Transformation Efficiency of RasQ61 Mutants Linked to Structural Features of the Switch Regions in the Presence of Raf

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SUMMARY

Transformation efficiencies of Ras mutants at residue 61 range over three orders of magnitude, but the in vitro GTPase activity decreases 10-fold for all mutants. We show that Raf impairs the GTPase activity of RasQ61L, suggesting that the Ras/Raf complex differentially modulates transformation. Our crystal structures show that, in transforming mutants, switch II takes part in a network of hydrophobic interactions burying the nucleotide and precatalytic water molecule. Our results suggest that Y32 and a water molecule bridging it to the γ-phosphate in the wild-type structure play a role in GTP hydrolysis in lieu of the Arg finger in the absence of GAP. The bridging water molecule is absent in the transforming mutants, contributing to the burying of the nucleotide. We propose a mechanism for intrinsic hydrolysis in Raf-bound Ras and elucidate structural features in the Q61 mutants that correlate with their potency to transform cells.

INTRODUCTION

Ras is the canonical member of a large superfamily of small, monomeric GTPase proteins that function as “molecular switches” in a number of signaling pathways in the cell (Barbacid, 1987). Ras cycles between the inactive GDP- and the active GTP-bound forms through large conformational changes near the nucleotide-binding site, localized to the switch I (30–38) and switch II (59–72) regions (Campbell et al., 1998). Both switch regions are generally involved in interactions with downstream effector proteins and with proteins that mediate the state of the switch. H-Ras has a low intrinsic rate of GTPase activity that is enhanced by at least 3 orders of magnitude in the presence of GTPase activating proteins (GAPs), resulting in the signal transduction switch being turned off (Scheffzek et al., 1997). The switch is turned on by Guanine nucleotide exchange factors (GEFs) that accelerate the rate of nucleotide release to allow for loading with GTP (Sprang, 1997).

Mutations in codons 12, 13, or 61 convert the ras gene into an active oncogene (Adari et al., 1988). These mutant proteins are constitutively active, resulting in unregulated cell proliferation and tumor formation. In particular, Q61L has one of the highest transformation efficiencies of any gain-of-function mutant. Not all substitutions for Q61, however, result in the potent transforming efficiency of cells containing the Ras mutants, although they all decrease the in vitro GTPase activity of Ras about 10-fold (Der et al., 1986). The Q61L, Q61V, and Q61K mutant Ras variants transform NIH 3T3 cells nearly 300-fold and 1000-fold more efficiently than the Q61G and Q61E mutants, respectively. Interestingly, Q61I is an order of magnitude less efficient than the L, V, and K mutants, and thus it is only moderately transforming. Since the interaction of Ras-GTP with its effector Raf kinase mediates one of the major pathways through which Ras is involved in the control of cell proliferation (Hanahan and Weinberg, 2000), it is relevant to observe the influence of the mutations on switch II given the switch I conformation present in the Ras/Raf complex.

NMR spectroscopy experiments have shown that, in solution, the switch I and switch II regions in Ras bound to the GTP analog, GppNHp, are dynamic and interconvert between two or more stable conformers in the millisecond timescale (Ito et al., 1997). Indeed, 31P NMR experiments, in which the environment of the nucleotide phosphorus atoms can be monitored, show two distinct conformational states for the protein regions surrounding the triphosphate group of the nucleotide (Geyer et al., 1996). State 1 has been linked to a conformation in which Y32 is directed away from the nucleotide, and state 2 has been linked to one in which Y32 is close enough to the nucleotide for the phosphate groups to experience a chemical shift due to the aromatic ring system (Geyer et al., 1996). These conformations are referred to throughout this article as the open and closed conformations, respectively. Interestingly, 31P NMR experiments with Ras-GppNHp in complex with RasGAP shows Ras-GppNHp in state 1, consistent with the crystal structure of the complex, in which Y32 is in an open conformation and interacts intimately with RasGAP (Scheffzek et al.,
In the complex with Raf kinase-Ras binding domain (Raf-RBD), Ras-GppNP has present entirely in state 2 (Geyer et al., 1996). Not surprisingly, the crystal structure of the Ras-RBD complex, in which Ras is a mutant form of Rap containing the E30D, K31E double mutation that makes its effector region identical to that of Ras, shows Y32 closed over the nucleotide (Nassar et al., 1995).

A recently published series of crystal structures focuses on the effects of switch II mutants on Ras-GppNP, including two that crystallize with the symmetry of space group R32: A59G (Hall et al., 2002) and Q61G (Ford et al., 2006). In this crystal form, switch I is in the closed conformation, with Y32 near the nucleotide, as observed in the interaction with Raf kinase. Switch II is unhindered by crystal contacts. This is in contrast to the previously published structures of wild-type Ras-GppNP and its mutants from crystals with the symmetry of space group P3_21, in which both switch regions are modulated by crystal contacts and switch I has Y32 in an open conformation (Krengel et al., 1990). We discovered the R32 crystal form with wild-type Ras in similar (but different) crystallization conditions, and we have used it to explore the possibility that conformational properties of RasQ61 mutants might have a role in the potency of its oncogenic phenotype. The crystal structures of wild-type Ras-GppNP of three highly transforming mutants, and of a moderately transforming mutant from crystals with the symmetry of space group R32, together with a set of experiments showing a marked effect of Raf on the GTPase activity of RasQ61L, resolve the paradox associated with the wide range of transformation efficiencies of RasQ61 mutants.

RESULTS

The construct of H-Ras used in the present studies contains the catalytic domain with 23 residues truncated from the C terminus and is referred to simply as Ras throughout this article. The structures of wild-type Ras-GppNP; of the strongly transforming RasQ61L-GppNP, RasQ61V-GppNP, and RasQ61K-GppNP; and of the moderately transforming RasQ61I-GppNP were solved to 1.4 Å, 2.0 Å, 1.6 Å, 1.35 Å, and 1.9 Å resolution, respectively, from crystals with symmetry of the space group R32. In addition, the structure of RasQ61I-GppNP was solved to 1.45 Å resolution from crystals with P3_21 symmetry. The mutants were chosen based on the published transformation efficiency of codon 61 ras mutants (Der et al., 1986). L, V, and K are the three most highly transforming mutants among six observed to produce foci at high efficiencies in NIH 3T3 cells. Of the moderately transforming mutants, Ile was chosen because its transformation efficiency is in the middle of this group’s range. The published structure of the Q61G mutant (Ford et al., 2006) is used to represent the weakly transforming category. Diffraction data for all structures were collected at 100K at the SER-CAT synchrotron beamline 22-ID, APS (Argonne, IL). Table 1 shows the data collection and refinement statistics for the six crystallographic models presented in this study.

The Crystal Structure of Wild-Type Ras-GppNP with Switch I in the Closed Conformation

The structure of Ras-GppNP has switch I in the closed conformation, with Y32 stacked over the nucleotide. Its side chain hydroxyl group interacts with the γ-phosphate of GppNP through a water molecule, precisely as observed in the Ras-GppNP-Ras-RBD complex (Nassar et al., 1996). Superposition of the switch I regions based on alignment of the nucleotide in these two structures yields a Cα root-mean-square deviation (rmsd) of 0.34 Å. The crystal contacts in the R32 form result in a switch I conformation that mimics the Ras/Raf-RBD interface, including a Ca^2+ ion provided by the crystallization mother liquor making similar interactions to those of Lys84 in Ras (Figure 1). This closed form of Ras-GppNP precludes binding of RasGAP in the catalytically productive conformation observed in the Ras-RasGAP complex, consistent with the complete shift to the open form in the presence of GAP (Geyer et al., 1996). Interestingly, the previously published canonical structure (as exemplified by PDB code: 1CTQ) (Scheidig et al., 1999) is very similar to the GAP-bound form of Ras (PDB code: 1WQ1) (Scheffzek et al., 1997). Most importantly, switch I and, in particular, Y32 are in the same open conformation and superimpose well in the two structures.

While switch I has well-defined electron density, switch II in wild-type Ras-GppNP is completely disordered, with no electron density for residues 61–68 and with only main chain electron density for Q70 and Y71. At the beginning of the switch, G60 is well ordered and makes its usual amide interaction with the γ-phosphate of the nucleotide, and beyond the switch R73 is anchored by crystal contacts. Residues 61–68 are not included in the model, and residues 70 and 71 are modeled as alanine in our wild-type structure.

Strongly Transforming Mutants of RasQ61: Q61L, Q61V, and Q61K

Unlike the disordered switch II seen in wild-type Ras-GppNP, the strongly transforming Q61 mutants have an ordered switch II region (Figures 2A–2C). Surprisingly, switch II in the L, V, and K mutants is found in a very different conformation than previously observed for the L mutant (Krengel et al., 1990) and is unique compared to all currently available structures of Ras. There is good electron density for nearly the entire switch II; there are some weak areas along Ala66 and Met67, and no electron density for the side chain atoms of residues E62 and E63, which are modeled as alanine. In each of the mutant structures, switch I and switch II come together, resulting in close interaction between Y32, P34, I36, L/V/K61, and Y64 to form a hydrophobic cluster over the nucleotide and the associated precatalytic water molecule (Figure 3).

In the RasQ61K-GppNP structure, the aliphatic portion of K61 participates in the hydrophobic cluster, and its positively charged amino group is exposed to solvent. Surface
accessibility calculations with a probe of radius equal to 1.4 Å (Lee and Richards, 1971) show that the hydrophilic complex of the nucleotide and water molecule is completely buried in all three mutant structures (Figures 4A–4C). The buried surface area of the nucleotide and water molecule, the B factors for the mutant side chains, and the distances between residue 61 and neighboring residues in the hydrophobic cluster are shown in Table 2 for all mutant structures presented in this article.

Switch II in the canonical structures (P3(2)21) and in the Ras/RasGAP complex forms an α helix that spans residues 62–73 (PDB code: 1CTQ). In our structures of the transforming mutants containing the hydrophobic cluster, the helix is observed only for the second part of the switch, spanning residues 68–73. The first part of switch II forms a type III β turn with residues 61–64, representing the i, i+1, i+2, and i+3 positions, respectively, and a good H-bond between the C=O of residue 61 and the NH group of Y64 (Chou and Fasman, 1977) (Figure 3). The unwinding of the helix allows for extension of switch II toward switch I and for positioning of the side chains of residues 61 and 64 to form the hydrophobic cluster. Residue 61 is central in this cluster, and its side chain makes key van der Waals interactions to isolate the nucleotide from bulk solvent; its amide N atom H-bonds to the precatalytic water molecule, and its carbonyl group is involved in the H-bond between residues i and i+3 in the type III β turn (Table 2).

### Table 1. Data Collection and Refinement Statistics

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Rsym = ∑[II − ⟨CI⟩]/∑[CI], Rwork = ∑[Fobs − |Fc|]/∑[Fobs], calculated by using 90% of the reflections against which the model was refined. Rfree = ∑[Fobs − |Fc|]/∑[Fobs], calculated by using a test set consisting of 10% of the total reflections, randomly selected from the original data set. Parentheses include information for the highest-resolution shell.

The Moderately Transforming Mutant RasQ61I

The RasQ61I-GppNHp mutant crystallized in the presence of CaCl₂ under conditions similar to those used to obtain crystals of the wild-type and of the Q61L, Q61V, and Q61K mutants with symmetry of space group P32. Interestingly, however, the vast majority of the RasQ61I-GppNHp crystals (about 80%) obtained under these conditions are of the canonical crystal form with symmetry of space group P3(2)21. This was a surprise, since the crystallization conditions are different from those that normally
yield the canonical form. In the more prominent form with P3_21 symmetry, switch I has Y32 in the open conformation, switch II is disordered from residues 61–67 (removed from the model), and the end of the switch forms a helix, as previously observed for the Q61L mutant in the canonical form (Krengel et al., 1990). Although the relative prominence of the two crystal forms in the crystallization drops cannot be simply correlated with the equilibrium constant between the open and closed forms of switch I in solution, the presence of Ile at position 61 appears to be less favorable to the closed form than the L, V, and K residues that result in strongly transforming mutations.

The structure of RasQ61I-GppNHp obtained from the R32 crystal form shows that switch II is highly ordered (Figure 2D), and that the I61 residue is involved in a hydrophobic cluster virtually identical to that seen in the strongly transforming mutants (Figure 3), with similar burying of the nucleotide and precatalytic water molecule (Figure 4; Table 2). A superposition of this structure onto the RasQ61V shows that the Cγ1 and Cγ2 atoms in the two mutants superimpose very well (Figure 3). Comparison with the RasQ61K structure shows good overlap of the Cβ, Cγ1, and Cδ atoms in I61 with the Cβ, Cγ, and Cδ atoms of K61. Interestingly, in the Q61L and Q61K structures, in which there is no branching from Cβ, the backbone of the Type III turn residues is in the order of 0.5 Å closer to switch I than in the Q61V and Q61I, in which the Cγ2 methyl group protrudes in the direction of the turn, forming a tight van der Waal’s interaction within about 3.9 Å. However, the Cδ groups in K61 and I61 make tight van der Waal’s contact with P34 within the hydrophobic cluster. In RasQ61V, the close contact between the Cγ1 group and the Type III turn can be somewhat relieved due to the absence of a Cδ group, which provides more room toward switch I. In RasQ61L and RasQ61K, the presence of Cδ is compensated for by a lack of strain toward the Type III turn. I61, however, encroaches both toward the turn and toward switch I. This may result in a more strained structure, which, though it can exist, is less favored than in the strongly transforming mutants, thus resulting in a high prominence of the crystals with Y32 in the open conformation. Our results for RasQ61I suggest that the transforming power of the mutants at position 61 is correlated with the ability to form the hydrophobic cluster in the context of the switch I conformation seen in the Ras/Q61 complex (Figure 5A).

The Hydrophobic Cluster Is Observed in a Noncatalytic Form of Ran GTPase

A search through all of the GTPase structures in the Protein Data Bank revealed a striking similarity between the switch II conformation in transforming Ras61 mutants and that found in the Ran-GppNHp/importin-β complex (PDB code: 1IBR) (Vetter et al., 1999) (Figure 3). Superposition of the nucleotide in the RasQ61L and Ran structures yields an average Cα rmsd of 0.48 Å for switch II residues 60–65, and the arrangement of the active site is very similar in the two structures (Figure 5B). Inhibition of the intrinsic Ran-GTP hydrolysis reaction by importin-β is an important aspect in the spatial control of nuclear import of proteins (Gorlich et al., 1997; Vetter et al., 1999). Importin-β that has released a cargo protein is transported by Ran-GTP from the nucleus to the cytoplasm, where RanBP1 and other factors aid in the release of importin-β from Ran-GTP, and GAP-catalyzed GTP hydrolysis converts Ran to the GDP-bound form (Floer et al., 1997). It is critical to this transport mechanism that Ran remain in the GTP-bound form until it reaches the cytoplasm, consistent with the experimental finding that Ran-GTP bound to importin-β is catalytically inactive (Gorlich et al., 1997). We call the associated structure the noncatalytic conformation of switch II. This noncatalytic conformation in wild-type Ran is stabilized by interactions with importin-β, but in Ras it is attained by the capacity of residue 61 to stabilize the hydrophobic cluster with switch I in the Ras/Raf complex.

Raf Impairs Intrinsic Hydrolysis in RasQ61L, but Not in Wild-Type Ras

As a test of the idea that it is in the context of the Ras/Raf complex that the RasQ61 mutants exhibit their oncogenic phenotypes, a set of experiments were performed to...
qualitatively assess the effect of Raf on the hydrolysis of GTP by RasQ61L relative to its effect on the wild-type protein. There are two domains in Raf known to interact with Ras. The first is the RBD, mentioned in the Introduction, which interacts preferentially with Ras-GTP (or a GTP analog). The second is the cysteine-rich domain (CRD), known to mediate activation of the Raf kinase and to bind Ras independent of the state of bound nucleotide (Thapar et al., 2004). In our experiments, we used a construct of C-Raf containing residues 52–196, including both the RBD and CRD domains fused at the N terminus to a 54 residue GB1 domain (referred to as Raf from here on).

Ras was purified from E. coli cells in the GDP-bound form, and the GDP was exchanged for GTP by following a previously published procedure (Cheng et al., 2001). Samples of freshly prepared RasQ61L-GTP were allowed to hydrolyze at room temperature either in the presence or absence of stoichiometric amounts of Raf. The samples were left to react for 6–7 hr, beyond the time expected for completion of hydrolysis in the wild-type, which has a half-life of 25 min at 37°C (Herrmann et al., 1995). Raf was added to the free RasQ61L sample before analysis by gel-filtration chromatography. This step is important because Raf binds with nanomolar affinity to Ras-GTP and with only micromolar affinity to Ras-GDP, providing excellent separation between the two forms in gel-filtration chromatography (Herrmann et al., 1995). The result for the RasQ61L/Raf hydrolysis is shown in Figure 6A. The first peak corresponds to elution of the RasQ61L/Raf complex, and the second peak represents free Ras (in the GDP-bound form). This is shown by the SDS gel in the inset to Figure 6A. The elution profile for the RasQ61L/Raf mixture soon after the GDP/GTP exchange reaction, at the beginning of hydrolysis (not shown), is the same as at the end of the incubation period, indicating that the RasQ61L-GDP was initially there due to incomplete exchange, rather than resulting from GTP hydrolysis by RasQ61L/Raf. In the presence of Raf, the RasQ61L mutant is essentially unable to hydrolyze GTP within the time frame of the experiment. In contrast, Figures 6B–6D representing the RasQ61L, wild-type Ras/Raf, and wild-type Ras reactions, respectively, show corresponding peaks in similar elution volumes, but the relative intensities of the peaks are reversed compared to those in the RasQ61L/Raf situation. In the absence of Raf, RasQ61L is able to hydrolyze GTP, and at the end of 7 hr the results are qualitatively indistinguishable from those for wild-type

Figure 2. Electron Density for Switch II Residues 61–70
(A) RasQ61L-GppNHp.
(B) RasQ6V-GppNHp.
(C) RasQ61K-GppNHp.
(D) RasQ61L-GppNHp (R32).
All panels show final 2Fo – Fc electron density maps contoured at the 1σ level.
Structure
GTP Hydrolysis in RasQ61 Mutants Impaired by Raf

Figure 3. Superposition of the Switches in Transforming Ras-GppNHp Q61 Mutants, and Ran-GppNHp/Importin-β
The comparison includes residues 32–35 and 61–64 in Ras and residues 40–43 and 69–72 in Ran. GppNHp is in yellow. The mutant structures are colored as follows: Q61L, cyan; Q61K, green; Q61I, magenta; Q61V, orange. Ran is in light gray. Wat175 corresponds to the precatalytic water molecule. Red, dashed lines represent hydrogen bonds.

Water Molecules near the γ-Phosphate in Wild-Type Ras-GppNHp
A thorough analysis of water molecules in the active site of Ras with Y32 in an open conformation has previously been published based on the Ras-GppNHp (PDB code: 1CTQ) and Ras-GTP (PDB code: 1QRA) structures, both solved from the canonical crystal form with P3_2_1 symmetry (Scheidig et al., 1999). Except for the water molecules that coordinate the Mg^{2+} ion, the arrangement of active site water in our wild-type Ras-GppNHp structure, in which Y32 is closed over the nucleotide, is significantly different from that seen in the open form and exactly as observed for the Raps-GppNHp/Raf-RBD structure (Figure 5A) (PDB code: 1GUA) (Nassar et al., 1995). In our wild-type structure, two water molecules H-bond to the least buried oxygen atom of the γ-phosphate (O1G). The first is the so-called precatalytic water molecule proposed to be the nucleophile in the hydrolysis reaction. It is 2.7 Å from the O1G atom of the γ-phosphate and 2.9 Å from the carbonyl oxygen atom of T35. The precatalytic water molecule is analogous to Wat175 in the Ras-GTP structure (1QRA), not far from the position found in the GAP-catalyzed transition state mimic (Scheffzek et al., 1997). The second water molecule in the active site is 2.6 Å from the O1G atom and 2.5 Å from the hydroxyl group of Y32, bridging between the two groups (Figure 5A). In the previously published, weakly transforming mutant RasQ61G-GppNHp (PDB code: 1ZW6) (Figure 4E) as well as in our wild-type Ras-GppNHp structure (Figure 4F), a water channel links the γ-phosphate to the bulk solvent. It has been suggested that a water channel plays an important role in both facilitating a shift of the precatalytic water molecule for inline nucleophilic attack on the γ-phosphate and in providing a path for product release (Pasqualato and Cherfils, 2005). The water channel is completely absent in the strongly transforming Q61L, Q61V, and Q61K mutants (Figures 4A–4C, respectively) as well as in the closed conformation of the moderately transforming mutant Q61I (Figure 4D).

Water Molecules near the γ-Phosphate in the Ras Mutants
The structures of the Q61L, Q61V, Q61K, and Q61I mutants of Ras-GppNHp reveal a somewhat different water arrangement. The precatalytic water molecule is found in the same location as in wild-type, and it makes nearly ideal H-bonds to the O1G atom of the γ-phosphate and to the carbonyl oxygen atom of T35 (Table 2). In addition, this water molecule makes a good H-bond to the backbone amide of residue 61 in the respective structures and is isolated from bulk solvent by the hydrophobic cluster that closes over the γ-phosphate group, obstructing the water channel that exists both in the wild-type and in the weakly transforming Q61G mutant. The bridging water molecule is completely absent in our four transforming mutant structures, and, instead, there is a direct H-bond between the hydroxyl group of Y32 and the O1G atom of the γ-phosphate. This is exactly the situation observed in the Ran/importin-β structure, in which the hydroxyl group of Y32 makes a 2.9 Å H-bond to the γ-phosphate oxygen atom of GppNHp (Figure 5B).

DISCUSSION
Since the transformation experiments with RasQ61 mutants (Der et al., 1986), the crystal structures of several oncogenic Ras mutants have been solved, including those of RasQ61L (highly transforming) and RasQ61H (moderately transforming) (Krengel et al., 1990). However, these structures were derived from crystals with symmetry of space group P3_2_1, which most closely mimics the configuration of the GAP-bound Ras structure. Although these structures offer important insights into the overall reduced activity of these mutants, they do not explain the large variation in transformation efficiency between different mutations at residue 61. In thinking about transformation, it is particularly important to understand how the structural features of switch II might affect intrinsic hydrolysis in the Ras/Raf complex, in which switch I is locked in the closed conformation with Y32 linked to the γ-phosphate through the bridging water molecule (Nassar et al., 1996) and with RasGAP unable to bind Ras (Moodie et al., 1995). There is strong experimental evidence that switch II is not involved in the Ras/Raf interaction (Thapar et al., 2004), and it is therefore a reasonable assumption that it may be disordered in the complex. This situation is very closely mimicked in the context of the crystalline
environment with symmetry of space group R32: switch I is in the conformation found in the Raps/Raf complex, and switch II is disordered in the wild-type protein.

Proposed Contribution of Y32 and the Bridging Water Molecule to Intrinsic Catalysis in Ras

Two critical residues in GAP-catalyzed GTP hydrolysis are RasQ61 and RasGAP R789 (Arg finger) (Scheffzek et al., 1997). A role for Q61 as a general base for activation of the catalytic water is unlikely, but it is thought to be a critical residue in positioning the water molecule during the reaction (Maegley et al., 1996). The role of the Arg finger, inserted into the active site, is to stabilize the negative charge that develops on the bridging oxygen between the \( \beta \)- and \( \gamma \)-phosphate atoms of the nucleotide during catalysis (Kosloff and Selinger, 2001; Li and Zhang, 2004). In the RasGAP-catalyzed reaction, Y32 is in an open conformation, interacting intimately at the Ras/RasGAP interface, where it is not involved directly in catalysis. In the presence of Raf, catalysis must occur with Y32 in the pocket that is occupied by R789 in the Ras/RasGAP complex. In this situation, it is much more likely that Y32 is involved in the catalytic mechanism.

There is evidence in the literature that the base that activates the water molecule for nucleophilic attack in the hydrolysis reaction is the \( \gamma \)-phosphate of GTP itself, in a substrate-assisted catalytic mechanism in which the abstracted proton ends up being shared by the \( P_i \) and the \( \beta \)-phosphate of the GDP leaving group in the product (Kosloff and Selinger, 2001; Pasqualato and Cherfils, 2005). One objection to this mechanism is that electron density would be stabilized at the \( \gamma \)-phosphorous atom, which is inconsistent with a reaction mechanism in which the outcome is an increase in negative charge at the oxygen bridging the \( \beta \)- and \( \gamma \)-phosphorus atoms (Maegley et al., 1996). In the GAP-catalyzed reaction, in which the transition state and product are highly stabilized by the Arg finger in the Ras/RasGAP complex, this may be a negligible effect, but in intrinsic catalysis it could be more of a problem. This problem would be alleviated, however, if the abstracted proton were to become part of a hydrogen-bonding network in which it could be donated to an...
electronegative acceptor. This is exactly the situation observed in our wild-type Ras-GppNHp structure and in the Raps-GppNHp/Raf-RBD complex, in which the O1G oxygen atom of the γ-phosphate is 2.7 Å from the precataytic water molecule and 2.6 Å from the water molecule bridging to the hydroxyl group of the Y32 side chain (Figure 5A). During the course of the reaction, there is inversion of the configuration at the γ-phosphate. The arrangement of the bridging water molecule and Y32 allows sufficient flexibility for this water to accompany the reaction coordinate, following the dynamic charge shifts that must occur during the reaction, and facilitates transfer of the proton to the β-phosphate as negative charge accumulates there in the transition state and as GDP is formed. This situation would be one in which the interaction between R789 and both the β- and γ-phosphate oxygen atoms seen in GAP-catalyzed hydrolysis would be mimicked, albeit in a much weaker form, by the bridging water molecule and Y32 together during intrinsic catalysis. In this context, the importance of Y32 is two-fold: the lone pair of electrons on its hydroxyl group can accept an H-bond from the bridging water molecule, which increases the tendency of this molecule to accept the H-bond from the O1G γ-phosphate oxygen atom; and the Y side chain has the ability to precisely position the bridging water molecule within the active site.

In the ground state, the precataytic water molecule donates an H-bond to the carbonyl oxygen atom of T35 (2.9 Å). It is not in line with the γ-phosphorous atom, but it is shifted toward the O1G oxygen of the phosphate group (Pasqualato and Cherfils, 2005). Q61 is disordered in the ground state. During the reaction, as a hydrogen is abstracted by the γ-phosphate O1G atom and donated in an H-bonding interaction to the bridging water molecule, the catalytic water is activated and the H-bond to the carbonyl group of T35 is no longer favored. This perhaps facilitates the shift in position necessary for inline nucleophilic attack on the γ-phosphate, and as the reaction proceeds Q61 may interact with the newly formed transition state, as it does in the Ras/RasGAP transition state mimic (Scheffzek et al., 1997).

### The Structure of Switch II in Transforming Mutants Is Consistent with a Noncatalytic Ras

The crystal structures presented here show that, in the context of a closed conformation of switch I, with Y32 over the nucleotide, transformation is highly correlated with the ability of the mutated residue to form the hydrophobic cluster. There are two important features of this conformation that are consistent with a noncatalytic version of the GTPases. One is that the hydrophobic cluster completely shields the γ-phosphate and precataytic

### Table 2. Buried Surface Areas, Average B Factors, and Interatomic Distances for RasQ61 Mutants in the Noncatalytic Conformation

<table>
<thead>
<tr>
<th>Residue</th>
<th>Q61L Ras-GppNHp</th>
<th>Q61V Ras-GppNHp</th>
<th>Q61K Ras-GppNHp</th>
<th>Q61I Ras-GppNHp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buried surface area</td>
<td>934.87 Å²</td>
<td>926.71 Å²</td>
<td>934.35 Å²</td>
<td>936.03 Å²</td>
</tr>
<tr>
<td>Average B factor (residue 61)</td>
<td>34.7 Å³</td>
<td>34.7 Å³</td>
<td>28.13 Å³</td>
<td>27.15 Å³</td>
</tr>
<tr>
<td>Distance (Å)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61 Cα ... Tyr64 Cα</td>
<td>4.5</td>
<td>n/a</td>
<td>3.8</td>
<td>4.6</td>
</tr>
<tr>
<td>61 Cβ ... Tyr64 Cβ</td>
<td>4.2</td>
<td>3.7</td>
<td>3.5</td>
<td>3.8</td>
</tr>
<tr>
<td>61 Cα ... Pro34 Cα</td>
<td>4.0</td>
<td>n/a</td>
<td>3.6</td>
<td>3.8</td>
</tr>
<tr>
<td>61 C=O ... H-N Tyr64</td>
<td>2.9</td>
<td>2.9</td>
<td>2.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Tyr32 Cα ... Pro34 Cα</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
<td>3.7</td>
</tr>
<tr>
<td>Tyr32 Cβ ... Pro34 Cβ</td>
<td>4.3</td>
<td>4.3</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Tyr32 Cα ... Pro34 Cβ</td>
<td>3.3</td>
<td>3.3</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Tyr32 CZ ... Gly13 Cα</td>
<td>3.9</td>
<td>3.9</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>V61 Cα ... H-N Glu63;</td>
<td>n/a</td>
<td>3.9</td>
<td>n/a</td>
<td>3.9</td>
</tr>
<tr>
<td>I61 Cα ... H-N Glu63</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>61 N-H ... Wat175</td>
<td>3.0</td>
<td>2.9</td>
<td>2.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Thr35 C=O ... Wat175</td>
<td>2.7</td>
<td>2.8</td>
<td>2.8</td>
<td>2.7</td>
</tr>
<tr>
<td>O1G ... Wat175</td>
<td>2.8</td>
<td>2.8</td>
<td>2.8</td>
<td>2.7</td>
</tr>
</tbody>
</table>

a The buried surface area was obtained by using the Lee & Richards buried surface accessibility calculation in CNS.
b The average B factor was calculated by using all atoms from each residue.
c The closest distance was measured between Tyr64 and Pro34, and this was modulated by different orientations of the aromatic ring of Tyr64 relative to Q61.
d Q61V and Q61I are the only mutants with carbon atoms that contact the upper portion of the switch. Distances are given in Å. A lack of interaction is indicated by “n/a.” Wat175 is the precataytic water molecule.
GTP Hydrolysis in RasQ61 Mutants Impaired by Raf

Intrinsic Catalysis In Vitro versus Transformation Efficiency in Cells

Over 20 years ago, it was proposed that residue 61 represents a strong conformation-determining region in Ras that modulates differences in affinity for effectors or regulatory molecules in accordance with the observed transforming potency (Der et al., 1986). However, in spite of a large number of biochemical and structural studies on Ras, its mutants, and related GTPases, there has been no explanation for the fact that all mutations at residue 61 show an ~10-fold decrease in Ras intrinsic GTPase activity measured in vitro, whereas there is a 1000-fold range in vivo transformation efficiency. The structures presented here shed light on this problem, supporting the idea that the conformation of residue 61 is indeed associated with transformation efficiency in the cell.

We propose that the key to understanding the differences in the in vitro versus the in vivo results is to consider that intrinsic hydrolysis was measured in vitro in the absence of Raf, with switch I in equilibrium between two or more conformations, as shown by NMR (Geyer et al., 1996). In this situation, the hydrophobic cluster, which involves Y32 and other switch I residues, is unstable and does not result in a noncatalytic Ras. The nature of the mutation may then be negligible, and the uniform reduction in GTPase activity is due to detrimental changes that occur when the Q61 side chain is removed. In the in vivo experiments, the situation is much more complex, and we propose that it is in the Raf-bound state that the identity of the mutation becomes important. This idea is supported by our hydrolysis experiments showing a strong damping effect of Raf on the RasQ61L GTPase reaction, which was not observed for wild-type Ras (Figure 6). When bound to Raf, Ras is entirely in state 2, and Y32 is in the closed conformation (Geyer et al., 1996). This provides a stable docking surface composed of Y32 and P34 with which the aliphatic portions of a variety of side chains at position 61 can interact to form the noncatalytic conformation. This is completely consistent with the finding that V, L, K, A, C, and R are strongly transforming mutations (Der et al., 1986). All of these, except for K and R, have side chains with hydrophobic character (Radzicka and Wolfenden, 1986).

water molecule from the bulk solvent (Figure 4), so that even if the reaction were to occur, the release of the P₁ would be severely hindered. In contrast, the wild-type and the weakly transforming mutant RasQ61G (Ford et al., 2006) have an open water channel into the active site (Figure 4). The second feature of the conformation found in the transforming mutants is that the hydrogen-bonding network in the active site is changed by Y32 making a direct H-bond to the γ-phosphate O₁G atom (excluding the bridging water molecule) and by the formation of a good H-bond between the backbone amide group of residue 61 and the precatalytic water molecule (Figures 3 and 5; Table 2). In this situation, the precatalytic water molecule could still donate a hydrogen bond to the O₁G atom, which could, in turn, be donated to the hydroxyl group of Y32. However, because of the resonance involving the aromatic ring, the hydroxyl oxygen atom is sp² hybridized, and its H-atom and lone pair tend to stay in the plane of the ring (Thanki et al., 1988). The H-bonding orientation is therefore highly controlled by the overall rotational freedom of the Y32 side chain. This side chain, in turn, is sandwiched between P34, G13, and the sugar moiety of the nucleotide in a restricted conformation that would not favor transfer of the abstracted hydrogen atom to the β-γ bridging oxygen during the reaction. Interestingly, the hydroxyl oxygen atom is positioned to interact ideally with the O₁G atom of the γ-phosphate, which is in the plane of the ring in the ground state.

In the noncatalytic conformation, the buried precatalytic water molecule still donates an H-bond to the backbone carbonyl group of T35 and to the O₁G atom of the γ-phosphate, but, in addition, it accepts an H-bond from the backbone amide of residue 61. This new H-bond is likely to diminish the nucleophilicity of the water molecule, helping to stabilize the entire system in a nonreactive ground state. Taken together, the features resulting from formation of the hydrophobic cluster create a strongly anticatalytic situation, in which the H-bonding network is ideal in the ground state, the precatalytic water is more difficult to activate, and the entire complex is trapped in the active site.
and, based on the common noncatalytic conformation, are expected to fit well into the hydrophobic cluster. The R side chain has an aliphatic portion that is one carbon shorter than that of K; however, it is probably positioned similarly, with its charged head group toward the bulk solvent. On the other hand, Y, W, and F are too bulky to significantly sample the noncatalytic conformation and, accordingly, have much weaker transformation efficiencies (Der et al., 1986). We propose that the moderately transforming mutants N, H, I, M, and T have side chains that frequently sample the noncatalytic conformation but are either a little too bulky or too polar for optimal stabilization of the hydrophobic cluster. Gly is the most weakly transforming of all the mutations, whereas Pro and Glu are equivalent to the wild-type and essentially show no transformation unless highly overexpressed (Der et al., 1986). Gly, with no side chain, cannot contribute to the hydrophobic cluster. In this structure, there is a direct H-bond between Y32 and the O1G atom of the γ-phosphate, a feature that perhaps explains the measurable, although weakly transforming phenotype, of the mutant. Glu would be expected to be similar to Q in size, shape, and the ability to participate in intrinsic catalysis (Frech et al., 1994). Interestingly, P would be somewhat strained, with a φ dihedral angle of about −85° in the noncatalytic conformation of switch II, given that its ideal φ angle is −60°. However, even if it were able to attain this conformation, its backbone N atom could not donate an H-bond to the precatalytic water molecule, disrupting a component of the anticatalytic arrangement observed for the transforming mutants. These two features together could be sufficient to destabilize the noncatalytic conformation in the Q61P mutant, opening the water channel to the active site.

Our results are consistent with a scenario in which the transformation efficiency of the mutant is directly correlated with its ability to stabilize the noncatalytic conformation in the context of the Ras/Raf complex, rather than one in which each mutant stabilizes a different conformation that is more or less transforming. They offer a rationale for the previously obtained transformation efficiencies observed for RasQ61 mutants and provide a mechanism to explain how the conformation first observed and proven to be noncatalytic in the Ran/importin-β complex can result in a severely impaired enzyme in the context of the Ras/Raf complex.

EXPERIMENTAL PROCEDURES

All of the experiments were done with a truncated version of H-Ras, containing residues 1–166. Dr. Sharon Campbell (UNC, Chapel Hill) provided the expression systems for wild-type Ras, the RasQ61L mutant, and the C-Raf construct containing the RBD and CRD domains (residues 52–196). The QuikChange II Site-Directed Mutagenesis Kit from Stratagene was used, by following the manufacturer’s instructions, to obtain RasQ61V, RasQ61K, and RasQ61I. The DNA coding for each protein was cloned into the pET21A(+) vector (Novagen) and transformed into E. coli BL21 cells (Novagen) for expression and purification.
described (Buhrman et al., 2003). The GDP was exchanged for the GTP analog, GppNHP, by following published procedures (Stumber et al., 2002). Protein in a buffer solution containing 20 mM Tris (pH 8.0), 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 5% glycerol, and 20 μM GppNHP was concentrated and used immediately for crystallization or was stored in 50 μl aliquots at −80 °C. Crystals were grown by the hanging-drop vapor-diffusion method at 18 °C. The initial crystallization drops contained 4 μl protein solution and 4 μl reservoir solution. For crystals of wild-type Ras-GppNHp, the purified protein solution was concentrated to 15–20 mg/ml and the reservoir solution consisted of 200 mM CaCl2, 20% PEG 3350 (PEG Ion Screen #7 from Hampton Research). For crystals of the Q61L mutant, the purified protein was concentrated to 10–15 mg/ml, and the reservoir solution consisted of 200 mM CaCl2, 25% PEG 3350, and 1 mM DTT. Crystals grew in 5–10 d to an average size of 0.5 mm3 and were flash frozen in liquid nitrogen directly from the crystallization drop without additional cryoprotectant.

The final protein concentration for RasQ61K was 12 mg/ml in a buffer solution containing 20 mM HEPES (pH 7.5), 20 mM MgCl2, 50 mM NaCl, and 1 mM DTT (stabilization buffer). Crystals were obtained by using the hanging-drop vapor-diffusion method at 18 °C, in drops containing 2 μl protein solution and 2 μl reservoir solution (500 μl PEG Ion Screen #7 and 200 μl stabilization buffer). Crystals of Q61K took 3–5 d to form. Crystals were exchanged into a cryoprotectant solution consisting of 800 μl PEG Ion Screen #7 and 200 μl PEG 400 immediately prior to flash freezing in liquid nitrogen.

The final protein concentration for the Q61V mutant was 5 mg/ml in stabilization buffer. Crystals were obtained from wells containing 400 μl PEG Ion Screen #7, 100 μl stabilization buffer, and 150 μl PEG 400. They grew in 3–5 d to an average size of 0.1 mm3 and were cryo-protected as with Q61K.

The final protein concentration for the Q61L mutant was 12 mg/ml in stabilization buffer. Crystals of Q61L were obtained from wells containing 500 μl PEG Ion Screen #7 and 150 μl 50% PEG 6000 or PEG 8000. Crystals of Q61L grew in 7 d. The cryo-protectant for the RasQ61L crystals was a solution of 500 μl PEG Ion Screen #7, 200 μl stabilization buffer, 100 μl 50% PEG 6000, and 200 μl glycerol.

For the hydrolysis experiments, Ras-GDP was exchanged for Ras-GTP by following a published procedure (Cheng et al., 2001). The protein was kept at 4 °C for immediate use in the experiments.

Data Collection and Structure Refinement

High-resolution data for the wild-type Ras and the Q61 mutants were collected at 100K on the Ser-CAT ID-22 beamline at APS (Argonne, IL), by using a Mar CCD detector. The X-rays were tuned to a wavelength of 1.0 Å. Exposure was from 1 to 3 s with an oscillation angle of 1° and a crystal-to-detector distance of 120 mm. The data were processed with HKL2000 (Ohwino and Minor, 1997).

The structure of H-Ras 166 (PDB code: 1CTQ) with all non-protein atoms and residues 61–71 deleted from the model and with atomic B factors set at 1.0 Å was used as an initial search model for molecular replacement by using the program Crystallography and NMR System (CNS) (Brünger et al., 1998). CNS was also used for all reciprocal space refinement, and 10% of the unique reflections was set aside for the calculation of Rfree (Kleywegt and Brünger, 1996). The best molecular replacement solution was applied to generate a model used for rigid-body refinement at 2.5 Å, followed by rigid-body refinement at 2.0 Å, simulated annealing, energy minimization, and group B factor refinement in CNS prior to generation of Fσ – F and F – F electron density maps. Manual rebuilding was done in O (Jones et al., 1991) and COOT (Emsley and Cowtan, 2004). CNS was used in successive rounds of energy minimization and individual B factor refinement. The GppNHP molecule was added to the model early in the refinement. Water molecules and ions were added in successive rounds of manual rebuilding. The final model for the wild-type protein was used to phase the RasQ61L mutant. The final model for the RasQ61L mutant structure was used to phase RasQ61K, RasQ61V, and RasQ81I. The initial search model (PDB code 1CTQ) was used to phase the RasQ611 mutant structure from crystals with the symmetry of the P321 space group.

C-Raf(RBD-CRD): Expression and Purification

The C-Raf(S2–196) construct was expressed from a Protein G expression vector (GE2) with an N-terminal GB1 tag for increased solubility and an N-terminal His tag for affinity purification. After standard overexpression in E. coli, Raf was purified by affinity chromatography, by using a 5 ml nickel-NTA column (Amersham Pharmacia). Protein was solubilized in Buffer A (20 mM HEPES [pH 7.5], 500 mM NaCl, 30 mM imidazole, 5 mM b-mercaptoethanol) and eluted from a gradient (80% in 80 ml) of increasing Buffer B (20 mM HEPES [pH 7.5], 50 mM NaCl, 500 mM imidazole, 5 mM b-mercaptoethanol). Raf elutes from 50%–70% Buffer B. Raf protein was pooled, dialyzed into Buffer A minus the imidazole, concentrated to 3 mg/ml by placing the dialysis bag on a bed of polyethylene glycol (MW: 20,000), and stored at –80 °C.

Hydrolysis Experiments

The hydrolysis reactions were performed at room temperature in a total volume of 2.5 ml Hydrolysis Buffer (50 mM Tris [pH 7.5], 10 mM DTT, 50 mM NaCl, 10 mM MgCl2) and allowed to proceed for 6–7 hr. The experiments were repeated independently to ensure reproducibility. Either RasQ61L or wild-type Ras was present initially at a concentration of 8–10 μM before exchange to GTP and was allowed to incubate in two sets of parallel experiments: one in which a stoichiometric amount of Raf was added at the onset of the reaction, and one in which it was added 30 min before being transferred to 4 °C, a temperature at which the reaction in all cases was slowed down for analysis. Samples were run on 5-100 gel-filtration columns pre-equilibrated in Hydrolysis Buffer + 5% glycerol at 4 °C before and after the incubation period. Gel filtration with Ras-GDP/Raf showed a small amount of complex.

ACKNOWLEDGMENTS

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REFERENCES


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Accession Numbers
Coordinates and structure factors have been deposited in the PDB with the following accession codes: wild-type Ras, 2RGE; RasQ61L, 2RGD; RasQ61V, 2RGC; RasQ61K, 2RGB; RasQ61I (R32), 2RGA; RasQ61I (P3221), 2RGG.