The small GTPases K-Ras, N-Ras, and H-Ras have distinct biochemical properties determined by allosteric effects

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H-Ras, K-Ras, and N-Ras are small GTPases that are important in the control of cell proliferation, differentiation, and survival, and their mutants occur frequently in human cancers. The G-domain, which catalyzes GTP hydrolysis and mediates downstream signaling, is 95% conserved between the Ras isoforms. Because of their very high sequence identity, biochemical studies done on H-Ras have been considered representative of all three Ras proteins. We show here that this is not a valid assumption. Using enzyme kinetic assays under identical conditions, we observed clear differences between the three isoforms in intrinsic catalysis of GTP by Ras in the absence and presence of the Ras-binding domain (RBD) of the c-Raf kinase protein (Raf-RBD). Given their identical active sites, isoform G-domain differences must be allosteric in origin, due to remote isoform-specific residues that affect conformational states. We present the crystal structure of N-Ras bound to a GTP analogue and interpret the kinetic data in terms of structural features specific for H-, K-, and N-Ras.

The Ras isoforms, K-Ras4B (referred to here as K-Ras), N-Ras, and H-Ras are GTPases that play key roles as regulators of signal transduction pathways involved in cell growth, differentiation, migration, and apoptosis (1). All Ras proteins are anchored to the membrane via post-translational modifications at their C-terminal hypervariable regions (HVR)3 that guide localization into distinct membrane compartments (2, 3). The catalytic domain, consisting of residues 1–166, actively propagates signaling when bound to GTP and is inactive when bound to GDP, with the P-loop (residues 10–17), switch I (residues 30–40), and switch II (residues 60–76) forming the active site for GTP hydrolysis, as well as an interaction surface for different effectors and regulators (4). The catalytic domain comprises two lobes (Fig. 1). The active site structural elements are found within the first half of the catalytic domain (residues 1–86), referred to as the effector lobe, which is 100% identical in sequence between the Ras isoforms (5, 6). The effector lobe contains three of the five highly conserved motifs found in small GTPases (7, 8). The backbone amides of the P-loop (GXXXXGKS/T) interact with the α- and β-phosphates of the nucleotide, whereas Thr-35 and residues 57–60 of the DXXG motif are important for Mg2+ coordination (9, 10).

On the opposite side of the G-domain is the allosteric lobe, comprising the second half of the catalytic domain (residues 87–166). The allosteric lobe contains the NKXD (residues 116–119) motif responsible for the specificity of the guanine nucleotide (11), and the EXSAK (residues 143–147) motif, aided by effector lobe residue Phe-28, helps to stabilize nucleotide binding. The allosteric lobe contains hot spots of protein–ligand interactions (5) that bind membrane components (12) and harbors all of the residue differences between the Ras G-domains, with sequence identity of 90% between allosteric lobes of the three Ras proteins.

The isoforms of Ras exist in a diverse ensemble of conformations, regulated by the nucleotide-bound state (13). In the GTP-bound state, switch I in the active site interconverts between open (state 1) and closed (states 2) conformations (Fig. 1), as assessed by NMR (14, 15). States 1 and 2 are associated with different biochemical activities, and the equilibrium between these states differs among small GTPases (15, 16). State 1 favors nucleotide exchange but inhibits interaction with effector proteins (16). In contrast, state 2 is associated with effector binding and GTP hydrolysis (15).

In the Ras–Raf–RBD complex, with switch I stabilized in state 2, switch II can attain two states corresponding to catalytically incompetent (T state) and catalytically active (R state) conformations (Fig. 1) (17). The allosteric lobe contains an allosteric site that binds Ca2+ and a negatively charged ligand to promote a disorder to order transition in switch II through a shift in helix 3/loop 7 toward helix 4, placing Gln-61 at the catalytic center (18). We associate this ordered state with an increase in intrinsic hydrolysis in the presence of Raf (17, 19–21).

Regulation of the signaling state of Ras is a result of activation by guanine nucleotide exchange factors that catalyze the loading of GTP (22) and deactivation by GTPase-activating proteins.
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Figure 1. Catalytic G-domain of Ras showing the two lobes and conformational states. Three models are superimposed: H-Ras with switch I in state 1 and the allosteric switch (helix 3/loop 7) in the R state (magenta, PDB code 4EFL); H-Ras with switch I in state 2 and the allosteric switch in the T state (gray, PDB code 2RGE); and H-Ras with switch I in state 2 and the allosteric switch in the R state (orange, PDB code 3K8Y). The effector lobe is shaded in gray, and the allosteric lobe is shaded in green. Tyr-32, which is close to the γ-phosphate of the nucleotide, and Thr-35, which interacts with the Mg\(^{2+}\) ion when switch II is in state 2, are both turned away from the active site in state 1. The conformational change in going from T state to R state is indicated by an arrow. In the T state, helix 3 clashes with switch II, promoting a disordered conformation, whereas in the R state there is room for the switch II helix to form.

(GAPs) that increase the GAPase activity of Ras (23). Although GAP is effective in complex with the wild-type Ras proteins, oncogenic mutants are insensitive to regulation by GAPs (24). Raf is a unique effector protein in that it interacts with Ras at switch I, but not switch II, and binds with nanomolar affinity (21, 25). In this context, switch II is free for allosteric modulation by Ca\(^{2+}\) binding in the allosteric site, and we hypothesize that this leads to specific regulation of intrinsic hydrolysis in the context of Raf signaling, a mechanism that is independent from regulation by GAPs (17–21, 26, 27). This scenario is consistent with the observation that binding of Raf-RBD to Ras fully inhibits GAP activity (28) and that GAP is generally a poor competitor of Ras–effector interactions (28, 29). We have experimentally linked allosteric modulation with signaling through the Ras/Raf/MEK/ERK pathway in NIH-3T3 cells and shown that GAP hydrolysis is completely abrogated in the Q61L oncogenic mutant in complex with Raf (19). It is clear that both GAP-catalyzed and intrinsic hydrolysis of GTP on Ras play important and unique roles in signal attenuation.

Little effort has been made to place the biochemical activities of the Ras isoforms in their individual contexts, and nearly all of the structural biology and biochemistry work has used H-Ras as a model for all three isoforms. In fact, there are no published crystal structures of wild-type K-Ras or N-Ras in the GTP-bound form, although there are structures of a few K-Ras oncogenic mutants bound to GTP analogues, a structure of N-Ras-GDP, and several structures of K-Ras and its mutants in the GDP-bound form, often bound to inhibitors of nucleotide exchange (30). This lack of structural information for wild-type K-Ras and N-Ras in the GTP-bound active state leaves H-Ras as the representative of all three isoforms for discerning mechanisms at the molecular level.

Early work on full-length H-Ras and N-Ras hinted at differences in intrinsic hydrolytic activity (31, 32). These results have received little attention, as the experimental studies were performed under diverse reaction conditions. More recently, molecular dynamics simulations of Ras in its nucleotide-free (6) and nucleotide-bound (33, 34) forms show that H-, K-, and N-Ras differ in their overall dynamics. However, the assumption that the identical active sites in the Ras isoforms leads to identical kinetics has prevailed, with an exclusive reliance on H-Ras for linking biochemical and structural properties.

Here, we present a systematic study of intrinsic hydrolytic reactivity of H-, K-, and N-Ras based on single-turnover experiments performed under the same conditions for the three wild-type isoforms alone and in the presence Raf-RBD. Determination of the hydrolysis rate constants for Ras loaded with radioactive GTP shows that there are significant differences in the biochemical activities between the isoforms. The rate constants for hydrolysis catalyzed by K-Ras and N-Ras are about one-third of that observed for H-Ras. Raf-RBD has little or no effect on hydrolysis for H-Ras and N-Ras, but it increases the rate constant for K-Ras to the value obtained for H-Ras. In addition to hydrolysis rate constants, we present the crystal structure of N-Ras in its GTP-bound state for a detailed comparison with H-Ras and K-Ras and test the effects on hydrolysis of specific mutants in the allosteric lobe that differ among the isoforms. Although we find that these point mutants do not significantly affect the GTP hydrolysis rate constant on H-Ras, we suggest key structural features that together may drive differences in allosteric modulation between the isoforms.

Results

Kinetics of GTP hydrolysis on H-, K-, and N-Ras in the absence and presence of Raf-RBD

Single-turnover experiments were performed to obtain rate constants for hydrolysis of GTP catalyzed by Ras, using a discontinuous assay based on the production of \(^{32}\)P, as described previously (27, 35). Briefly, Ras is loaded with [\(\gamma\)-\(^{32}\)P]GTP (PerkinElmer Life Sciences); samples are removed from the reaction mixture at specific time points; the \(^{32}\)P product is isolated using an organic extraction method (36, 37), and the radioactivity is measured with a scintillation counter (300SL, LabLogic). All hydrolysis experiments were performed in triplicates. Combined experimental data, i.e. \(^{32}\)P product concentrations versus reaction time, were subjected to global nonlinear regression analysis (38) by using the software package DynaFit (39, 40). The data were fit to a single-step, first-order kinetic model represented by Equation 1, where \(P_i\) is the experimentally observed radioactive inorganic phosphate.
buffer protonation enthalpies, and this issue has been extensively discussed elsewhere (44, 45). For the K-Ras and Raf-RBD interaction, the \( K_d \) is 98 ± 2 nm. This and all other thermodynamic parameters obtained for the interaction between K-Ras and Raf-RBD are similar to those obtained for H-Ras, with a nearly identical \( \Delta G^0 \) of -9.56 kcal/mol. The N-Ras–Raf-RBD interaction has a lower affinity, with \( K_d \) of 205 ± 8 nm, very similar \( \Delta H^0 \) values, but lower \( \Delta S^0 \), implying that binding between N-Ras and Raf-RBD is less entropically favorable than for the other two isoforms.

### Crystal structure of wild-type N-Ras bound to the GTP analogue GppNHp

To correlate our kinetic data with Ras structural features, we first identified specific locations of isoform variation in H-, K-, and N-Ras. A direct comparison of G-domain sequences shows that there are 17 residue positions in the allosteric lobe that differ in at least one of the three isoforms and that they cluster near the highly conserved nucleotide-binding NKFold and EXSAK motifs characteristic of small GTPases (46), as well as on helix 3 (Fig. 3a). Of these 17 residues in the allosteric lobe, 9 of them (53%) are unique to N-Ras, 7 (41%) are unique to H-Ras, and only 5 (29%) are unique to K-Ras.

In H-Ras, residue differences cluster at residues 121–122 (loop 8) and 126–128 (helix 4). In N-Ras the differences cluster at residues 94–95 (helix 3) and 131–132 (helix 4). In contrast, K-Ras lacks two or more consecutive sequence variations, with its five unique residues dispersed throughout the allosteric lobe (residues 95, 107, 122, 153, and 165). Interestingly, there are only two positions that differ in all three isoforms: residue 95 in helix 3 and residue 122 in loop 8. By mapping these sequence variations onto the respective isoforms in Fig. 3b, it becomes clear that the isoform differences cluster architecturally around the conserved allosteric site that we propose modulates hydrolysis in the presence of Raf (helix 3, loop 7, and helix 4) (19, 21).
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**Table 3. Amino acid sequence differences between H-, K-, and N-Ras.**

| HRAS | KRAS | NRAS |
|------|------|------|
| MTEYKLVV | MTEYKLVV | MTEYKLVV |
| GAVGKY8 | GAVGKY8 | GAVGKY8 |
| DQSLTIQHVF | DQSLTIQHVF | DQSLTIQHVF |
| DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA | DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA | DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA |
| β1 | β1 | β1 |
| α1 | α1 | α1 |
| G2 | G2 | G2 |
| MTEYKLVV | MTEYKLVV | MTEYKLVV |
| GAVGKY8 | GAVGKY8 | GAVGKY8 |
| DQSLTIQHVF | DQSLTIQHVF | DQSLTIQHVF |
| DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA | DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA | DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA |
| β2 | β2 | β2 |
| α2 | α2 | α2 |
| G3 | G3 | G3 |
| MTEYKLVV | MTEYKLVV | MTEYKLVV |
| GAVGKY8 | GAVGKY8 | GAVGKY8 |
| DQSLTIQHVF | DQSLTIQHVF | DQSLTIQHVF |
| DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA | DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA | DGSFLDILTAQGEEYSAMRDQIMRTGEGFLCVFA |
| β3 | β3 | β3 |
| α3 | α3 | α3 |
| G4 | G4 | G4 |
| INNTKSFEDIHQREIQKRDSDVPVLVNGKCDLAAKTRVESQAOQLLARSGYIGPIETSAKTRQGVVEDAFYTLVREIRQH | INNTKSFEDIHQREIQKRDSDVPVLVNGKCDLAAKTRVESQAOQLLARSGYIGPIETSAKTRQGVVEDAFYTLVREIRQH | INNTKSFEDIHQREIQKRDSDVPVLVNGKCDLAAKTRVESQAOQLLARSGYIGPIETSAKTRQGVVEDAFYTLVREIRQH |
| α4 | α4 | α4 |
| G5 | G5 | G5 |
| NNXD | NNXD | NNXD |
| NH2D | NH2D | NH2D |
| NH3 | NH3 | NH3 |
| X | X | X |
| DN H | DN H | DN H |
| SAK | SAK | SAK |
| NKCD | NKCD | NKCD |
| motif | motif | motif |
| G1 | G1 | G1 |
| P-loop | P-loop | P-loop |
| G2 | G2 | G2 |
| switch I | switch I | switch I |
| G3 | G3 | G3 |
| switch II | switch II | switch II |
| G4 | G4 | G4 |
| NKD and E-XS-XK motifs | NKD and E-XS-XK motifs | NKD and E-XS-XK motifs |
| G5 | G5 | G5 |
| motifs | motifs | motifs |
| G1 | G1 | G1 |
| is the P-loop; G2 is switch I; and G3 is switch II. In the allosteric lobe, G4 and G5 are the NKD and E-XS-XK motifs, respectively, important for binding the guanine base of the nucleotide. | is the P-loop; G2 is switch I; and G3 is switch II. In the allosteric lobe, G4 and G5 are the NKD and E-XS-XK motifs, respectively, important for binding the guanine base of the nucleotide. | is the P-loop; G2 is switch I; and G3 is switch II. In the allosteric lobe, G4 and G5 are the NKD and E-XS-XK motifs, respectively, important for binding the guanine base of the nucleotide. |

**Figure 3. Amino acid sequence differences between H-, K-, and N-Ras.**

A, sequences of the three Ras isoforms are shown, numbered every 10 residues. Amino acid residues common to H-, K-, and N-Ras are in black. Residues that are different in any isoform are shown in green in the H-Ras sequence (top row). Residues in K-Ras that are the same as in H-Ras are shown in green in the K-Ras sequence, and those that are different from H-Ras are shown in yellow (middle row). Residues in N-Ras that are the same as in H-Ras are shown in green, and those that are the same as in K-Ras are shown in yellow, and those that are unique to N-Ras are in cyan (bottom row). The G elements found in all GTPases are shown in red with boxes above the Ras sequences labeled G1–G5. In the effector lobe, G1 is the P-loop; G2 is switch I; and G3 is switch II. In the allosteric lobe, G4 and G5 are the NKD and E-XS-XK motifs, respectively, important for binding the guanine base of the nucleotide.

and the NXXD and E-XS-XK motifs that directly affect nucleotide binding (loop 8, helix 4 and helix 5) (10). The locations of these residues in relation to the allosteric site and active-site motifs implicate these regions as differentiating the kinetics we observed for H-, K-, and N-Ras isoforms.

A significant number of crystal structures of Ras and its mutants have been deposited in the PDB (13, 47). However, to correlate the kinetic differences with structural features it is desirable to compare crystal structures of each of the isoforms in their active GTP, or GTP-analogue, states. Although we could find such structures of H-Ras and K-Ras in the PDB, the only available crystal structure of N-Ras is bound to GDP. We therefore solved the crystal structure of the G-domain of N-Ras bound to the GTP analogue GppNHp (Table 3). N-Ras-GppNHp crystallized with symmetry of the P3221 space group, with similar packing as H-Ras-GppNHp in the same crystal form (PDB code 1CTQ) (48). We use our N-Ras structure as the basis for comparison with the structures of H- and K-Ras in the GTP-bound form.

The crystal structure of N-Ras-GppNHp has switch I ordered in the active site, with Tyr-32 interacting with the γ-phosphate of a neighboring symmetry-related molecule of N-Ras. The conformation of switch I is nearly identical to that in H-Ras (PDB code 1CTQ), as is the P-loop and N terminus of switch II (Fig. 4a). Interestingly, the nucleophilic water molecule (i.e. Wat-175 in H-Ras) (48) is not present in our structure, and the side chains of Gln-61, Glu-62, and Glu-63 are disordered. The N termini of switch II in N-Ras and H-Ras are very similar in the two structures, up to residue Tyr-64. Starting at residue 65, the backbone of switch II in N-Ras begins to adopt a more open conformation that is farther from helix 3 and different from H-Ras (Fig. 4b). Although Arg-68 is well ordered in the H-Ras structure and makes van der Waals interactions with Tyr-71, it is partially disordered in N-Ras where Tyr-71 is disordered and...
turned out toward the solvent. The side chains for residues 69–74 at the end of helix 2 in switch II are also disordered. The main chain atoms in the two structures converge again starting at residue 75. In agreement with a poorly ordered N-Ras switch II, helix 3 is shifted away from the allosteric site to favor the T state (Fig. 4). The shift to the T state in helix 3 toward switch II is also seen for K-Ras (data not shown). These observations are consistent with the drop in $k_{\text{hyd}}$ for K- and N-Ras in our hydrolysis experiments, i.e., both N- and K-Ras appear to favor the T state more so than H-Ras.

Table 3

| Property                                      | Value  |
|-----------------------------------------------|--------|
| Wavelength (Å)                                | 1.54   |
| Resolution range (Å)                          | 28.5–1.67 (1.73–1.67)  |
| Space group                                   | P3,21  |
| Unit cell $a, b, c$ (Å)                       | 30.01, 39.01, 159.2 |
| $\alpha, \beta, \gamma$ (°)                  | 90.90, 120 |
| Total reflections                             | 17,229 (1,459)* |
| Unique reflections                            | 195,722 |
| Multiplicity                                  | 11.4   |
| Completeness (%)                              | 98.7 (87.7)* |
| Mean I/O                                      | 24.0 (1,312)* |
| Wilson B factor                               | 26.4   |
| $R_{\text{merge}}$ (%)                       | 0.079  |
| $R_{\text{work}}$ (%)                        | 18.8 (26.8)* |
| $R_{\text{free}}$ (%)                        | 22.8 (30.6)* |
| No. of non-hydrogen atoms                     | 1434   |
| Macromolecules                                | 1281   |
| Ligands                                       | 33     |
| Water                                         | 120    |
| Protein residues                              | 166    |
| Root mean square (bonds)                      | 0.007  |
| Root mean square (°)                          | 1.11   |
| Ramachandran favored (%)                     | 97     |
| Ramachandran outliers (%)                    | 0      |
| Clash score                                   | 4.3    |
| $B$-factor (average)                          | 28.5   |
| Macromolecules                                | 27.7   |
| Ligands                                       | 25.4   |
| Solvent                                       | 36.7   |

* Parentheses refer to data in highest resolution shell.

Isoform-specific residues near the allosteric site

The allosteric switch centers on interactions between helix 3 and switch II (18) and appears to be modulated by isoform-specific residues. N-Ras residue Asn-94 forms an H-bond with Tyr-137 (2.8 Å), which is not seen with His-94 in the H-Ras structure with PDB code 1CTQ (48). This interaction in N-Ras creates a water network that is also seen in some structures of H-Ras (i.e., PDB code 3K8Y) (18), connecting Arg-97, Lys-101, and Glu-98 together in the allosteric site (Fig. 5a). The two conformations that have been observed for His-94 in H-Ras allow either interaction with Tyr-137, whereas Glu-98 makes a salt bridge to Lys-101 as observed for Asn-94 in the N-Ras structure, or with Glu-98, in which case His-94 sequesters Glu-98 from making the salt bridge with Lys-101 (Fig. 5a). Residue 94 is also a histidine in K-Ras, and in the structure with PDB code 3GFT (Structural Genomics Consortium) it is in an intermediate position between those described for H-Ras and N-Ras. It is likely the case that the two conformations are accessible but populated differently in the three isoforms.

Both helix 3 and loop 7 are shifted toward switch II in N-Ras, and residue Tyr-166, a residue unique to this isoform, appears to play a role in stabilizing this T state conformation (Fig. 5b). In wild-type H-Ras, His-166 forms peripheral H-bonds with the side chain and backbone of Asp-108, and it is often disordered in other crystal forms of H-Ras. The same situation is seen for K-Ras in the structures with PDB code 3GFT, in which residue 166 is also a histidine. Although Tyr-166 in N-Ras and His-166 in H- and K-Ras both have planar aromatic rings, the tyrosyl moiety reaches out closer to loop 7. Here, Tyr-166 forms a direct H-bond with Ser-106 instead of Asp-108. This new H-bond changes the interactions between helix 5 and loop 7, pushing loop 7, and by extension helix 3, toward switch II, a conformation characteristic of the T state.
Residue 95, which is a glutamine in H-Ras, a histidine in K-Ras, and a leucine in N-Ras, is uniquely positioned to influence the conformational states of residue 94 (Fig. 5c). For example, the Leu-95 residue in its most favored rotamer as found in the N-Ras structure sterically impedes the second conformation of Asn-94 due to its side-chain branch at C\textsubscript{\(\gamma\)}. His-95 in K-Ras may do the same, although its polar side chain could be in a conformation where it H-bonds with polar or charged switch II residues. In contrast, the branch at C\(\delta\) for Gln-95 in H-Ras allows the dual conformation of His-94. Residue 95 also faces the N terminus of switch II, thereby providing an isoform-specific interaction surface between helix 3 and switch II, near the active site.

**Impact on conserved nucleotide-binding motifs**

A second area of isoform-specific binding residues is between the evolutionarily conserved NK\(\text{X}\)D (residues 116–119) and E\(\text{X}\)SAK (residues 143–147) motifs (8), located near the gua-
nine moiety of GTP near the N termini of helix 3 and helix 4 (Fig. 3).

Toward the N terminus of helix 3, N-Ras has distinct residues at positions Ser-87 and Ala-91 compared with H-Ras and K-Ras, in which these residues are Thr-87 and Glu-91. In N-Ras, Ser-87 is found in a dual conformation that mimics the threonine conformer found in H- and K-Ras. In all three isoforms, residue 87 H-bonds with the backbone carbonyl and side-chain hydroxyl group of Thr-124 in loop 8 (Fig. 6). Loop 8 contains residues 116–125, including the conserved NKXD motif (116–119) followed by a number of isoform-specific sequence variations. Loop 8 leads to helix 4, whose first few residues (126–128, 131–132, and 135) also show sequence variation and are close to the EXSAX motif (Fig. 3). Although proline at residue 121 in K- and N-Ras is likely to decrease the dynamics of loop 8, the conformation of loop 8 is overall similar to that seen for H-Ras with its alanine at residue 121. Loop 8 is exposed to solvent, and the hydroxyl-containing side chains of Ser-122 and Thr-124 in K- and N-Ras are expected to form more favorable solvent-related interactions as compared with Ala-122 in H-Ras.

Loop 8 sequentially links β5 at the protein core to helix 4 (Figs. 3 and 6b). The side chain of Arg-123 extends between these two elements to make a salt bridge with Glu-143, from the EXSAX motif in β6, over loop 8 (Fig. 6b). This salt bridge links the nucleotide-binding motifs NKXD in loop 8 and EXSAX, thereby stabilizing the active site. Intriguingly, residue 141 appears to affect the geometry of interaction between Arg-123 and Glu-143. In H-Ras, the salt bridge is in ideal geometry with both nitrogen atoms of the Arg-123 guanidinium group and both oxygen atoms of the Glu-143 carboxylate forming a head-to-head interaction (Fig. 6b). Residue Tyr-141 hydroxyl group in this structure makes an H-bond with the side chain of Glu-143. In both K-Ras and N-Ras, residue 141 is a phenylalanine that sterically interacts with the carboxylate of the Glu-143 side chain. This interaction results in a shift of Glu-143 away from Phe-141, replacing a highly conserved water molecule found in H-Ras, such that only one of its side-chain oxygen atoms interacts with one of the nitrogen atoms of the Arg-123 guanidinium moiety (Fig. 6b). The other nitrogen atom interacts with a nearby water molecule that is H-bonded to the conserved guanine-binding residues, thereby stabilizing the active site.

H-Ras point mutants do not have altered hydrolysis rate constants

Having identified areas of isoform-specific residues based on structural comparisons, we mutated H-Ras at three key residues discussed above to residues found in N-Ras, and the
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resulting rate constants were obtained by single-turnover experiments. Residues 95 on helix 3, 166 near the allosteric site, and 141 near the Arg-123-Glu-143 salt bridge were tested on H-RasQ95L, H-RasH166Y, and H-RasY141F mutants, respectively. The rate constants obtained from the single-turnover experiments are shown graphically in Fig. 7 and numerically in Table 1. The raw data with progress curves fitted to the first-order kinetic model are shown in the supplemental data. The rate constants of 0.019, 0.019, and 0.015 min$^{-1}$ for H-RasQ95L, H-RasH166Y, and H-RasY141F, respectively, show a minimal effect, if any within the experimental error, compared with the rate constant of 0.016 min$^{-1}$ obtained for wild-type H-Ras. Thus, although collectively these residues may contribute to the differences in rate constants observed for the isoforms, individually they do not significantly alter the rate constant for H-Ras. This supports the idea that isoform-specific residues play a role in the context of the collective residue differences in the allosteric lobe between the isoforms, rather than functioning in isolation to impact individual structural elements.

Discussion

H-, K-, and N-Ras are biochemically distinct

We performed single-turnover experiments on H-Ras, K-Ras, and N-Ras using the natural substrate GTP, and we found that the isoforms show biochemical differences that have previously gone unappreciated.

Single-turnover experiments using Ras pre-loaded with some form of labeled GTP have been the norm in Ras enzymology for the past 2 decades (15, 19, 31, 32, 43, 49–62). Early work on the GTP hydrolysis catalyzed by H-Ras and to a lesser extent by N-Ras was performed by using $^{32}$P-labeled GTP at the $\gamma$-phosphate position, until the fluorescent N-methylanthraniloyl (mant) nucleotide derivatives became available in the early 1990s to measure the kinetics of hydrolysis and nucleotide exchange on Ras (63). These assays, dependent on the detection of fluorescence changes due to the microscopic environment of the mant probe, became the dominant strategy through which biochemical studies are performed to this day. However, the mant nucleotides have recently been shown by NMR to alter the active site environment in unpredictable ways (57). Therefore, the rate constants obtained using this method may not reflect the enzyme activity with GTP, as conformational states may be altered in subtle ways that affect hydrolysis. One could envision, for example, the mant moiety having a dominant effect, masking isoform differences and resulting in similar rate constants for the three Ras proteins. The progress of GAP-catalyzed hydrolysis of GTP bound to H-, K-, and N-Ras can be fitted to a single exponential, with higher rate constants, which were found to be similar between the three isoforms (49). The experiments used mant-GTP, which also affects GAP-catalyzed hydrolysis reactions (57), and it would be interesting to see if the results hold when using [$γ$-$^{32}$P]GTP. Enzyme-coupled reactions provide an alternative (59, 60, 63, 64), but this method reports on GTPase product release rather than on cleavage of the phosphodiester bond (64, 63). In general, previously published studies on Ras hydrolysis are variable with respect to nucleotide pre-loading time, nucleotide identity, reaction temperature, and whether or not free nucleotide was removed prior to the reaction. Most importantly, they focus on one of the isoforms at a time, primarily H-Ras, with the assumption that results obtained for one of the isoforms are valid for the other two.

To determine first-order (exponential) rate constants with sufficient precision, it is important for the reaction progress curves associated with GTP hydrolysis to progress sufficiently past the initial quasi-linear region, such that the (exponential) curvature of the kinetic trace is clearly defined. In general, first-order exponential rate constants are determined with confidence when experimental kinetic traces progress to ~2-fold past the half-life $t_1/2 = \ln(2)/k_{\text{hydr}}$ (66). The original hydrolysis experiments published for H-Ras in the late 1980s used a [$γ$-$^{32}$P]GTP discontinuous assay, referencing a 1983 paper on determination of GTP hydrolysis rate constants for EF-Tu, in which hydrolysis was carried out for 60 min with time points collected every minute (67). The rate constant for N-Ras was measured by a different group at the time using $^3$H instead of $^{32}$P to follow the reaction in a discontinuous assay for 250 min, with one time point measured at 360 min to determine that there was no longer any GTP, i.e. that the reaction had essentially gone to completion (32). This corresponds to about two half-lives for N-Ras at 37 °C. In our previous experiments with H-Ras, we collected hydrolysis data points for 100 min (18, 27), i.e. approximately two half-lives for this isoform. Here, we extended the experiments to 240 min, taking into account the lower hydrolytic rate constant for N-Ras. The reaction rate constants with associated half-lives shown in Table 1, this represents 2 half-lives for N-Ras and K-Ras and 6 half-lives for H-Ras. In the presence of Raf-RBD, we collected data for 5, 4, and 2 half-lives for H-, K-, and N-Ras, respectively. Thus, we have confidence in our rate constants.

In this study, we used a radiometric assay adapted from earlier Ras literature (36, 37), where the substrate is GTP itself. We
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By comparing the single-turnover reaction rate constants of the Ras isoforms alone and in the presence of Raf-RBD, we are able to obtain useful information about the nature of the conformational change that occurs for each isoform, because Raf-RBD binds switch I, bringing associated residues into a conformation that is optimal for catalysis. Thus, it is possible to determine the extent to which the state 1 to state 2 shift contributes to the overall reaction rate. State 1 is an open and non-catalytic conformation of switch I, whereas state 2 is a closed conformation necessary for hydrolysis (Fig. 1) (15, 16). Although state 2 is the dominant switch I conformation at equilibrium in the GTP-bound state of H-Ras (15), the equilibrium between the two states in K- and N-Ras has not yet been published. Because Raf-RBD does not affect the reaction rate constants for H-Ras or N-Ras, we conclude that the transition from state 1 to state 2 is not a limiting step for hydrolysis in these two isoforms. For K-Ras, however, state 2 stabilization through the interaction with Raf-RBD increases the rate constant to nearly the value obtained for H-Ras, supporting a rate-determining role for the state 1 to state 2 transition in K-Ras. Interestingly, K-Ras and H-Ras have very similar affinities for Raf-RBD, suggesting a low energy barrier for the transition between the two switch I conformational states in the presence of Raf. The affinity of N-Ras for Raf-RBD is about half of that observed for the other two isoforms. This implies that in N-Ras, the conformational transition necessary to attain a state 2 Raf-RBD-binding conformation is energetically costlier than in either H-Ras or K-Ras. Our ITC experiments determined that this is an entropic cost (Table 2).

There are several isoform-specific residues near the nucleotide-binding pocket that may play a role in the conformational transitions associated with the active site. One is Tyr-141 in H-Ras, and in K-Ras and N-Ras this residue is Phe-141. This combined with the presence of Pro-121 in these two isoforms may affect the configuration of the salt bridge between Arg-123 and Glu-143, which link the NKxD and EYSAK conserved active-site motifs. In N-Ras, the lower affinity for Raf-RBD may also have a contribution from Leu-95 in helix 3, which affects the conformation of switch II (18). Although the Raf-RBD binds only at switch I, the two switches are conformationally linked, with helix 3 and switch II driving the conformation of switch I (71).

Our single-turnover data are consistent with a state 1 to state 2 transition responsible for the lower rate constant in K-Ras compared with H-Ras. In N-Ras, this transition is not rate-limiting, because Raf-RBD stabilizes the state 2 conformation without any effect on the rate constant, which remains low. Therefore, unlike the situation for K-Ras, its lower rate constant must be due to conformational transitions elsewhere on the protein, perhaps including the above-suggested effect of Leu-95 on helix 3 and switch II. Interestingly, based on our crystal structures, the helix 3 conformation also appears to be influenced by residue 166, which is the bulkier Tyr-166 in N-Ras but is His-166 in H-Ras and K-Ras. N-Ras Tyr-166 pushes into loop 7, which could help stabilize the kink of helix 3 toward switch II that is essential to the T state conformation (Figs. 4b and 5b). This is not observed in the H-Ras–GppNHp structure in the same crystal form (PDB code 1CTQ) (48), which is found in the R state. A population shift toward the T state would mean that the catalytic conformation is accessed more rarely in N-Ras than in H-Ras and K-Ras, consistent with a smaller N-Ras rate constant in the presence of Raf-RBD. The interactions between His-94/Asn-94 and Glu-98 (which is sequestered from forming a salt bridge with Lys-101) or between His-94/Asn-94 and Tyr-137 (in which case Glu-98 interacts with Lys-101) (Fig. 5a) provide a mechanism for linking the allosteric site, helix 3, switch II, and switch I in different ways and may also contribute to the differences in rate constants observed for H-, K-, and N-Ras. It is important to stress that these proposed residue contributions to the differences in rate constants between the isoforms occur within the context of multiple isoform-specific residues, as our H-Ras point mutant experiments show no significant effects on the rate constant.

Overall, the fact that binding of Raf-RBD does not affect the hydrolysis rate constants for H-Ras and N-Ras suggests that conformational transitions other than state 1 to state 2 must be rate-limiting for these isoforms. These conformational transitions are much slower for N-Ras than for H-Ras as reflected in their relative rate constants. For K-Ras, once switch I is in state 2, the other conformational transitions occur at similar rates to those in H-Ras, as indicated by their similar rate constants in the presence of Raf-RBD.
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H-bonding networks linking effector and allosteric lobes modulate isoform-specific behavior

Ras in its GTP-bound form is a flexible molecule capable of accessing a range of conformational states, particularly with respect to switch I and switch II regions that form the active site (70). These, in turn, are linked to the isoform-specific allosteric lobe through networks of communication that lead to functionally significant areas associated with the membrane and dimerization to activate Raf (18, 27, 30, 72). Critical elements of the networks involving the switch regions are conserved, implying that these mechanisms are common to the three isoforms. However, they are modulated differently by surrounding residues in H-, K-, and N-Ras, leading to differences in dynamics (6, 34) and kinetics of GTP hydrolysis as observed in this study. These differences are also likely to impact dimerization and the way in which the G-domain interacts with the membrane, modulating interactions specific to each isoform (12). They could impact isoform-specific biological functions in ways that have not yet been determined (73).

Switch I connects to nucleotide-sensing residues in helix 5 through Cys-118, the X residue in the NKXD motif conserved across small GTPases, where Asp-119 makes nucleotide-specific interactions with the guanine base and is critical for nucleotide binding (Fig. 6a) (34). Cys-118 is linked to Glu-143 in the EKSAX motif through a water molecule that is highly conserved in Ras structures bound to GTP analogues (27), and from Glu-143, the water-mediated network continues on to the helix 5 residues. Thus, Cys-118 and Glu-143 are critical elements in the water-mediated network of communication between switch I and nucleotide-sensing residues in helix 5, and they are adjacent to residues that directly interact with the nucleotide. These two highly conserved elements are linked through loop 8 and helix 4, two of the most variable regions between the Ras isoforms (Fig. 3A), opening possibilities for isoform-specific variations in active-site conformations. The fact that Glu-143 links switch I to helix 5 residues, coupled with its interaction with Arg-123 in loop 8 as part of stabilizing the SAK residues at the nucleotide-binding site, explains the strictly conserved nature of Glu-143 across all GTPases. We have shown that perturbing the Arg-123–Glu-143 salt bridge in H-Ras by mutation of Tyr-141 to Phe does not by itself affect the hydrolysis rate constant for H-Ras. However, we propose that the isoform-specific variations found in loop 8 and helix 4, as well as at residue 141, collectively impact the nucleotide exchange reaction and the population of conformational states associated with switch I.

The switch II conformation is modulated by an allosteric mechanism through which it is linked to a Ca$^{2+}$-binding site in the allosteric lobe, promoting the shift in helix 3 and loop 7 toward helix 4, characteristic of the T to R transition necessary for ordering switch II residues for GTP hydrolysis (Fig. 1) (17). The shift to the R state involves straightening the kink in helix 3 that is present in the T state around residues 90–95, where the H-bond between the carbonyl group of residue 90 and the amide of residue 94 is disrupted. This is precisely at another hot spot of Ras isoform-specific differences (Fig. 3), poised to affect the network of communication between switch II and the allosteric site. We have identified residue differences at positions 94 and 95 and propose that their local interactions affect the conformational equilibrium between T and R states associated with enzyme catalysis (Fig. 5c). In N-Ras, further stabilization of the non-catalytic T state appears to be aided by Tyr-166 as discussed above. It is intriguing, but not surprising, that residue 95, adjacent to the helix 3 kink, and residue 122, adjacent to the Arg-123 salt bridge, are the two positions in Ras where residues are different in all three isoforms.

The Ras allosteric networks are joined at the active site, where they affect each other through the interdependence previously established between switch I and switch II (71). Thus, although described here through local interactions, the effects of isoform-specific differences are global, and at least in terms of affecting hydrolysis rate constants, they likely occur in the context of multiple sites.

We have shown here that the isoform-specific sequences in the allosteric lobe have an impact on GTP hydrolysis and interaction with an effector protein, which must be due to allosteric effects on dynamics and conformational states, given the identical active sites. Although it is not currently possible to directly link the unique hydrolysis rate constants to biological function in the cell, these differences are likely to contribute to the higher level of complexity emerging from current studies in the Ras field. A significant population of state 1 on K-Ras, for example, could facilitate regulation by the positively charged C-terminal HVR as proposed previously (74), whereas the low hydrolysis rate constants observed for N-Ras in the presence of Raf-RBD might contribute to sustained signaling through the MAPK pathway in skin legions (75).

As a corollary to the differences we have observed between the three isoforms of Ras, it is likely that oncogenic mutants affect the H-, K-, and N-Ras functions in significantly different ways within the contexts of their isoform-specific sequences, structures, and dynamics. It is not sufficient to study each mutant within its local context in the active site. We have already shown that the Q61L mutant profoundly affects the dynamics of the entire H–Ras–Raf-RBD complex (21). It is likely that localization and other large-scale biological contexts play a significant role in the selection of particular mutations in the Ras isoforms associated with specific cancers (76), but it is now clear that conformational states at the active site are affected by sequence at the molecular level in a global scale, with great sensitivity to any changes that perturb the finely tuned equilibrium of conformational states inherent in each of the Ras isoforms.

Experimental procedures

Protein expression and purification

Hydrolysis and crystallization experiments were performed using the catalytic G-domain (residues 1–166) of human H-, K-, and N-Ras (EC 3.6.5.2). Protein expression and purification of wild-type Ras isoforms was performed as described for H-Ras (27, 77), with no modifications for K-Ras or N-Ras. The Ras proteins were stored at −80 °C for hydrolysis experiments in their GDP-bound state (78). For crystallization of N-Ras, GDP was exchanged for the GTP analogue GppNHp, using methods...
Previously described (26, 77), and concentrated to 3.5 mg/ml before storage at $-80^\circ$C. In all cases, the purified proteins were flash-frozen in 25–55-$\mu$l aliquots. Protein concentrations for biochemical experiments were determined using the Bradford assay just prior to use (79).

For purification of the RBD (residues 51–131) of the c-Raf kinase (EC 2.7.11.11), Escherichia coli BL21 cells transfected with DNA coding for Raf-RBD in the pET21 vector system were grown in Luria Broth (LB) containing ampicillin (50 mg/lite) at 37 $^\circ$C, and protein was expressed using 0.5 mM isopropyl 1-thio-β-d-galactopyranosidase at 37 $^\circ$C for 4 h. Pellets were thawed and solubilized in buffer A (20 mM HEPES, pH 7.0, 20 mM NaCl, 1 mM DTT) with standard protease inhibitors. Solubilized cells were sonicated and subjected to a 0.02% v/v polyethyleneimine bulk cut on ice. After polyethyleneimine precipitation, the protein sample was clarified again using centrifugation and syringe filtered with a 0.45-$\mu$m membrane before use in chromatography. The protein was purified by cation-exchange and size-exclusion chromatography, in that order, using an ÄKTA FPLC system (GE Healthcare). Cation exchange was done using a HiPrepTM SPFF column pre-equilibrated with buffer A, and elution was performed using a 0–70% buffer B (same as buffer A but with 1 mM NaCl). Size-exclusion chromatography was run in 20 mM HEPES, pH 7.5, 50 mM NaCl, and 20 mM MgCl$_2$. Pure Raf-RBD was pooled and concentrated to 8–11 mg/ml, flash-frozen in 25–55-$\mu$l aliquots, and stored at $-80^\circ$C. Protein purity was assessed using 15% SDS-polyacrylamide gel.

**Intrinsic hydrolysis experiments**

Single-turnover experiments were performed for the truncated H-, K-, N-Ras, alone and in the presence of 5-fold molar excess Raf-RBD (27, 36, 37). Given the high affinities shown in Table 2, this should result in all of the Ras proteins bound to Raf-RBD. Briefly, 5 $\mu$M Ras-GDP was incubated with 50 nM [γ-32P]GTP (PerkinElmer Life Sciences) in the presence of 1 mM EDTA for 5 min. The reaction was started by addition of 4 $\mu$l of this protein solution to 16 $\mu$l of hydrolysis buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl$_2$, 2 mM DTT, at 37 $^\circ$C, with Ras and [γ-32P]GTP at final concentrations of 1 $\mu$M and 10 $\mu$M, respectively.

To allow sufficient reaction time, such that the exponential shape of reaction progress curves became sufficiently developed, single-turnover hydrolysis experiments were performed by collecting experimental data up to $t_{max} = 300$ min, at the following time points: 0, 5, 10, 15, 20, 25, 30, 35, 40, 60, 75, 90, 120, 150, 180, 210, 240, and 300 min. However, the $t = 300$-min data points were excluded from analysis, because of excessive experimental noise. The 32P product was isolated using an organic extraction method (36, 37), and the radioactivity was measured with a scintillation counter (300SL, LabLogic). The $k_{syn}$ values correspond to best-fit values from global fit (38) of combined triplicate experiments. Details are found in the supplemental data.

The single-turnover experiments were performed both in the absence and in the presence of Raf-RBD for the wild-type isoforms, but only in the absence of Raf-RBD for the H-Ras mutants. Each set of experiments was performed on 3 different days, with each day focused on one of the triplicates for H-, K-, and N-Ras (for the wild-type isoforms) or H-RasQ95L, H-RasH166Y, and H-RasY141F (for the mutants) performed side by side.

**Isothermal titration calorimetry measurements of Ras isoforms in the presence of Raf-RBD**

Thermodynamic properties of the Ras–Raf-RBD interaction were measured using a low volume isothermal titration calorimeter (Nano ITC, TA Instruments). H-, K-, and N-Ras and Raf-RBD were extensively dialyzed into filtered and degassed buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl$_2$, 0.5 mM tris(2-carboxyethyl)phosphine, and 0.05% n-octyl β-d-glucopyranoside. Protein concentrations were calculated using an average of absorbance at 280 nm ($A_{280}$) for accuracy. In all measurements, 300 $\mu$l of 30–80 $\mu$m of the appropriate Ras isoform was placed in the cell and titrated with Raf-RBD at an ~10-fold higher concentration to achieve saturation. Because Ras and Raf-RBD interact with a 1:1 stoichiometry, the reverse experiment of 30–80 $\mu$m Raf-RBD titrated with ~10-fold Ras was also conducted for each isoform to ensure data accuracy in the determined thermodynamic values. The background heat produced by ligand titration into buffer alone was subtracted from each titration experiment. All binding experiments were performed in triplicate.

Data analysis was conducted via manufacturer software (NanoAnalyze, TA Instruments) to give the stoichiometry ($n$), association constant ($K_a$), and enthalpy change ($\Delta H^0$) for the Ras–Raf-RBD interaction. The dissociation constant ($K_d$) was determined as the inverse of $K_a$. Total free energy change ($\Delta G^0$) upon complex formation can be calculated from the thermodynamic relationship $\Delta G^0 = -RT \ln K_a$, whereas the entropy change upon binding is dependent on the relationship $\Delta G^0 = \Delta H^0 - T\Delta S^0$. These values allow for complete thermodynamic characterization of the interaction between each Ras isofrom G-domain and Raf-RBD in solution.

**Crystallization, data collection, and structure refinement for wild-type N-Ras-GppNHp**

Purified N-Ras–GppNHp stored at $-80^\circ$C in 20 mM HEPES, pH 7.5, 50 mM NaCl, 20 mM MgCl$_2$, and 1 mM DTT at a concentration of 3.5 mg/ml was thawed in ice. Crystals were grown using the hanging drop vapor diffusion method in 24-well plates with drops of 1.5 $\mu$l of reservoir solution. The reservoir contained 200 $\mu$l of 0.2 M magnesium acetate tetrahydrate, 0.1 M sodium cacodylate trihydrate, pH 6.5, 20% w/v PEG 8000 (Hampton Research Crystal Screen number 18). Crystals were grown for 1 week at 18 $^\circ$C and cryoprotected in a solution containing 70% mother liquor and 30% glycerol. Crystals were immediately flash-frozen in liquid nitrogen for data collection on a MicroMax-007 Rigaku X-ray generator mounted on an R-Axis IV++ detector. X-ray diffraction data were merged and scaled using HKL3000 (80). Molecular replacement and structure refinement were performed using the PHENIX suite of programs (81) with the H-Ras-GppNHp coordinates with PDB code 1CTQ used as the initial phasing model. The structure of N-Ras-GppNHp was solved at a resolution of 1.67Å, with data collection and refinement statistics shown in Table 3.
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Author contributions—C. W. J. conceived the project, designed experiments, purified protein, performed and analyzed hydrolysis experiments, collected X-ray data, refined and analyzed the Ras structures, and wrote the paper. D. R. purified protein, performed and analyzed hydrolysis experiments, optimized N-Ras–GppNHp crystals, collected X-ray data, and refined N-Ras crystal structure. J. A. P. collected and analyzed ITC data. S. S. purified protein and found initial crystallization conditions for N-Ras–GppNHp. R. K. helped to analyze results of hydrolysis experiments. P. K. designed and analyzed the hydrolysis experiments using DynaFit4. C. M. conceived the project, designed experiments, analyzed results, and wrote the paper. All authors approved the final version of the manuscript.

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