Biochemical Classification of Disease-associated Mutants of RAS-like Protein Expressed in Many Tissues (RIT1)*

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RAS-like protein expressed in many tissues 1 (RIT1) is a disease-associated RAS subfamily small guanosine triphosphatase (GTPase). Recent studies revealed that germ-line and somatic RIT1 mutations can cause Noonan syndrome (NS), and drive proliferation of lung adenocarcinomas, respectively, akin to RAS mutations in these diseases. However, the locations of these RIT1 mutations differ significantly from those found in RAS, and do not affect the three mutational “hot spots” of RAS. Moreover, few studies have characterized the GTPase cycle of RIT1 and its disease-associated mutants. Here we developed a real-time NMR-based GTPase assay for RIT1 and investigated the effect of disease-associated mutations on GTPase cycle. RIT1 exhibits an intrinsic GTP hydrolysis rate similar to that of H-RAS, but its intrinsic nucleotide exchange rate is ~4-fold faster, likely as a result of divergent residues near the nucleotide binding site. All of the disease-associated mutations investigated increased the GTP-loaded, activated state of RIT1 in vitro, but they could be classified into two groups with different intrinsic GTPase properties. The S35T, A57G, and Y89H mutants exhibited more rapid nucleotide exchange, whereas F82V and T83P impaired GTP hydrolysis. A RAS-binding domain pulldown assay indicated that RIT1 A57G and Y89H were highly activated in HEK293T cells, whereas T83P and F82V exhibited more modest activation. All five mutations are associated with NS, whereas two (A57G and F82V) have also been identified in urinary tract cancers and myeloid malignancies. Characterization of the effects on the GTPase cycle of RIT1 disease-associated mutations should enable better understanding of their role in disease processes.

Small GTPase proteins regulate signal transduction through a conserved “switch” mechanism (1). The GTP-bound state of these proteins adopts an activated conformation that can bind to and activate downstream effector proteins, thus driving signal transduction. Hydrolysis of GTP to GDP then terminates the signaling event (Fig. 1A). GTPase proteins have the ability to hydrolyze GTP (intrinsic hydrolysis), and to become reactivated by releasing GDP and binding a new molecule of GTP (intrinsic exchange), although both of these processes occur slowly. Most GTPases can be rapidly inactivated by GTPase-activating proteins (GAPs), which catalyze nucleotide hydrolysis, whereas guanine nucleotide exchange factors (GEFs) promote their reactivation by accelerating the exchange of GDP for GTP. Mutations in small GTPases or their GAPs and GEFs can disrupt this GTPase cycle and result in diseases such as cancer and developmental disorders (2, 3).

Recent genetic analyses of Noonan syndrome (NS) patients have shown that mutations in RIT1 can cause this condition (Fig. 1B and Table 1) (4–11). NS is a “RASopathy” characterized by short stature, distinctive facial features, and heart defects, caused by mutations in several RAS signaling pathway genes, including PTPN11, SOS1/2, RAF1, KRAS, NRAS, or BRAF (12–14). RIT1 belongs to the RAS subfamily, sharing ~44% sequence identity with H-, K-, and N-RAS, but is distinct in that it has a unique N-terminal extension of unknown function, and its C-terminal hypervariable region lacks prenylation and palmitoylation motifs, although reportedly, it can be recruited to the plasma membrane through a C-terminal polybasic region (15, 16). There are three splice variants of RIT1: isoform 2 contains 18 extra N-terminal residues relative to RAS; an additional exon in isoform 1 further extends the N terminus by an additional 17 amino acids; and isoform 3 initiates at a Met downstream of the G1 box and thus lacks a critical component of the nucleotide binding site (Fig. 1B). In this study, the residue numbering is based on RIT1 isoform 2, consistent with previous studies. RIT1 activates some of the same effector proteins as RAS, including RAF kinases and RAL GEFs (15), however, potential GAPs and GEFs for RIT1 remain unknown.

An engineered hyperactivated RIT1 mutant (Q79L, which corresponds to RAS Q61L) has been reported to transform

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6 The abbreviations used are: GAP, GTPase-activating proteins; RIT1, RAS-like protein expressed in many tissues; GEF, guanine nucleotide exchange factors; RBD, RAS-binding domain; HSQC, heteronuclear single-quantum coherence; NS, Noonan syndrome; GTPγS, guanosine 5’-3-O-(thio)triphosphate; GMP-PNP, guanosine 5’-β,γ-imido-triphosphate.
NIH3T3 cells, suggesting that RIT1 signaling could promote oncogenesis (17, 18). Subsequently, RIT1 mutations were identified as driver mutations in ~2% of lung adenocarcinomas (19), and more recently RIT1 mutations have been reported in a wide variety of human cancers, including myeloid malignancies, hepatocellular carcinoma, breast cancer, and others (20–23). The RIT1 disease-associated mutations reported to date (summarized in Fig. 1B and Table 1) are dispersed widely throughout the gene and affect codons distinct from the three mutational hot spots in H-, K-, and N-RAS (i.e., Gly-12, Gly-13, and Gln-61), making it difficult to predict their effects on the GTPase cycle (17, 18). Subsequently, RIT1 mutations were identified as driver mutations in ~2% of lung adenocarcinomas (19), and more recently RIT1 mutations have been reported in a wide variety of human cancers, including myeloid malignancies, hepatocellular carcinoma, breast cancer, and others (20–23). 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TABLE 1
RIT1 cancer and NS-associated mutations identified in patients

| RIT1 residues | Cancer-associated somatic mutations (No.) | NS-associated germline mutations (No.) | Corresponding KRAS4B residues | Conserved with KRAS4B? |
|---------------|------------------------------------------|---------------------------------------|-------------------------------|------------------------|
| Ser-19        | Leu (2)                                  | Asn (1)                               | Pro (1), Ser (1), Thr (5)     | Met-1                  | No                     |
| Lys-23        | Glu (2)                                  | Lys-K5                                |                              |                        | Yes                    |
| Gly-30        |                                          | Gly-12                                |                              |                        | Yes                    |
| Gly-31        |                                          | Gly-13                                |                              |                        | Yes                    |
| Gly-33        | Trp (1)                                  | Gly-15                                |                              |                        | Yes                    |
| Lys-34        | Thr (1)                                  | Lys-16                                |                              |                        | Yes                    |
| Ser-35        |                                          | Ser-17                                |                              |                        | Yes                    |
| Thr-38        | Ala (2)                                  | Thr-20                                |                              |                        | Yes                    |
| Gln-40        | Leu (3)                                  | Gln-22                                |                              |                        | Yes                    |
| Arg-45        | Gln (2)                                  | His-27†                               |                              | Asp-33                 | Conservative           |
| Asp-51        | Val (1)                                  | D51Y (2)                              |                              | Ser-39                 | Conservative           |
| Ala-57        | Gal (1)                                  | Gly (24)                              |                              |                        |                        |
| Leu-74        | Met (1)                                  | Leu-56                                |                              |                        | Yes                    |
| Ala-77        | Pro (3), Ser (2)                         | Ala-59                                |                              |                        | Yes                    |
| Gly-79        |                                          | Gln-61                                |                              |                        | Yes                    |
| Glu-81        | Gln (2), Gly (1)                         | Gly (1), Gln (1)                      |                              | Glu-63                 | Yes                    |
| Phe-82        | Cys (4), Leu (3), Val (1), Ile (1)       | Ile (1), Leu (17), Val (6), Ser(1)    |                              | Tyr-64                 | Conservative           |
| Thr-83        | Pro (2)                                  |                                      |                              | Ser-65                 | Conservative           |
| Ala-84        |                                            |                                        |                              |                        |                        |
| Asp-87        | Asn (1)                                  | Asp-69                                |                              |                        | Yes                    |
| Tyr-89        |                                         | Tyr-71                                |                              |                        | Yes                    |
| Met-90        | Ile (10)                                 | Met-72                                |                              |                        | Yes                    |
| Gly-95        | Ile (5)                                  | Gly-77                                |                              |                        | Yes                    |
| Phe-108       | Leu (2)                                  | Phe-90                                |                              |                        | Yes                    |
| Arg-112       | Cys (2)                                  |                                         |                              |                        |                        |
| Arg-122       | Leu (3),* (2), Qln (1)                   |                                         |                              |                        |                        |
| Gly-133       | Glu (1)                                  |                                         |                              |                        |                        |
| Arg-154       | Leu (1)                                  |                                         |                              |                        |                        |
| Arg-168       | His (2)                                  |                                         |                              |                        |                        |
| Asp-172       | Glu (1)                                  |                                         |                              |                        |                        |
| Asp-173       | Asn (1)                                  |                                         |                              |                        |                        |
| Arg-180       | Leu (1)                                  |                                         |                              |                        |                        |
| Arg-183       | His (1)                                  |                                         |                              |                        |                        |
| Ala-192       | Thr (3)                                  |                                         |                              |                        |                        |
| Phe-211       | Leu (2)                                  |                                         |                              |                        |                        |
| Asp-216       | Tyr (2)                                  |                                         |                              |                        |                        |

* Nonsense mutation.
NA, not applicable.

Table 1 shows biochemical classification of RIT1 with respect to KRAS4B residues. Strength and high pH (450 mM NaCl, pH 8) was required to prevent precipitation of RIT1 during concentration of the final NMR sample. These conditions were used throughout the purification as well as the GTPase assays described below. The size exclusion chromatography elution profiles revealed that addition of 5 μM GDP to the running buffer reduced aggregation of the protein (data not shown), and RIT1-GDP eluted as a monomer under these conditions as measured by in-line multi-angle light scattering (Fig. 2A).

High quality 1H/15N HSQC spectra of RIT1 (0.8 mM) were obtained for both the GMP-PNP-bound state and the GDP-bound state under the optimized buffer conditions (Fig. 2B). Standard triple-resonance backbone experiments were used to assign 125 cross-peaks for the GDP-bound form (Table 2). To initiate nucleotide exchange, a 10-fold molar excess of the non-hydrolysable GTP analog GTPγS was added to GDP-loaded GTPase samples, and HSQC spectra were collected in succession to monitor the reaction. Compared with H-RAS, RIT1 exhibited ~4-fold faster nucleotide exchange (Fig. 3A and Table 2). To measure intrinsic nucleotide hydrolysis, the samples were loaded with GTP before collection of successive HSQC spectra (Fig. 2C). RIT1 exhibited a 2.2-fold faster hydrolysis rate relative to H-RAS (Fig. 3B).

The Effect of Disease-associated Mutations on RIT1 GTPase Cycle—Next, we investigated how engineered and disease-associated mutations affect the GTPase cycle of RIT1. We chose to study six mutations that have been associated with NS (S35T, A57G, F82V, T83P, Y89H, and G95A) and an engineered mutation, Q79L. Two of these mutations (A57G and F82V) have also been found in human cancers. Three of these mutations, S35T, A57G, and Y89H, increased the intrinsic nucleotide exchange rate by ~4-fold, the Q79L and T83P mutations doubled the exchange rate, and F82V had no appreciable effect (Fig. 3, C and D). We then examined the effects of these mutations on GTP hydrolysis, beginning with RIT1 Q79L, an engineered mutation of the putative catalytic residue corresponding to RAS Gln-61 (27). As expected, the Q79L mutation strongly impaired GTase activity, reducing the hydrolysis rate by 17-fold. Of the disease-associated mutations, F82V and T83P had the largest effect on GTP hydrolysis, reducing the rate by ~3-fold, fol-
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The ratio of the exchange to hydrolysis rates \((k_E/k_H)\) for RIT1 is 2-fold higher than that of H-RAS under matched high salt conditions, implying a higher basal activation level. Here we propose a formula (see “Experimental Procedures”) to predict the fraction of H-RAS, RIT1, and their mutants in the activated state, on the basis of their intrinsic exchange and hydrolysis rates (Fig. 4B). The predicted basal activation level of H-RAS was 30% at 100 mM NaCl; however, the effects of high salt (450 mM NaCl) on \(k_E\) and \(k_H\) increased this value to 84% (Table 2). In the same high salt buffer, 90% of wild-type RIT1 was predicted to be in the activated state, primarily because it exhibits faster exchange (Fig. 4B). However, the basal activation level of RIT1

followed by S35T (2.4-fold decrease). The A57G and Y89H mutations each caused a modest (1.5-fold) reduction in hydrolysis rate (Fig. 3, E and F).

The RIT1 disease-associated mutants investigated in this study exhibited either a faster nucleotide exchange rate, a slower hydrolysis rate, or both (Fig. 4A), strongly suggesting that they would exist in a more highly activated state in vivo. We propose that these mutants can be classified into two groups according to their biochemical properties: fast-exchangers versus slow-hydrolyzers. The fast-exchanger group includes S35T, A57G, and Y89H, whereas the slow-hydrolyzer group includes F82V and T83P, as well as the engineered mutant Q79L.

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would probably be lower under physiological salt concentrations, which we were unable to investigate due protein precipitation (extrapolating the effect of salt on H-RAS suggests ~44% basal activation level of RIT1).

The activation of RIT1 was further increased by mutation. The engineered mutant Q79L was the most highly activated mutant, with nearly complete GTP loading due to its impaired ability to hydrolyze GTP. Of the disease-associated mutants, the fast-exchangers were predicted to exhibit higher overall levels of activation than the slow-hydrolyzers. Mutations that perturb both exchange and hydrolysis (S35T and T83P) lead to particularly high levels of activation (Fig. 4, A and B). Using data from our previously published characterization of the intrinsic exchange and hydrolysis rates of H-RAS, we used the same formula to predict the fraction of activated H-RAS oncogenic mutants (25). The H-RAS mutants G12V and Q61L are slow-hydrolyzers, whereas G13D is a fast-exchanger. The impact of these H-RAS mutations on the intrinsic hydrolysis and exchange rates (Fig. 4, C and D) increases the basal activation state from 30 to 70~99% at 100 mM NaCl. The G13D substitution introduces a negative charge in the phosphate-binding site that renders binding of nucleotide less favorable (28). Affinity...
for nucleotide can also be reduced through an engineered H-RAS mutation (F28L) that disrupts a hydrophobic interaction with the base (29). This approach has been extended to produce analogous fast-exchange mutants of RhoA, Rac1, and Cdc42, which better mimic deregulated oncogenic RHO-GEF activity than mutants with impaired GTP hydrolysis (30). Thus, mutations that decrease nucleotide affinity can increase nucleotide exchange and the activation of small GTPases. It is interesting that three NS-associated mutations in RIT1 (S35T, A57G, and Y89H) behave like G13D and F28L in H-RAS, whereas the other two RIT1 NS mutations studied (F82V and T83P) are akin to G12V and Q61L in H-RAS. Furthermore, characterization of RASopathy-associated germ-line K-RAS mutations revealed subsets with fast intrinsic nucleotide exchange or slow hydrolysis (31). Thus, the proposed formula uncovered unexpected similarities between RIT1 and RAS mutants.

**Effects of Disease-associated Mutations on Basal Activation of RIT1 in Cells**—It is important to note that the activation state of RIT1 in *in vivo* is likely to be substantially modulated by GAPs and/or GEFs, and the effect of RIT1 mutations on sensitivity to GAP/GEF activities. Currently, no such regulators have been identified, and this question awaits further investigation. Nevertheless, the overall activation state of a GTPase protein can be estimated by the fraction that can be pulled down through a GTP-dependent manner (data not shown), thus we used BRAF RBD for RIT1 pulldown assays.

Surprisingly, the engineered mutation of the catalytic residue (Q79L) did not significantly affect the amount of RIT1 pulled down by the RBD (Fig. 5), although a similar result was reported previously by Shi and Andres (32). Among five disease-associated RIT1 mutants tested, three of them (A57G, T83P, and Y89H) were pulled down ≥2-fold more than wild-type by BRAF-RBD (Fig. 5). The F82V mutant exhibited modest activation, whereas S35T was not pulled down to a greater extent than wild-type RIT1 (Fig. 5). Thus the predicted GTP loading of RIT1 mutants based on their intrinsic hydrolysis (Fig. 4) and exchange rates is not strongly correlated with the RIT1 activation state inferred from the results of the BRAF-RBD pulldown assay, suggesting that mutational effects occur on multiple levels in cells. For example, some mutations may alter the sensitivity of RIT1 to the actions of GAPs or GEFs in cells, which were not present in the GTPase assay. Furthermore, different mutations may differentially impact the affinity of RIT1 for the BRAF RBD probe used to assess activation in the cell, akin to the previously reported effects of the H-RAS G12V mutation on ARAF and RGL1 RBD binding (33). It is important to note that the binding affinity for RAF-RBD is reduced by most of the germ-line and cancer-associated K-RAS mutations that have been studied, including those that are distal from the RBD-binding site (28, 31). The activation state of such mutants would be underestimated in RBD pulldown assays. The effect of Q79L mutation is somewhat puzzling, because there is evidence that this RIT1 mutation enhances phosphorylation of RAF and ERK (4, 19, 32) in the absence of any substantial increase in binding to BRAF (32), suggesting that additional regulatory mechanisms may contribute to RIT1-induced ERK activation.
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Structural Considerations of RIT1 Disease-associated Mutations—To deepen our understanding of the mechanistic rationale for the GTPase cycle defects of the RIT1 mutants, we modeled them using the structure of RIT1-GDP (PDB code 4KLZ) recently solved by Siegal and co-workers,7 and gained insight into these mutations from the extensive literature available on characterization of RAS mutations.

In RAS, the G13D mutation accelerates nucleotide exchange by destabilizing nucleotide binding. A recent crystal structure of K-RAS G13D revealed that the negative charge of the Asp substitution in proximity to the nucleotide phosphates creates unfavorable electrostatic interactions (28). The fast-exchanger RIT1 mutants each appear to destabilize nucleotide binding through different mechanisms. The side chain hydroxyl of RIT1 Ser-35 is highly conserved in RAS GTPases, and is involved in coordinating the Mg2+ ion that is required for high affinity nucleotide binding. The S35T mutation appears to destabilize nucleotide binding, either by affecting the rotameric dynamics of the hydroxyl group or by introducing steric hindrance in the nucleotide binding site (Fig. 6, A–D). A decrease in nucleotide affinity would accelerate nucleotide exchange, consistent with the gain-of-function phenotype of S35T reported for ELK transactivation (4). It is possible that the S35T mutant may be highly activated in the GTP-rich cellular environment, but due to its reduced affinity the nucleotide may be lost during washing in the RBD pulldown assay, thus hindering the interaction. Interestingly, threonine is found in this position in many GTPases (ARF, RHO, and RAB subfamilies), but introduction of a more negatively charged residue would accelerate nucleotide exchange, consistent with the gain-of-function phenotype of S35T reported for ELK transactivation (4), its expression at near physiological levels did not reduce ERK phosphorylation (5).

The A57G and Y89H mutations, which cause fast-exchange, are found in/near switch I and switch II, respectively (corresponding to RAS Ser-39 and Tyr-71, Fig. 1C), and the structure of wild-type RIT1-GDP suggests that these disease-associated mutations might perturb Van der Waals interactions between these hydrophobic side chains and Leu-74 (Fig. 6, E and F). Disruption of these interactions could destabilize Switch I or Switch II, respectively, thereby disrupting nucleotide binding and promoting nucleotide exchange (Fig. 6, E and F). The A57G and Y89H mutations were highly activated in the RBD pull-down, and have been shown to enhance activation of ERK (5, 8) and ELK (4, 9).

The RIT1 G95A protein exhibited severely reduced stability in vitro, and although some protein was present in the soluble fraction of Escherichia coli lysate, it precipitated during purification. Interestingly, of four NS-associated mutants expressed in Flp-In T-Rex 293 cells by Chen et al. (5), only G95A exhibited reduced expression and did not enhance EGF-induced ERK activation, whereas the other NS mutations (and Q79L) expressed more highly than wild-type and increased ERK activation. By contrast, transient overexpression of RIT1 G95A enhanced ELK transactivation and induced more developmental defects in zebrafish embryos than wild-type (4), suggesting that the intrinsic instability of this mutant protein might be overcome by higher expression levels in zebrafish. The instability of this mutant might be explained by impaired nucleotide binding, akin to other nucleotide-free GTPases such as the RAS homolog enriched in brain (RHEB) mutant D60K, which fails to bind GDP and GTP (36) and exhibited markedly reduced stability and solubility in E. coli expression. RIT1 Gly-95 is located in a glycine-rich hinge region connecting Switch II to β4. The corresponding glycine residues in RAS (Gly-77), together with Gly-75, is thought to confer flexibility on Switch II (37); thus the G95A mutation might impair nucleotide binding by restricting the conformation of Switch II. Notably G95A is the most frequent RIT1 mutation associated with NS and thus is very likely causative, although the underlying mechanism remains unclear. To date, this mutation has not been reported in cancer.

In RAS, Gln-61 is a key catalytic residue for hydrolysis of GTP. The RAS Gln-61 side chain forms an H-bond with a nucleophilic water molecule, which becomes activated to attack the γ-phosphate. The Q61L mutation displaces the bridging water and significantly decreases GTP hydrolysis (38, 39). The loss of RIT1 GTPase activity caused by the engineered mutation of the corresponding residue (Gln-79) suggests conservation of this type of mechanism, although a structure of RIT1 in the GTP-bound form is needed to reveal the details. The two RIT1 mutations that produce slow GTP hydrlyzers, F82V and T83P, are both in close proximity to Gln-79, suggesting that their impact on nucleotide hydrolysis might stem from perturbation of the precise positioning of Gln-79. These two mutations could be analogous to RAS G12V, which impairs GTP hydrolysis by interfering with the positioning of Gln-61 and the bridging water (38). Mutations of RIT1 Phe-82 to Val or Leu and T83P have been shown to increase phosphorylation of

7 D. M. Shah, M. Kobayashi, P. H. Keizers, A. W. Tuin, E. Ab, L. Manning, A. A. Rzepiela, M. Andrews, F. J. Hoedemaeker, and G. Siegal, unpublished data.
ERK (5), and activation of ELK (4, 9). In addition to fast nucleotide exchange, the S35T mutant also exhibited reduced GTPase activity, which is likely related to perturbation of the Mg\(^{2+}\) ion.

Comparison of the Mutational Profiles of RIT1 and RAS—It is interesting to note that among the disease-associated RIT1 mutations reported to date, there is only a single example (10) involving a codon that corresponds to the three RAS hotspots (i.e. RIT1 Gly-30, Gly-31, and Gln-79). Mutation of codon 79 (CAG) of the cDNA impaired GTP hydrolysis by altering the catalytic Gln residue; however, in the RIT1 gene this codon forms the splice site at the 3’ end of exon 4, as noted previously (19). Mutations that affect the second or third position of this codon would alter the consensus AG splice site motif, likely disrupting mRNA splicing and yielding a non-functional polypeptide. Likewise, mutation of the first position could lead to premature termination (TAG), or a very rare splice site (GAG) (40) encoding a Q79E mutation, which by analogy to H-RAS Q61E might accelerate intrinsic nucleotide hydrolysis (41). Of the nine possible point mutations of codon 79, only one (i.e. AAG) would be predicted to produce an activated RIT1 protein (Q79K); however, AAG is also rarely found at splice sites. The engineered RIT1 mutation G30V was shown to be less effective than Q79L or NS-associated mutations at inducing ELK transactivation (4). As this manuscript was in revision, a RIT1 G31R mutation (corresponding to RAS Gly-13) was identified in a patient with Noonan syndrome (10), however, its biochemical and functional properties remain to be characterized. It is not clear why RIT1 Gly-30 and Gly-31 are infrequently mutated relative to RAS, and whether local DNA sequence or structure of the RIT1 gene might render these codons less susceptible to mutation than RAS Gly-12/Gly-13.

Compared with the oncogenic hot spots in RAS isoforms, cancer-associated mutations of RIT1 are much more dispersed throughout the sequence (Fig. 1B and Table 1), resembling the spectrum of RASopathy-associated germ-line RAS mutations (19, 21, 23). Four of the cancer-associated RIT1 mutations affect residues (K23E, Q40L, D172E, and M90I) corresponding to germ-line and low-frequency somatic K-RAS mutation sites (K5N, Q22E, Q22R, D153V, and M72L) (21, 42). There are several examples of the same RIT1 mutation occurring in both NS and cancer (e.g. A57G, A77P, E81G, F82L, F82V, M90I), suggesting that mutations associated with the two diseases do not lead to distinct biochemical properties. The most frequently mutated RIT1 site is Phe-82, where mutations to Cys, Leu, Ile, and Val have been identified in multiple cancer types, whereas Leu and Val substitutions have been found in NS patients. Mutation of the corresponding site in H-RAS (Tyr-64) or RAP1A (Phe-64) impairs both intrinsic and GAP-mediated GTP hydrolysis (43).

The high level of RIT1 activation that results from its intrinsic hydrolysis and exchange rates underscores the importance of identifying potential GAP proteins that inactivate RIT1 in the cell, although none have been identified to date. Resistance to the catalytic activity of GAPs is the major mechanism by which oncogenic RAS mutants maintain high activation levels in cells (39, 44), whereas germ-line RASopathy-associated RAS mutations are highly variable in their impact on GAP sensitivity, ranging from undetectable to profound impairment (31, 45). Differential sensitivity to GAP activity might underlie some of the discordance between in vitro activation of RIT1 mutants and the RBD pulldown assays from cell lysates, although this remains to be investigated if a GAP is identified. RIT1 mutants that are resistant to GAP activity would be dependent on their intrinsic GTPase activity for inactivation.

Some cancer-associated RIT1 mutations (M90I and A77S) have been validated as oncogenic drivers (19), but most remain to be investigated. The cancer-associated mutations are biased toward highly conserved residues, suggesting that many are likely to perturb RIT1 function (Table 1).

Functional Implications of Divergent RIT1 Residues—An alignment of the sequences of RIT1 and three RAS isoforms...
reveals some key amino acid substitutions in the switch regions and the conserved nucleotide-binding G-box motifs (Fig. 1C), which could be responsible for the faster intrinsic exchange observed with RIT1. Compared with RAS, RIT1 has substitutions of residues within or immediately adjacent to all five G-box motifs. Most of these substitutions occur in sites that are fully conserved between H-, K-, and N-RAS, but exhibit some divergence across the RAS superfamily, consistent with our finding that RIT1 nucleotide binding is modestly destabilized, but not completely disrupted. Interestingly, at many of these sites (e.g. RIT1 codons His-50, Ala-57, Ala-80, Phe-82, Ser-136, and Ala-166), the RIT1 side chains are less bulky than those of RAS, potentially reducing the surface area in contact with the nucleotide.

Previous mutagenesis studies of some of these sites in H-RAS shed light on how some of the divergent residues might contribute to the biochemical properties of RIT1. Mutation of H-RAS Ser-39 or Lys-147 have been shown to destabilize nucleotide binding (46–48), and both of these positions bear alanine substitutions in RIT1 (Ala-57 and Ala-166, respectively). H-RAS Lys-147 lies within the G5 box and interacts with Phe-28 and Asp-119 through Van der Waals and polar interactions, stabilizing their side chains in conformations that can form ring stacking and ionic interactions, respectively, with the nucleotide base (Fig. 7A). H-RAS Ser-39 is adjacent to the G2 box in switch I, where it forms interactions with Asp-54 and Leu-56 that restrain the switch in a conformation whereby key G2 residues interact with the nucleotide phosphate groups (Fig. 7D). Substitution of these lysine or serine residues with alanine in RIT1 destabilizes these interactions, indirectly distorting the nucleotide binding site (Fig. 7, B and C).

H-RAS mutagenesis experiments suggest that the RIT1 substitutions in the G3 and G4 boxes (i.e. E62A and C118S, respectively) are unlikely to contribute to faster nucleotide exchange, but provide insight into other functional properties of RIT1. In H-RAS, neither the E62A nor the C118S mutation (analogous to RIT1 Ala-80 and Ser-136) directly impacts intrinsic nucleotide exchange. However, RAS E62A abolishes SOS-mediated nucleotide exchange, and this was identified as a highly conserved residue that is required for the GEF-mediated activation of most small GTPases (49, 50). Substitution of the corresponding residue of RIT1 with Ala, together with the rapid intrinsic nucleotide exchange rate, suggest that RIT1 might not be regulated by a GEF in the cell, or that any potential GEF may function through a non-canonical mechanism. Interestingly, SOS and EPAC (RAS- and RAP-GEFs, respectively) are required for RIT1 activation in cells, however, they fail to activate RIT1 in vitro (51, 52). RAS can be regulated through modification of Cys-118 by nitric oxide, which destabilizes nucleotide binding and promotes activation by accelerating nucleotide exchange (50). Notably, the oncogenicity of K-RAS is reduced by a C118S mutation (53, 54). Hence, the presence of serine at the corresponding position in RIT1 (Ser-136) argues that RIT1 would not be regulated by nitric oxide.

Classification of RIT1 GTPase mutants according to their hydrolysis and exchange properties could be of clinical significance for treatment of RIT1-mutant cancers. It was recently appreciated that specific K-RAS mutations are associated with different responses to targeted therapies. Colon cancer patients with K-RAS G13D, but not Gly-12 mutations benefit from the EGFR inhibitor Cetuximab (55–57), whereas non-small-cell lung cancers with G12C or G12V mutations responded better to a MEK inhibitor than those with other K-RAS mutations (58). The G13D mutation is associated with increased EGF receptor expression and p53 phosphorylation, which are strikingly different compared with codon 12 and 61 mutations (59). The number of patients identified with RIT1 mutant tumors to date is small, and specific targeted therapies for these patients have not been identified. Nevertheless, stratification of patients with specific mutations (or the two classes of mutations defined here) could be important for future analyses of the impact of RIT1 mutation status on clinical outcomes.

**FIGURE 7. Structural analysis of divergent residues of RIT1 relative to RAS.** The crystal structure of RIT1-GDP is shown (PDB code 4KLZ). Substitutions in the G2 and G5 motifs of RIT1 may destabilize nucleotide binding, resulting in faster nucleotide exchange. Panels A and B, RAS Lys-147 is involved in a stabilizing network of side chain interactions (A), which is disrupted by substitution with Ala (A166) in RIT1 (B). Panels C and D, RAS Ser-39 (G2) stabilizes a conformation of switch I that makes favorable interactions with the nucleotide (C), however, this network is disrupted by substitution with Ala (A57) in RIT1 (D).
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Experimental Procedures

Protein Preparation—Human H-RAS (residues 1–171) and RIT1 isoform 2 (residues 17–191) were expressed as His-tagged proteins in E. coli (BL21) from the pET28a vector described previously (60). RIT1 mutations (S35T, A57G, Q79L, F82V, T83P, Y89H, and G95A) were introduced using QuikChange site-directed mutagenesis (Stratagene). Bacteria were grown in minimal M9 media, supplemented with \(^{15}\)NH\(_4\)Cl and (\[^{13}\]C)glucose to prepare samples for resonance assignment, and protein expression was induced with 0.25 mM isopropyl 1-thio-\(\beta\)-d-galactopyranoside at 15 °C overnight. Proteins were purified using nickel-nitritotriacetic acid resin, and His tags were removed by thrombin. The thrombin-cleaved proteins were further purified through Superdex-75 size exclusion chromatography (GE Healthcare). Following purification from E. coli, H-RAS and RIT1 are in the GDP-bound form. Depending on the particular experiment, purified proteins were preloaded with GMP-PNP, GTP\(\gamma\)S, or GTP (Sigma) in the presence of EDTA, which was removed along with the excess nucleotide using a G25 resin PD10 desalting column (GE Healthcare). A construct encoding residues 150–233 of the RBD of BRAF was expressed as His-tagged protein, which was purified as previously described (33).

NMR Spectroscopy—Protein samples were prepared in a sample buffer containing 20 mM Hepes (pH 8), 450 mM NaCl, 5 mM MgCl\(_2\), 2 mM tris(2-carboxyethyl)phosphine hydrochloride, and 5 mM GDP. The final protein concentrations used in the real-time NMR assay and backbone chemical shift assignment experiments were 0.4 and 0.8 mM, respectively. For GTP hydrolysis assays, proteins were loaded with GTP by adding a 10-fold molar excess in the presence of EDTA, and excess reagents were removed by passage through a desalting column (PD MidiTrap G-25, GE Healthcare). For nucleotide exchange assays, we found it necessary to add 1 molar eq (0.4 mM) of GDP to the sample to stabilize some of the RIT1 mutants (e.g. RIT1 A57G precipitated within 15 min at room temperature in the absence of additional nucleotide); therefore, all samples were treated in this manner. Nucleotide exchange was initiated by adding a 10-fold molar excess of GTP\(\gamma\)S to mimic physiological conditions. RIT1 G95A precipitated at 4 °C even in the presence of excess GDP, and could not be purified, suggesting that its nucleotide binding may be severely impaired.

All NMR data were acquired at 25 °C on an 800 MHz Bruker AVANCE II spectrometer equipped with a 5-mm TCI CryoProbe or a 600 MHz Bruker AVANCE III spectrometer with a TXI 1.7-mm CryoProbe. Triple resonance HNCA/CB/CA, HNCC, HNCO, and HNCACO spectra, as well as \(^{1}H-^{15}N\) HSQC spectra, were collected for the backbone chemical shift assignments. To monitor the GTPase reaction, \(^{1}H-^{15}N\) HSQC spectra, were collected for the backbone chemical shift assignments. To monitor the GTPase reaction, \(^{1}H-^{15}N\) HSQC spectra were collected in succession with 2 scans (5 min) for nucleotide exchange assays and 8 scans (20 min) for GTP hydrolysis assays. The spectra were processed with NMRPipe (61), and analyzed with SPARKY (62). Pairs of peaks that exhibited distinct chemical shifts in each nucleotide-bound state and were well resolved from other peaks were selected as reporters of reactions rates. The fraction of RIT1 in the GDP-bound state was calculated from \(I_{\text{GDP}}/(I_{\text{GDP}} + I_{\text{GTP}})\), where \(I\) is the peak intensity of reporters, plotted against time, and fit to a one-phase exponential decay curve using Prism (GraphPad software). The error was estimated as described previously (24).

Formula Used for Calculating Level of Intrinsic Activation of GTPase Proteins—We developed a formula to predict the fraction of RIT1 bound to GTP at steady state when GTP hydrolysis and nucleotide exchange occur concurrently in the presence of a constant 10:1 ratio of GTP to GDP, which is consistent with the cellular GTP to GDP ratio. At steady state, the rate of GTP hydrolysis is equal to the rate of nucleotide exchange. As hydrolysis and exchange are first order reactions, the rates of which depend on the concentration of reactants, this leads to Equation 1 (\(k_p\), exchange rate constant; \(k_{\text{hyd}}\) hydrolysis rate constant; \(F_{\text{GTP}}\) fraction of GTP-bound RIT1 in total RIT1; \(F_{\text{GDP}}\) fraction of GDP-bound RIT1 in total RIT1). This equation demonstrates that intrinsic GTP loading of small GTPases is a function of the ratio of the exchange and hydrolysis rates (\(k_p/k_{\text{hyd}}\) in this isolated system. For this reason, the intrinsic activation level of a small GTPase can be estimated on the basis of these rate constants. This level of intrinsic activation does not consider the activities of GEF-mediated activation or GAP activity. To date, no RIT1 GAPs or GEFs have been reported.

Cell Lines, Transfections, and Assays of Cellular RIT1 Activation—HEK293T cells maintained in DMEM plus 10% FBS and antibiotics were transiently transfected using jetPRIME transfection reagent (Polyplus Transfection, Illkirch, France) according to the manufacturer’s protocol 1 day after seeding. 24 h after transfection, cells were starved in serum-free DMEM overnight before harvesting. The GST-BRAF-RAS binding domain (RBD) pulldown assay was performed as described previously (63). Lysates containing 600 μg of total protein were incubated with 10 μg of GST-BRAF-RBD mixed with 10 μl of 50% slurry of glutathione resin (GeneScript) in a total volume of 600 μl for 45 min at 4 °C. The resin was collected and washed three times before bound proteins were released by boiling the resin in Laemmli sample buffer for 5 min. Samples were then resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and analyzed by immunoblotting to probe for FLAG-tagged RIT1 (anti-FLAG, clone M2, Sigma) in the pulldown and whole cell lysate, as well as ERK2 (anti-ERK1/2, Millipore) in the whole cell lysate, and GST-RBD (anti-GST, Novagen) in the pulldown to ensure equal loading. IRDye infrared secondary antibodies were used for visualization in the Odyssey Infrared imaging system (Li-Cor Biosciences), followed by quantification using Odyssey version 3.0 software. Ratios of each FLAG-RIT1 mutant band in the pulldown relative to whole cell lysate were calculated and normalized to that of wild-type RIT1.

\[
\begin{align*}
\frac{k_{\text{GDP}} \times [\text{RIT1}_{\text{GTP}}]}{k_{\text{GTP}} \times [\text{RIT1}_{\text{GDP}}]} &= \frac{k_p \times [\text{RIT1}_{\text{GTP}}]}{k_{\text{hyd}} \times [\text{RIT1}_{\text{GDP}}]} \\
\frac{k_{\text{GDP}} \times [\text{RIT1}_{\text{GTP}}]}{[\text{RIT1}_{\text{GDP}}]} &= \frac{k_p \times [\text{RIT1}_{\text{GTP}}]}{[\text{RIT1}_{\text{GDP}}]} \\
F_{\text{GTP}} &= \frac{k_p}{(k_p + k_{\text{hyd}})} \\
F_{\text{GDP}} &= 1 / [1 + (k_p/k_{\text{hyd}})]
\end{align*}
\]

EQUATION 1.
Author Contributions—Z. F., C. B. M., and M. I. conceived the project and designed the experiments. Z. F. conducted most of the experiments and J. C. Y. performed Western blot analysis. J. C. Y., Y. X., and B. G. N. contributed new reagents/analytic tools. Z. F., C. B. M., M. T. M.-J., G. M. C. G.-S., M. J. S., T. N., B. G. N., and M. I. analyzed the results. Z. F., C. B. M., and M. I. wrote the paper.

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