes, and enzymatic functions make in vitro profiling of numerous GTPase activities together a massive challenge. For NMR-based analyses, uniformly labeled samples of multiple GTPases result in excessive crowding of resonance peaks, as demonstrated in Fig. 1C using HRAS, KRAS, and NRAS. To observe multiple GTPases concurrently required a selective isotopic labeling approach with single $^{15}$N-amino acids. Fig. 1, D and E, show isotopically labeled RAS isoforms ([$^{15}$N]Ile HRAS, [$^{15}$N]Thr KRAS, and [$^{15}$N]Leu NRAS) in both the GDP-bound and GTP-bound conformations (using nonhydrolyzable GMPPNP). The reduction in spectral complexity allowed us to monitor nucleotide exchange, GTP hydrolysis, or effector binding in mixtures of even the most highly related GTPases.

We performed exchange and hydrolysis assays on individual selectively labeled GTPases to profile their kinetic activity in isolation. NMR spectra and nucleotide exchange plots for [$^{15}$N]Tyr HRAS, [$^{15}$N]Thr KRAS, [$^{15}$N]Leu NRAS, [$^{15}$N]Leu RALA, [$^{15}$N]Val RHEB, [$^{15}$N]Val RHOG, and [$^{15}$N]Leu RAC1 are displayed in Figs. S1 and S2, A–G. Kinetics for these reactions are detailed in Table S1 (as for all individually measured GTPase kinetics). Exchange was initiated by the addition of a 10:1 molar ratio of the GTP analog GTPγS, used to reflect the cellular GTP:GDP ratio. Measured exchange rates for HRAS match what has been calculated by RT-NMR using uniformly labeled samples (21). As individual GTPases reach an activation plateau (i.e., nucleotide exchange no longer proceeds), they exhibit differential GTPγS loading that reflects their activation state at equilibrium. We report these data in Table S1 as “% Activated.” Furthermore, as we control the GTPγS:GDP ratio at 10:1, we can also calculate the preference of these GTPases to each nucleotide, reported as “GDP Preference.” We observed that the three RAS isoforms and RHEB exhibited a much higher ratio of GTPγS-bound (∼65–75%) in these conditions compared with RALA (38%) or RHOG (49%), a direct reflection of differential nucleotide affinities. We next measured intrinsic GTP hydrolysis for each RAS isoform ([$^{15}$N]Tyr HRAS, [$^{15}$N]Thr KRAS, and [$^{15}$N]Leu NRAS) (Fig. S2, H–J). The measured rate for HRAS matches what was previously determined on uniformly labeled protein (21), and NRAS and KRAS exhibited near identical rates. Overall, the selective-labeling approach provides an opportunity to precisely measure GTPase activity while reducing spectral complexity.

Dependence of nucleotide exchange on Mg$^{2+}$

To begin assaying multiplexed GTPases, we first focused on the three highly related RAS isoforms. There has been little attempt to study differences in nucleotide exchange and/or GTP hydrolysis rates for the RAS isoforms in their individual contexts; however, one study found that the intrinsic rates of GTP catalysis differed across isoforms using [$^{32}$P]GTP single turnover (29). Due to the inefficiency of selective labeling, monitoring HSQC chemical shifts of the three GTPases required each at a concentration of 300 μM. Interestingly, this meant we could assay GTPase activity at concentrations that should drive RAS dimerization. The existence of KRAS homodimers remains contentious, with proposed dissociation binding constants ranging from low μM (30) to mM (31). Recent biophysical data dispute the existence of RAS dimers even at high concentrations (32, 33). Performing these assays at total GTPase concentrations approaching 1 mM would resolve whether G-domain oligomers can influence RAS activation in solution. Initial multiplexed nucleotide exchange assays (GDP-to-GTPγS) at a
10:1 molar excess of GTPγS showed exchange rates substantially faster than those measured for GTPTases individually at comparatively lower concentrations. To resolve this, we considered that exchange assays with each RAS isoform alone at various concentrations (150, 250, or 350 μM) showed that rates increase with protein concentration (Fig. 2, A–C). Mg2+ cofactor is absolutely required for nucleotide binding to RAS GTPTases, and both RAS (34) and RHO (35) nucleotide exchange is highly dependent on Mg2+ concentration. The majority of GTPTase kinetic studies use MgCl2 at a steady concentration of 5 mM, and we postulated that increasing protein concentrations lower the [Mg2+]:[GTPTase] ratio, leading to Mg2+ scarcity and faster exchange rates. To test this, we measured nucleotide exchange of NRAS at 200 or 350 μM in 5 mM MgCl2, again showing that the rate increases at a higher concentration (Fig. 2D). When the assay was repeated at 15 mM MgCl2, exchange rates were identical. Thus, Mg2+ availability is a key determinant of GTPTase exchange, which otherwise proceeds independently of protein concentration even approaching 1 mM. When we performed a “reverse exchange

Figure 1. NMR spectroscopy to monitor activity of multiple, highly similar small GTPTases simultaneously. A, amino acid sequence alignment of the complete RAS and RHO subfamily small GTPTases. GTPTases used in this study are highlighted. The scale represents divergence time based on amino acid substitution rate. B, available structures of GTPTases used in this study. Root mean square deviations (R.M.S.D.) between these GTPTases and KRAS demonstrate structural homology. C, HSQC overlay of uniformly 15N-labeled HRAS, KRAS, and NRAS loaded with GDP. D, HSQC overlay of selectively labeled RAS isoforms loaded with GDP; [15N]Ile-labeled HRAS (purple), [15N]Thr-labeled KRAS (green), and [15N]Leu-labeled NRAS (blue). E, mixture of selectively labeled RAS isoforms from D loaded with GDP (black spectrum) and GMPPNP (red spectrum).
assay (GTP\_S-to-GDP) at two different concentrations of HRAS (150 and 300 \( \mu \)M) and a MgCl\(_2\) concentration of 5 mM, we saw no effect on the rate (Fig. S2\_K). GDP disassociation is therefore more influenced by [Mg\(^{2+}\)] than GTP.

Interestingly, there are several recurrent oncogenic mutants of RAS that have been determined to function by rapid intrinsic exchange (21). As these mutations lie proximal to the nucleotide/Mg\(^{2+}\)-binding pocket, we were curious whether increasing [Mg\(^{2+}\)] may slow their intrinsic exchange rate.

We purified isotopically labeled KRAS proteins of two fast-exchange mutants, G13D (found within the P-loop) and Q61L (in the switch II region) (21, 36) (Fig. 2\_E). We performed nucleotide exchange assays at either 5 or 50 mM MgCl\(_2\) (Fig. 2\_F). There was no Mg\(^{2+}\) dependence on the exchange rate of either mutant, indicating that these amino acid mutations alter nucleotide affinity rather than disrupt coordination of the Mg\(^{2+}\) ion.

Finally, we tested whether increasing concentrations of RAS with a steady concentration of Mg\(^{2+}\) would lead to differences in GTP hydrolysis. Neither KRAS, HRAS, nor NRAS exhibited altered GTP hydrolysis rates with increasing protein concentration at a constant [Mg\(^{2+}\)] of 5 mM (Fig. 2, G–I). This is consistent with the absence of competing nucleotide in these assays and with data suggesting that GDP binding is more dependent on Mg\(^{2+}\) than is GTP binding (37).

**Multiplexed nucleotide exchange assays**

With a strategy for selective amino acid labeling and conditions optimized for simultaneously monitoring multiple GTPase activities, we performed a series of multiplexed nucleotide exchange assays. Fig. 3, A–C, show HSQC spectra depicting the amino acid labeling strategy and consequent GDP/GTP\_S-bound peaks used to concurrently measure exchange rates for HRAS, KRAS, and NRAS. Each isoform was at a concentration of 300 \( \mu \)M. Mixing multiple
GTPases at these concentrations did not lead to obvious binding or higher-order complexes, as no significant chemical shift perturbations or peak broadening was observed. Intrinsic exchange rates for each of the three isoforms were not changed from those measured for HRAS, KRAS, and NRAS alone at lower concentrations. Kinetic analyses and parameters for all multiplexed assays are detailed in Table S2. All three GTPases were 80–90% activated at equilibrium and demonstrated a minor preference for GDP over GTP$\gamma$S. We can conclude that neither the presence of alternative RAS isoforms nor high concentrations of these GTPases significantly impact their activation state.
We next examined the NRAS GTPase in parallel with a related GTPase, RALA, and a RHO subfamily GTPase, RHOG.

**Fig. 3, F–J, depict the labeling strategy and multiplexed exchange.** RALA and RHOG exhibited slightly faster exchange rates than NRAS (1.4- and 1.3-fold, respectively), but their percent activation at equilibrium is significantly lower than that of NRAS (84% GTP-bound for NRAS, 46% for RALA, and 49% for RHOG). This reveals that RALA and RHOG have a greater preference for GDP over GTP compared with NRAS. These data highlight the need to directly monitor the nucleotide-bound state when quantifying GTPase activation, as kinetic rates alone can significantly misrepresent the total activated GTPase in a given system.

**Multiplexed GEF and GAP assays**

Our multiplexed NMR strategy presents an opportunity to observe the effects and specificity of exchange-promoting GEFs and hydrolysis-activating GAPs on mixtures of small GTPases (Fig. 4). We performed a GEF assay in a mixture of the three isoforms of RAS using a catalytic domain from the major RAS. We overlaid the GDP-loaded [15N] Tyr HRAS, [15N] Thr KRAS, and [15N] Leu NRAS at 150 μM. We observed several peaks exhibiting chemical shift perturbation that were circled (HRAS, THR, and TYR). Representative resonances (labeled "D" in C) for HRAS, KRAS, and NRAS across multiple time points of multiplexed exchange. NRAS Leu is unlabeled as the backbone is unassigned. E, intrinsic exchange curves of multiplexed HRAS, KRAS, and NRAS at 300 μM, each initiated by a 10:1 molar ratio of GTP·S in 10 mM MgCl₂. F, multiplexed exchange of KRAS and RAC1 at 300 μM, each initiated by an 8:1 molar ratio of GTP·S in 15 mM MgCl₂. G, multiplexed exchange of specifically labeled HRAS, RHOG, and RALA at 300 μM, each initiated by 8:1 molar ratio of GTP·S in 15 mM MgCl₂. H, multiplexed exchange of specifically labeled NRAS, RHOG, and RALA at 300 μM, each initiated by 8:1 molar ratio of GTP·S in 15 mM MgCl₂. Error bars represent S.D.

**Multiplexed SOS1 GEF Assay**

A, multiplexed SOS1 GEF assay with 1:15000 SOScat. Exchange was initiated by 8:1 GTP·S in 15 mM MgCl₂. B, multiplexed SOS1 GEF assay with 1:30000 SOScat. C, multiplexed SOS1 GEF assay with 1:8000 SOScat.
regulator SOS1 (SOS\textsubscript{cat} (21, 38)), added at a molar ratio of 1:15,000 (versus [total GTPase]) (Fig. 4B). We calculated similar increases in the SOS-catalyzed exchange rate for all three isoforms (4.5-fold increase for HRAS, 3.3-fold for KRAS, and 3-fold for NRAS), indicating that they have comparable sensitivities to this GEF.

To observe SOS specificity, we performed the same assay using multiplexed NRAS, RALA, and RHOG (Fig. 4C). In the presence of SOS\textsubscript{cat} we observed a significant increase in the exchange rate of NRAS relative to intrinsic exchange and no effect on the exchange rates of RALA or RHOG. We next performed a multiplexed assay using KRAS and RAC1, as SOS is reported to have GEF activity toward both. The activating GEF domains differ for each GTPase: the REM–CDC25 domains (SOS\textsubscript{cat}) mediate activity toward RAS (38, 39), and the DH–PH domains activate RAC1 (40, 41). The intrinsic exchange of KRAS proceeds 1.3-fold faster than that of RAC1, with percent activated at equilibrium measured at 83 and 65%, respectively (Fig. 4D). Upon addition of SOS\textsubscript{cat}, KRAS nucleotide exchange increases 2.5-fold, whereas RAC1 rates remained unchanged (Fig. 4E). Notably, the nucleotide-bound ratio of KRAS at equilibrium is not altered by the presence of SOS (83%), supporting the premise that GEFs do not actively exchange nucleotide but function by a passive mechanism.

We next employed our approach to measure GAP activation of GTPase hydrolysis by performing multiplexed assays with the three RAS isoforms. Calculated intrinsic hydrolysis rates for HRAS, KRAS, and NRAS were indistinguishable when measured in tandem (Fig. 4, F and G). In the presence of recombinant GAP-334 domain from the major RAS regulator p120GAP (added at a 1:5500), hydrolysis rates of each isoform were uniformly increased (2.3-fold for HRAS, 2.6-fold for KRAS, and 2.9-fold for NRAS (Fig. 4H)). These results indicate that the three isoforms share sensitivity to GAP activation, in addition to having comparable rates of intrinsic hydrolysis.

**Multiplexed GTPase assays with oncoproteins**

There are conflicting reports on WT RAS isoforms influencing the transformation potential of oncogenic mutants, and little is known about cross-talk between WT and mutant GTPases from a biophysical standpoint. We used multiplexed RT-NMR to concurrently monitor nucleotide exchange and GTP hydrolysis of WT RAS isoforms and several oncogene-derived mutants (Fig. 5A, KRAS G12C, KRAS Q61L, and HRAS G12V). HSQC spectral resolution of the activation state and nucleotide exchange assays on the individual oncoproteins are presented in Figs. 5, A–C, and S4, A–C. HRAS G12V and KRAS G12C have intrinsically slow nucleotide exchange compared with their WT counterparts, whereas KRAS Q61L exhibits rapid intrinsic exchange. This was also observed when these oncoproteins were multiplexed with the matched WT, whereby KRAS G12C intrinsically exchanges at a rate 2.9-fold slower than WT, HRAS G12V at a rate 1.6-fold slower, and KRAS Q61L at a rate 1.6-fold faster (Fig. S4, D–F). Significantly, the KRAS G12C variant reaches equilibrium at only 67% activated and HRAS G12V at only 36%. In contrast, KRAS Q61L reaches 87% activation under these conditions. We extended these analyses to measure GEF activity by adding SOS\textsubscript{cat}. The KRAS G12C mutant had a 1.5-fold slower exchange rate in the presence of SOS\textsubscript{cat} than WT KRAS, similar to the intrinsic difference (Fig. 5B). A multiplexed GEF assay with WT KRAS and the Q61L variant again demonstrated that Q61L exchanges at a faster rate than WT (1.3-fold) and reaches equilibrium at nearly 90% GTP\textsubscript{S}-bound (Fig. 5C). These data suggest that there is no biophysical interplay between WT and oncogenic RAS GTPases at even high concentrations of G-domain. They also imply that large pools of the RAS G12V or G12C variants likely remain GDP-bound even in the presence of activating GEF.

Impaired GTP hydrolysis is a key biochemical defect in oncogenic RAS GTPases. We determined that KRAS G12C has a 2.6-fold slower intrinsic rate of hydrolysis than WT KRAS when measured individually (Fig. S4G) or in a multiplexed assay (Fig. S4H). The KRAS Q61L variant showed effectively no intrinsic GTP hydrolysis over a 10-h time course (Fig. S4J). To monitor rates in the presence of GAP, we added GAP-334 to multiplexed samples. The addition of GAP at 1:5000 did not affect the GTP hydrolysis rate of either G12C or Q61L KRAS (Fig. 5, D and E), whereas the hydrolysis rate of WT KRAS increased 2.5-fold. Thus, the presence of neither WT KRAS nor GAP significantly alters the ability of these oncogenic mutants to hydrolyze GTP.

Genetic defects impacting small GTPase function in cancer are not limited to RAS proteins, so we sought to examine biophysical interplay between RAC1 and a RAC1 P29S mutant recurrently found in melanoma (Fig. 5F). A nucleotide exchange assay on the P29S variant alone demonstrated that this oncoprotein intrinsically exchanges 7-fold faster than WT RAC1 (Figs. S3D and S4J). A multiplexed approach with WT and P29S RAC1 provided the same result (Fig. 5G). Importantly, P29S RAC1 reaches almost 100% activation in these conditions, demonstrating a clear preference for binding GTP\textsubscript{S} over GDP, whereas WT RAC1 shows a 6-fold preference for GDP. The utility of an approach that directly monitors GTPase conformation is fully demonstrated in Fig. 5H and S4K. These NMR resonances demonstrate how poorly the HRAS G12V and KRAS G12C oncoproteins exchange over time in a 10-fold excess of GTP\textsubscript{S}, as compared with their WT counterparts, KRAS Q61L, or RAC1 P29S.

**Multiplexed effector binding assays**

The specificity of effector-binding domains for small GTPases is a question of enormous interest and one that has not been well-explored. Effectors targeting RAS subfamily GTPases typically comprise an RBD, and there are >50 potential RBDs in the human proteome (42). To directly observe effector specificity and competition for GTPase-binding partners in a complex system, we used NMR and multiple selectively labeled GTPases. We purified RBDs from two isoforms of the effector RAF kinases (ARAF and BRAF) and an RBD from the RAL effector RLIP76 (Fig. 6A) (43, 44). The RBD of ARAF was added to GMPPNP-loaded HRAS, KRAS, and NRAS and displayed uniform binding to all three isomers as determined by peak broadening and chemical shift perturbations (Fig. 6B). Next, we titrated the RBD of BRAF, and it also induced complete broadening across all three isoforms of RAS (Fig. 6C). To observe specificity across GTPase subfamilies, the BRAF RBD was titrated into a mixture of GMPPNP-loaded KRAS, NRAS, and
RHOG. Once again, severe peak broadening was observed for the KRAS and NRAS resonances, whereas peaks derived from RHOG were left unperturbed (Fig. 6D). The structurally unrelated RBD from RLIP76 was then titrated into a mixture of HRAS, KRAS, and RALA. Only RALA peaks displayed peak broadening, whereas HRAS and KRAS peaks were unperturbed (Fig. 6E). Finally, we looked to titrate an engineered monobody against RAS, NS1 (45), into a multiplexed mixture of HRAS, KRAS, and NRAS. This monobody was designed to interact with the distal site (from the nucleotide-binding pocket) of HRAS and KRAS to prevent its dimerization/clustering on the membrane. Upon titration of NS1 into the three isoforms of RAS, peak broadening was observed only in HRAS and KRAS (Fig. 6F), whereas NRAS-specific chemical shifts were unperturbed. Overall, the multiplexed NMR approach is a powerful technique to observe interactions and binding specificities of multiple, unmodified proteins simultaneously.

Discussion

Accurate data characterizing small GTPase nucleotide cycling are crucial to our understanding of both WT and mutant GTPase activation potential in cells. These are extremely challenging experiments that must consider codependent nucleotide and Mg²⁺ affinity, competing nucleotides and metal ions, and the distinct biochemical properties of a given GTPase. We have investigated the complete nucleotide exchange of WT and mutant GTPases in parallel using a selective-labeling approach coupled with RT-NMR. These multiplexed assays reveal details of GEF and GAP specificities, protein binding preferences, and nucleotide-dependent activation states that cannot be accurately assessed using conventional techniques.

Biochemical diversity in the three isoforms of RAS is an open question with large implications for developmental biology and...
cellular transformation. We found that intrinsic rates of exchange and hydrolysis are nearly identical across the three isoforms, as are their sensitivities to activation by SOS or inactivation by p120GAP. It appears that the core G-domains of HRAS, KRAS, and NRAS are biochemically equivalent and that biological differences are likely determined by post-translational modification, subcellular localization, or distinct effector–protein interactions. It will be interesting to determine whether these results hold true for other RAS-GEF proteins such as RASGRFs or RASGRPs (46, 47) and to examine GEF specificity against all 35 RAS subfamily GTPases (4).

Using high protein concentrations to profile GTPase kinetics revealed a strong dependence on Mg$^{2+}$ for nucleotide exchange. The Mg$^{2+}$ concentration in mammalian cells has been estimated at 17–20 mM; however, less than 5% of that is presumed free (48, 49). The cytoplasm (where membrane-tethered small GTPase proteins are exposed) is expected to

**Figure 6. Multiplexed GTPase binding interactions.** For each panel, the unbound-state spectrum of GTPase mixture is displayed in black. The spectrum of the GTPase mixture in the presence of each effector is colored as labeled in each panel. Peaks corresponding to each GTPase are highlighted by coloring described in legends (top). Displayed molar ratios are (effector):(total protein). A, GTPase–effector complex structures of HRAS and the RBD of RAF1, RALB and RLIP76 and the complex structure of HRAS with the engineered NS1 monobody. B, selectively labeled, GMPPNP-loaded H-, K-, and NRAS (black spectrum) were mixed with ARAF RBD (red). C, selectively labeled, GMPPNP-loaded H-, K-, and NRAS (black spectrum) were mixed with BRAF RBD (red). D, selectively labeled, GMPPNP-loaded KRAS, NRAS, and RHOG (black spectrum) were mixed with BRAF RBD (black). E, selectively labeled, GMPPNP-loaded KRAS, HRAS, and RALA (black spectrum) were mixed with RLIP76 RBD (blue). F, selectively labeled, GMPPNP-loaded H-, K-, and NRAS (black spectrum) were mixed with NS1 monobody (orange).
Multiplexed GTPase cycling and effector interactions

have only 0.5–1 mM free Mg²⁺, significantly less than used in most in vitro kinetic assays. Several thoughtful experiments from nearly three decades ago estimated the Mg²⁺ affinity for WT HRAS at 2.8 μM (50) and resolved that high Mg²⁺ concentrations in RAS exchange assays significantly slow GDP dissociation rates. Moreover, there are intriguing data that Mg²⁺–GTP affinity for HRAS is higher than that of Mg²⁺–GDP (37). This would be consistent with our observations of increased nucleotide exchange in conditions of Mg²⁺ scarcity, but how this affects RAS activity in cells is unknown. We can speculate that high-density RAS nanoclusters may be influenced by Mg²⁺ availability, which could act to promote nucleotide exchange.

Table S3 compiles the existing knowledge of RAS nucleotide binding and the little that is known about Mg²⁺ affinity. Most of these early data were generated by measuring retention of radiolabeled nucleotides on filters, but these detailed assessments of GTPase biochemistry and function should be reconsidered using modern approaches.

Multiple lines of evidence suggest functional interplay between WT and mutant RAS proteins, with claims that WT RAS can suppress oncogenic mutant activity (19, 51–53) and that mutants promote WT RAS activation (54, 55). Multiplexing GTPase mutants with their WT counterpart G-domains provided an opportunity to explore direct biophysical effects on hydrolysis or exchange. Our results corroborate observations that mutations at Gly-12 (Cys or Val) or Gln-61 (Leu) have unique hydrolysis or exchange. Our results corroborate observations that removal of specificities for multiple GTPases.

Experimental procedures

Plasmid constructs

HRAS WT and G12V (residues 1–171); KRAS WT, G12C, and Q61L (residues 1–171); the GAP-334 region of human p120GAP (residues 715–1047); and the SOS₁ domain of human SOS1 (residues 564–1049) were cloned into pET15b (Novagen/EMD Biosciences) with an N-terminal polyhistidine (His) tag. NRAS (residues 1–172), RHOG (residues 1–179), RALA (residues 1–183), and RAC1 WT and P29S (residues 1–177) were cloned into pDEST17 using Gateway Technology, with a thrombin cleavage site inserted between the His tag and GTPase. Mouse RHEB (residues 1–169), human BRAF RBD (residues 150–233), and human RLIP76 RBD (residues 395–517) were cloned as a GST fusion in pGEX-2T. RHEB constructs were a kind gift from Dr. Vuk Stambolic (University Health Network (UHN), Toronto, Canada). The NS1 monobody, expressed as a GST fusion protein, was a gift from Dr. Shohei Koide (NYU Langone).

Protein expression and purification

For unlabeled GST-tagged proteins, proteins were expressed in Escherichia coli BL21 codon+ cells in LB medium by induction with 0.25 mM isopropyl β-D-thiogalactopyranoside at 16 °C overnight. Uniformly ¹⁵N-labeled His- or GST-tagged proteins were expressed similarly but in minimal M9 medium supplemented with 1 g/liter ¹⁵NH₄Cl. Cells were lysed using sonication in 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 10% (v/v) glycerol, 0.4% Nonidet P-40, protease inhibitors, and either 1 mM DTT or 5 mM β-mercaptoethanol. Lysates were cleared by centrifugation and incubated with nickel-nitrilotriacetic acid or GSH resin for 1–2 h at 4 °C. After washing in high-salt buffer (20 mM Tris (pH 7.5), 500 mM NaCl, 5 mM MgCl₂, and 1 mM DTT or 5 mM β-mercaptoethanol), His-tagged proteins were eluted with 250 mM imidazole followed by thrombin cleavage. GST fusions were cleaved with thrombin directly on the GSH resin overnight at 4 °C. Cleaved proteins were then further purified through an S75-size exclusion column. WT GTPases purified from E. coli are predominantly in the GDP form. Proteins were preloaded with GMPPNP or GTPÀS when required. For nucleotide exchange, GTPases were incubated at 37 °C in the presence of 10 mM EDTA and a 10-fold molar excess (nucleotide:protein) of GMPPNP, GTPÀS, or GTP (Sigma-Aldrich) for 10 min. 20 mM MgCl₂ was then added to the sample, and the sample was placed on ice and immediately dialyzed at 4 °C or run through an S75 size-exclusion column. For hydrolysis assays, aliquots of GTP-loaded H₃, K₃, and NRAS were flash frozen and stored at −80 °C.

¹⁵N-Amino acid–selective labeling

Selectively labeled (¹⁵N)threonine, (¹⁵N)tyrosine, (¹⁵N)leucine, (¹⁵N)valine, and (¹⁵N)isoleucine) proteins were expressed by culturing cells in 37 °C in M9 medium supplemented with 1 g/liter ¹⁴NH₄Cl and a 100 mg/liter concentration of the 19 unlabeled amino acids. Cultures were grown to an OD₆⁰⁰ of 0.6–0.9, at which point 1 g/liter each of the 19 unlabeled amino acids and 100 mg/liter ¹⁵N-labeled amino acid were added. Amino acids were allowed to dissolve for 15 min, at which point 0.25
mm isopropyl β-D-thiogalactopyranoside was added to the culture, which was then incubated overnight at 16 °C.

**NMR spectroscopy**

NMR data were recorded at 25 °C on 600-MHz Bruker UltraShield spectrometer equipped with a 5-mm CryoProbe and 1.7-mm TCI MicroCryoProbe. All multiplexed assays were performed on the 1.7-mm cryoprobe. These samples of 40 μl were prepared with the composition and concentration of each small GTPase described under “Results” and/or in figure legends. Nucleotide exchange assays, GTP hydrolysis assays, and GTPase/effector titrations were typically performed in 20 mM Tris (pH 7.5), 100 mM NaCl, 5–15 mM MgCl₂, 1 mM DTT, and 10% D₂O. Two-dimensional [¹H-¹⁵N] HSQC spectra were collected sequentially to monitor kinetics. All multiplexed experiments used band-selective excitation short-transient (BEST) HSQCs ([59]. Spectra were processed with NMRPipe ([60]) and analyzed using NMRView ([61]). For intrinsic exchange and GEF assays, GTPγS was added at an 8- or 10-fold molar excess (GTPγS:Stotal protein), and SO₃-cat was added at molar ratios described in our results. To calculate the GDP-bound ratio ($I_{GDP}/(I_{GDP} + I_{GTP})$), peak intensities were extracted from each individual spectrum using NMRView. Exchange curves were plotted and fitted to a single-phase exponential decay function using GraphPad Software. For intrinsic GTP hydrolysis and GAP assays, peak intensities were extracted, and data were fit to a one-phase exponential association function. For effector/monobody titrations, unlabeled RBD or NSI monobody was titrated into selectively labeled mixtures of GTPases.

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