Substituting alanine for glycine at position 60 in v-H-Ras generated a dominant negative mutant that completely abolished the ability of v-H-Ras to transform NIH 3T3 cells and to induce germinal vesicle breakdown in Xenopus oocytes. The crystal structure of the GppNp-bound form of RasG60A unexpectedly shows that the switch regions adopt an open conformation reminiscent of the structure of the nucleotide-free form of Ras in complex with Sos. Critical residues that normally stabilize the guanine nucleotide and the Mg\(^{2+}\) ion have moved considerably. Sos binds to RasG60A but is unable to catalyze nucleotide exchange. Our data suggest that the dominant negative effect observed for RasG60A-GTP could result from the sequestering of Sos in a non-productive Ras-GTP-guanine nucleotide exchange factor ternary complex.

Ras is an essential component of signal transduction pathways that regulate growth, proliferation, differentiation, and apoptosis in response to the activation of membrane-bound receptors (1, 2). Dominant negative Ras mutants have been widely used to elucidate the role of Ras in a variety of signaling pathways. The asparagine for serine mutant at position 17, RasS17N, is probably the most frequently used dominant negative form of Ras (3) and the success of this mutant popularized the use of dominant negative mutants to study the signaling of other small GTPases. Despite this success, the exact molecular details by which dominant negative GTPases exert their inhibitory function are a matter of debate in the literature. It is widely accepted that RasS17N blocks the ability of endogenous Ras to function by sequestering and depleting the intracellular pool of available guanine nucleotide exchange factor (GEF),\(^1\) thereby blocking the activation of endogenous Ras (4–6). This argument is supported by the finding that overexpressing a dominant active form of Ras (e.g. RasG12V) or an activator domain usually abolishes the inhibitory effect of dominant negative Ras (3, 7). However, other explanations have been also proposed (8–9) including low affinity of RasS17N for GTP and the inability of GTP to induce the RasS17N conformation necessary for binding and activating downstream effectors (4, 10).

To complicate matters, the S17N mutant of Rap1A, which \textit{a priori} should behave like RasS17N, is unable to inhibit the activation of Rap1A by its exchange factor, C3G, \textit{in vitro} (11). Understanding at the molecular level how a dominant negative Ras functions should shed light on its cellular role and help in designing new tools to dissect the signaling of Ras and other small G-proteins. Because it inhibits the activation of endogenous Ras, dissecting the action of a dominant negative Ras should also better our understanding of the reaction of nucleotide exchange. So far, the structure of a dominant negative Ras complex is lacking in the literature.

The substitution of alanine for glycine at position 60 in v-H-Ras, v-H-RasG60A, generated a dominant negative mutant that completely abolished the ability of v-H-Ras to transform NIH 3T3 cells, to induce germinal vesicle breakdown in Xenopus oocytes, and to activate the Ser/Thr kinase Raf-1. Moreover, v-H-RasG60A inhibits the ability of v-H-Ras to induce oocyte germinal vesicle breakdown when co-injected (12–14). As with RasS17N, proper membrane localization of the G60A mutant is necessary for its dominant negative effect. Biochemical characterization showed that the G60A mutation does not alter the apparent affinity of v-H-Ras or c-H-Ras for GDP, GTP, the \textit{Saccharomyces cerevisiae} exchange factor SDC25C, or for GAP and NF-1. However, the mutation moderately reduced the ability of SDC25C to stimulate v-H-Ras exchange and abolished the ability of GAP or NF-1 to accelerate the rate of GTP hydrolysis. In addition, the G60A mutation slightly attenuated the binding of Ras to Raf but severely reduced the binding to RapGDS (14). How RasG60A reverts the transforming ability of constitutively active Ras is still unclear but conceptually, this reversal is likely to occur through sequestration of GEFs and/or downstream effectors into non-productive complexes.

In the present work, we have undertaken the structural and biochemical analysis of RasG60A to understand the origin of its dominant negative effects. We show that this mutant adopts a novel open conformation in the GppNp-bound form that bears

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\(^1\) The abbreviations used are: GEF, guanine nucleotide exchange factor; Ni-NTA, nickel-nitrilotriacetic acid; HPLC, high performance liquid chromatography; DTT, dithiothreitol; WT, wild-type; mant-, N-methylanthraniloyl; RafRBD, Ras binding domain of Raf kinase.
similarities to the structure of nucleotide-free Ras bound to the catalytic domain of Sos (15). Our structural and biochemical results suggest a new mechanism for the unique dominant negative effect of RasG60A.

MATERIALS AND METHODS

**Crystallography and Structure Determination**—Wild-type Ras and RasG60A mutant (residues 1–166) were expressed as His-tagged proteins (pProEx-HTb vector) in the Escherichia coli BL21(DE3) strain (30). Proteins were purified on a Ni-NTA column (Qiagen) followed by a Q-Sepharose (Sigma) and a gel filtration column. The GDP-bound nucleotide was exchanged to GppNp (31) and exchange was confirmed by use of an HPLC C18 reverse phase column. For diffraction experiments, crystals were grown at 20 °C by mixing 4 μl of 20 mg/ml RasG60A (in 20 mM HEPES, pH 7.5, 0.15 mM NaCl, 10 mM MgCl2) and 4 μl of a reservoir solution. For the GDP-bound form of RasG60A, the reservoir consisted of 30% (w/v) PEG4000, 0.2 mM magnesium sulfate, and 0.1 mM Tris-HCl, pH 8.0. For the GppNp-bound form of RasG60A, the reservoir consisted of 30% (w/v) PEG2000mm, 0.15 mM magnesium sulfate, 0.1 mM HEPES, pH 7.0, and 0.3% hydrogen peroxide. The GDP-bound form crystalized in space group R32 (a = 93.6 Å, c = 121.6 Å) with one copy in the asymmetric unit. The GppNp-bound form crystalized in space group I2222 (a = 181.4 Å, c = 121.3 Å) with one copy in the asymmetric unit. In each case, data were collected on beamline X26C at the National Synchrotron Laboratory Source, Brookhaven, on a 2k × 2k CCD detector (ADSC), processed with DENOZO, and scaled with SCALEPACK (32). The structures of the RasG60A mutant were solved by molecular replacement (33) with the deposited coordinates of wild-type Ras as search model (Protein Data Bank codes 5P21 and 4Q21 (16, 17)).

The GDP- and GppNp-bound structures were refined with the program CNS (34) or REFMAC (35) to 1.70- and 1.84-Å resolution to final crystallographic residuals Rwork = 22.8/23.9 and 18.9/24.4%, respectively. Stereochemistry was checked with the program PROCHECK (36). Data collection and refinement statistics are summarized in Table I. The final electron density maps of both RasG60A forms show Cys18 to be oxidized.

**GFP Assay**—0.7 ml of a 1 ml solution of the GppNp-bound form of Ras proteins in (10 mM HEPES, 2 mM MgCl2, 0.15 mM NaCl, pH 7.5, 10% D2O) was used for the NMR spectra. One-dimensional 31P NMR spectra were collected at 5 °C in a 5-mm broadband probe on a Bruker Avance 700 spectrometer at a phosphorous resonance frequency of 192.48 MHz; 16,584 or 47,104 transients were recorded after excitation with a 45° pulse at a repetition rate of 2 s per transient. A 10-Hz exponential apodization function was applied before Fourier transformation. The data were processed with the spectrometer software.

**Ras Activation Assay**—Cos1 cells were transiently transfected with the indicated pCMG (37) Ras constructs and allowed to express for 24 h. Cells were rinsed with phosphate-free Dulbecco's modified Eagle's medium (Invitrogen) and incubated with phosphate-free Dulbecco's modified Eagle's medium supplemented with 1 mM/μl 32PO4 (ICN) for 4 h prior to harvest. Cells were subsequently washed three times with ice-cold phosphate-buffered saline and lysed in 400 μl of ice-cold buffer containing 50 mM HEPES, pH 7.4, 0.1 mM NaCl, 1% Triton X-100, 5 mM MgCl2, 1 mM Mg-ATP hydrolysis buffer, 0.1% Triton X-100, 5 mM MgCl2 and 0.005% SDS. Bound nucleotides were eluted in 2 μl EDTA, pH 8.0, 2 mM dithiothreitol, and 0.2% SDS for 20 min at 68 °C. Nucleotides were resolved by thin layer chromatography on polyethyleneimine-cellulose plates (Machery-Nagel) in 0.75 M KH2PO4, pH 3.4, and quantified with a PhosphorImager (Amersham Biosciences). Percentage GTP was calculated with the formula [(GTP/GDP)3 + (GDP/2)]1/2 to 100, to account for differences in 32P incorporation within nucleotides.

**RESULTS**

**Comparison of GppNp-bound RasG60A and Wild-type Ras**—We have solved the crystal structure of the RasG60A mutant bound to the slowly hydrolysable GTP analog, GppNp. The final model is refined to 1.84-Å resolution with low crystallographic indicators and excellent stereochemistry (Table I). A section of the electron density around the GppNp is shown in Fig. 1A.

**Glycine 60 in Ras is located in the conserved DXXG motif of switch 2 that is part of a sharp flexible turn (LA) connecting strand β3 and helix α2 (16). Flexibility in this motif, which is important for Mg2+ ion coordination and γ-phosphate stabilization (16, 17), is essential for the proper cycling of Ras. Whereas the overall structures of the GppNp-bound forms of wild-type Ras (hereafter WT-Ras) and RasG60A superpose well outside the switch regions, switch 2 is extensively reorganized and represents a new conformation that has not been previously observed in structural studies of GDP- or GTP-bound Ras (Fig. 1B). Although switch 2 is poorly ordered in WT-Ras, its conformation is well defined in RasG60A. Residue 60 seems to be the initiator of the switch 2 restructuring. The (Φ, ψ) dihedral angles of this residue change from (−80°, −10°) in the WT-Ras structure to (−58°, 153°) in the RasG60A structure. The same rotation for residue 60 has been observed in the RasA59G mutant, which mimics the structure of an intermediate for GTP hydrolysis (18). This change does not disturb only the immediate surroundings of position 60 but propagates to the next eight residues (Gly60 to...
Arg68), which also undergo large shifts in their dihedral angles. One immediate consequence of switch 2 restructuring is the displacement of switch 2 residues away from the nucleotide (Fig. 1B). For example, the Co of residues Gln61 and Ala66 are shifted by 4.8 and 7.3 Å, respectively, relative to their positions in WT-Ras. Another consequence is a large change in solvent accessibil-

**TABLE I**

| Data collection and refinement statistics | GDP-bound form | GppNp-bound form |
|-----------------------------------------|---------------|-----------------|
| Resolution range (Å)                    | 29.7–1.7      | 31.6–1.84       |
| \(R_{sym}^{a}\), % overall (last shell)  | 8.2 (56.8)    | 8.7 (57.3)      |
| Completeness (%) overall (last shell)   | 99.5 (99.8)   | 97.5 (98.9)     |
| Multiplicity, overall (last shell)      | 11.5 (7.6)    | 4.4 (4.2)       |
| No. of unique reflections               | 22,655        | 15,186          |
| Protein atoms                           | 1,325         | 1,333           |
| Heterogeneous atoms                     | 30            | 34              |
| Solvent atoms                           | 68            | 98              |
| \(R_{free}^{b}\), % overall (last resolution shell) | 22.8 (29.6) | 15.3 (27.9) |
| Root mean square deviation in bond length (Å) | 0.007       | 0.014           |
| Root mean square deviation in bond angle (°) | 1.2          | 1.6             |
| Estimated coordinate error (Å)          | 0.23/0.22     | 0.24/0.24       |
| Ramachandran plane (%)                  | 91.4/8.6      | 94.1/5.9        |

\(a R_{sym} = \frac{\sum_{hkl} |I(hkl)| - I(hkl)/\sum_{hkl} I(hkl), \)

\(b R_{free} = \frac{\sum_{hkl} \left|F_{calc} - F_{obs}\right|}{\sum_{hkl} \left|F_{calc}\right|}, \) where \(T\) is the test set (39) obtained by randomly selecting 10% of the data. Last resolution shell is 1.81–1.70 Å and 1.89–1.84 Å for the GDP- and GppNp-bound forms, respectively.

d Estimated coordinate error calculated for the data in the 5.0 Å to the highest resolution range from the Luzzati/SIGMAA statistics.

Most favored/additional allowed regions.

**Fig. 1.** **A**, section of the electron density calculated with \((2F_{obs} - F_{calc})\) coefficients and phases derived from the final model is shown at 1σ cutoff around the GppNp. The nucleotide is shown in ball-and-stick, the Mg\(^{2+}\) ion and water molecules as cyan and red spheres. **B**, comparison of the GTP-binding site in RasG60A and WT-Ras. Water 33, which bridges the γ-phosphate and the main chain amide of Ala60 in RasG60A and water 175, the water molecule responsible for the nucleophilic attack on the γ-phosphate in WT-Ras are labeled with a W. The GppNp and the Mg\(^{2+}\) ion are in blue; red spheres represent water molecules; dotted green lines represent hydrogen bonds. **C**, surface representation of RasG60A and WT-Ras around the GppNp. Switch 1 is in wheat and yellow, switch 2 in lime and green. The GTP and the Mg\(^{2+}\) ion are in magenta and cyan. Generated with Molscript (38) and Pymol (pymol.sourceforge.net). **D**, change in solvent accessibility per amino acid resulting from the alanine for glycine substitution at position 60 (19). The change is expressed as a percent of the total surface area of the amino acid. Positive change represents increase in solvent accessibility caused by the mutation.
ity of switch 2 residues (Fig. 1, C and D). For example, Tyr64 is not solvent protected as in the wild-type structure, but is totally solvent exposed. The observed conformation of switch 2 is stabilized by a network of hydrogen bonds between the guanidinium group of Arg68 and an anion hole formed by the main chain carbonyls of Ala59, Ala60, and Glu63. In addition, a water molecule mediates the interaction of Arg68 with the main chain amide of Ser65. Consistent with previous reports (18), our results demonstrate that Gly60 serves as a pivot for switch 2 conformational changes and rationalize the conservation of a glycine at this position in all GTPases.

An unexpected feature of the GppNp-bound structure of RasG60A is the remodeling of switch 1 to a conformation that completely pulls it away from the nucleotide into the solvent (Fig. 2A). For example, the Cα of Phe28, the residue that normally stabilizes the guanine ring by stacking interactions in all nucleotide-bound forms of Ras, has moved 13.1 Å from its position in WT-Ras. Thr35, which coordinates the Mg2+ ion and the γ-phosphate in all GTP-bound structures of Ras, has also moved 3.5 Å (Cα) from the position it occupies in WT-Ras. This movement propels Thr35 into the solvent (Fig. 1D) and prevents it from directly coordinating the Mg2+ ion and the γ-phosphate. The temperature factors of switch 1 residues 30 to 37 are among the highest in the final model (50 Å² versus an average of 28 Å²) but the electron density of this region is clear enough to trace it. The observed conformation of switch 1 is stabilized by hydrophobic interactions between the phenyl group of Phe28 and apolar residues provided by crystal packing and by the strong hydrogen bonds Glu37 makes with Ala59 (2.9 Å) and Ser65 of switch 2 (2.6 Å), which are not present in WT-Ras. The strong interaction between Glu37 and Ser65 maintains the C terminus of switch 1 (residues 38–40) in a conformation similar to that of WT-Ras (Figs. 1B and 2E). Thus, the present conformation of switch 1 shows that when it adopts an open conformation, Ras has evolved a Phe28- and Thr35-independent way of stabilizing the nucleotide and the Mg2+ ion.

The remodeling of switch 1 and 2 affects nucleotide stabilization and Mg2+ ion coordination in an unprecedented way. Specifically, the nucleotide is more solvent exposed in RasG60A than in the wild-type structure (Fig. 1C). Surface accessibility calculations (19) show that the surface-exposed area of the GppNp has more than doubled between the RasG60A (191 Å²) and WT-Ras.
and the wild-type structure (85 Å²). The position of the phenyl group of Phe²⁸ is replaced in the RasG60A-GppNp structure by Lys¹⁴⁷ of the conserved 145SAK¹⁴⁷ motif (20). The long aliphatic side chain of Lys¹⁴⁷, which stabilizes the phenyl group of Phe²⁸ by stacking interactions in WT-Ras, now occupies the void left by the displacement of Phe²⁸. Consequently, the guanine base in RasG60A is stacked between the long side chains of Lys¹⁴⁷ and Lys¹⁴⁷ (Fig. 1, B and C). The interactions of Asn¹¹⁶ and Asp¹¹⁹ with the guanine base are conserved between WT-Ras and RasG60A. The ribose hydroxyls no longer interact with the protein main chain carbonyls, instead the OH²⁻ is within hydrogen-bond distance with the side chain amino group of Lys¹⁴⁷ (Fig. 1B). As a consequence, the ribose is more solvent exposed in RasG60A. The α- and β-phosphates have conserved interactions with the protein (16) with the exception that they are more solvent accessible (Fig. 1C). The γ-phosphate, which in WT-Ras makes direct hydrogen bonds to Lys¹⁴⁷ of the P-loop, Thr³⁵ of switch 1, and Gly⁶⁰ of switch 2 is more solvent exposed, does not interact with Thr³⁵, and interacts with the main chain amide of Ala⁶⁰ through a water molecule (Fig. 2C). In addition, there is no equivalent in the RasG60A structure for the water molecule responsible for the nucleophilic attack on the γ-phosphate (16). The Mg²⁺ ion makes conserved interactions in RasG60A with one important exception. The hydroxyl group of Thr³⁵ is not coordinating the metal ion, as is the case in all known GTP-bound structures of Ras, but is 6.4 Å from its position in WT-Ras. Instead, a water molecule completes the octahedral coordination of the Mg²⁺ (Fig. 2D). Thus, the G60A mutation, which confers a dominant negative characteristic for v-H-Ras, prevents Ras from adopting an active conformation and results in improper Mg²⁺-coordination.

Comparison of RasG60A-GppNp with Nucleotide-free Ras—The structure of the RasG60A mutant is reminiscent of nucleotide-free Ras (hereafter NF-Ras) in complex with Sos (15), which corresponds to a stable intermediate on the path for the catalyzed guanine nucleotide exchange reaction. In both structures, switch 1 adopts an “open” conformation that destabilizes the Phe²⁸/guanine base interaction and the coordination of the Mg²⁺ ion by Thr³⁵, whereas switch 2 is displaced from the γ-phosphate. In addition, Tyr⁶⁴ and Tyr⁷¹, which are at the heart of the Ras/Sos interface, adopt positions that are close to their positions in NF-Ras. However, the conformations of the switch regions in NF-Ras and RasG60A structures are not identical (Fig. 2). In particular, Phe²⁸ and Thr³⁵ are more distant from the nucleotide-binding site in NF-Ras than in the RasG60A structure (Fig. 2A). The side chain of Ala⁵⁹, which in NF-Ras sterically hinders the positioning of the Mg²⁺ ion to its binding site, has a wild-type conformation in the RasG60A structure (Fig. 2B). Met⁶⁷, which is buried at the Ras/Sos interface, is displaced 4.9 Å in the RasG60A structure from its position in NF-Ras. Very likely, the presence of the catalytic domain of Sos, which extensively interacts with residues of both switch regions, is responsible for the observed differences in the switch regions between RasG60A and NF-Ras.

Structure of RasG60A-GDP—To find out whether the switch regions adopt an open conformation in the inactive form of RasG60A, we solved its crystal structure. The RasG60A-GDP structure superposes well on the WT-Ras-GDP including the switch regions (17) (Table I). The GDP and the Mg²⁺ ion make conserved interactions with RasG60A including the Phe²⁸/guanine-ring stacking interaction. This observation shows that the open conformation is only characteristic of the triphosphate-bound form of the nucleotide and not the diphosphate.

Solution Studies—To further establish that the differences in the conformations of the switch regions between WT-Ras and RasG60A exist in solution and are not caused by crystal packing or other artifacts, we compared the one-dimensional 3¹P NMR solution spectra of GppNp-bound WT-Ras and RasG60A. The 3¹P resonances are sensitive to the environment of the bound GppNp and thus should provide information on the conformation of the switch regions. Fig. 3A shows that in the case of the wild-type protein, there are three distinct resonances for the α- and β-phosphates and a single resonance for the γ-phosphate. The data are consistent with at least three distinct states of the nucleotide-protein complex that exist in slow exchange. In the case of RasG60A, there is only a single resonance for each of the α-, β-, and γ-phosphates. The integrated intensities of the α-, β-, and γ-phosphate resonances are equal. The data are consistent with either one static conformation of the bound GppNp or multiple conformations in fast exchange. The comparison between the two NMR spectra suggests that in solution a large conformational change around the α- and β-phosphates of the GppNp takes place because of the glycine to alanine mutation. One plausible explanation for the observed differences in the 3¹P NMR spectra is that in solution, the switch regions of WT-Ras and RasG60A adopt different conformations.

To confirm further that the difference in the switch conformations exists in solution, we used the fluorescent nucleotide derivative mant-GTP. The mant moiety, which is attached to the ribose hydroxyls, is located in proximity of the polypeptide Phe²⁸ to Tyr⁴². We predicted from the GTP- and GDP-bound structures of RasG60A that following GTP hydrolysis, the switch 1 region would undergo substantial conformational changes until it reaches the GDP-bound conformation. During this transition, the hydrophobic mant moiety should experience an increase in fluorescence because it is moving from a polar and solvent-exposed to a more hydrophobic protein-like environment. To test this prediction, we loaded the Ras proteins with mant-GTP and followed the change in fluorescence because of GTP hydrolysis. Fig. 3B shows that, as predicted, GTP hydrolysis resulted in an increase in fluorescence in the case of RasG60A and in a decrease in the case of WT-Ras. The amplitude of the change is also different, suggesting that the extent of the change is more substantial in the RasG60A than in WT-Ras. Combined, the NMR and fluorescence data show that in solution the switch regions are consistent with the conformation observed in the crystal structure.

Biochemical Characterization of the RasG60A Mutant—In an effort to understand the functional significance of the conformation displayed by RasG60A, we characterized the biochemical properties of this mutant. The observed rate of intrinsic GTP hydrolysis at 37 °C (6.8 × 10⁻⁵ s⁻¹) is one-fifth of that for the WT-Ras (Fig. 4A). This value is ~7 times faster than the value initially reported at 30 °C (14). This discrepancy is probably because of the difference in temperature at which the experiments were conducted. This reduced GTPase activity can be attributed to the displacement of Gln⁶¹ from the catalytic site (Fig. 2B). This residue plays a critical role in orienting and stabilizing the water molecule responsible for the nucleophilic attack on the γ-phosphorus, identified as W175 in WT-Ras (16). The final electron density of the GppNp-bound form of RasG60A lacks density for a water molecule equivalent to W175. The only water that might attack the γ-phosphate, although not well positioned for an in-line attack, is the water situated between the γ-phosphate and Ala⁶⁰, W33 (Fig. 2C). To analyze the nucleotide binding status of this mutant in vivo, we transfected COS-1 cells with expression vectors encoding the wild-type, G60A, and the dominant active G12V mutant, and metabolically labeled the cells with ³²P⁴⁻. Nucleotide binding was determined by immunoprecipitation of Ras followed by thin layer chromatography analysis of the immunoprecipitant.
predominantly in the GTP-bound state, consistent with the observed reduction in GTPase activity of this mutant in vitro.

Next, we compared the abilities of WT-Ras and RasG60A to release guanine nucleotides by measuring the intrinsic dissociation rates of the fluorescent analog mant-GDP and mant-GTP (21). Fig. 5 and Table II show that the observed intrinsic dissociation rates of the GDP and GTP were not altered by the G60A mutation, consistent with previous nitrocellulose membrane binding measurements (14). An identical coordination of GDP and Mg\(^{2+}\) ion in WT-Ras and RasG60A structures justifies a similar rate of GDP dissociation. The fluorescence data, however, show that despite the structural differences in GppNp binding between WT-Ras and RasG60A, especially those related to Phe\(^{28}\), Thr\(^{35}\), the \(\gamma\)-phosphate, and the increase in solvent accessibility area of the GppNp, which predict that the triphosphate nucleotide is more easily detachable from RasG60A, the observed intrinsic rate of dissociation of the GTP was not affected by reorganization of the switch regions (Fig. 5B). The swinging of Lys\(^{147}\) in the RasG60A GppNp structure to stabilize the guanine ring by occupying the void left by Phe\(^{28}\)
is one likely explanation for the unchanged rate of dissociation of GTP between the wild-type and mutant protein.

To examine how the G60A mutation affects catalyzed nucleotide release, we compared the rate of nucleotide dissociation from WT-Ras and RasG60A in the presence of the catalytic domain of Sos. Whereas equimolar concentrations of Sos (1 μM) substantially accelerate the release of GDP and GTP from WT-Ras, they have a modest or no effect on both nucleotide-bound forms of RasG60A (Fig. 5 and Table II). Pull-down (Fig. 7) and gel filtration experiments (Fig. 6D) show comparable binding of Sos to both forms of RasG60A, albeit a reduced binding affinity. To rule out the possibility that a low binding affinity between RasG60A and Sos might be responsible for the defect in catalysis, we repeated the exchange experiments in the presence of a 10- and 100-fold excess of Sos. Fig. 5 and Table II show that despite the excess of Sos, the GEF is unable to accelerate nucleotide dissociation from RasG60A to the level of WT-Ras.

To test whether the binding of Sos to RasG60A results in the formation of a ternary RasG60A nucleotide-Sos complex, we purified the complexes of Sos and the GDP- or GppNp-bound forms of RasG60A on gel filtration (Fig. 6) and checked the nucleotide state of the resulting complexes by reverse phase HPLC. These experiments clearly show the presence of GppNp and, to a lesser extent, GDP in the RasG60A:Sos complexes (Fig. 6, B and C) demonstrating that the binding of Sos to RasG60A results in the formation of a stable RasG60A-GppNp:Sos in a 1:1:1 ratio and to a lesser extent a RasG60A-GDP:Sos complex (Fig. 6, E and F). In contrast, we did not detect any nucleotide in the case of the WT-Ras:Sos complex (Fig. 6, A and E). Combined, these data show that the binding of Sos to RasG60A stabilizes a protein-nucleotide ternary complex. Presently, atomic details of the ternary complexes between Sos and the GDP- or GTP-bound forms of RasG60A are not available to explain the better retention observed for the GppNp over the GDP in the Sos complexes. However, a likely explanation for the difference in nucleotide retention by the RasG60A:Sos complex comes from the comparisons of the crystal structures of NF-Ras and RasG60A. These comparisons propose that the absence of the γ-phosphate in RasG60A sterically hinders residues 59 and 60 from adopting nucleotide inhibitory positions as found in NF-Ras. In contrast, the absence of the γ-phosphate in RasG60A-GDP leaves enough room for residues 59 and 60 to adopt their corresponding NF-Ras positions.

It is now well established that binding of effector proteins to Ras requires a wild-type GTP-bound conformation of the switch regions (22–24). To examine the effects of the conformation adopted by the RasG60A mutant on the binding of downstream effectors, we have employed an in vitro pull-down assay using the Ras binding domain of Raf kinase (RafRBD). As illustrated in Fig. 7, a residual binding between RasG60A-GTP and RafRBD is observed at high Ras concentrations. This result is consistent with the observed conformation of switch 1 being unfavorable for effector binding (25) and contradicts the previous conclusion that the dominant negative phenotype of v-H-RasG60A is because of its sequestering of endogenous Ras downstream effectors including Raf kinase into non-productive complexes (13).
FIG. 6. Formation of a Ras-nucleotide-Sos ternary complex. Ras:Sos complexes were formed by incubating Ras and the catalytic domain of Sos as indicated in a 4:1 molar ratio in (10 mM HEPES, 0.15 mM NaCl, and 2 mM dithiothreitol) on ice for 1 h. The complex was then purified on a gel filtration column (Superdex 200, Amersham Biosciences) and immediately concentrated to ~10 mg/ml. 1.62 mg of RasG60A-Sos (A), 1.35 mg of RasG60A-GDP-Sos (B), or 1.0 mg of RasG60A-GppNp-Sos (C) complex were loaded on a reverse phase HPLC. The fluorescence of the eluant (y axis) was measured at 254 nm as a function of the retention time (x axis). The identity and mass of bound nucleotide was determined by comparing retention time, spectral profile, and integrated area of nucleotide peaks with known standards shown in gray, D, 12% SDS-PAGE of the purified complexes used for the HPLC experiments. Left to right, molecular weight standards (SDS-7, Sigma), WT-Ras:Sos, RasG60A-GDP:Sos, and RasG60A-GppNp:Sos. E, spectral scan between 220 and 320 nm of the HPLC peaks at 8.60 min in A (purple line), 8.60 min in B (black line), and of the GMP (red), GDP (blue), and GppNp (green) used as controls. Not shown is the spectral scan for C at 11.4 min, which overlaps with the green line. Note that the red and blue lines superpose. F, plot of the number of moles of Ras protein injected and of nucleotide retained on the HPLC C18 column in experiments A, B, and C. In each case, the number of moles of protein is normalized to 1.

DISCUSSION
Since their introduction in the late 1980s, dominant negative Ras mutants have served as powerful tools to dissect Ras signaling in a variety of biological systems. Despite this success, the molecular details of their mode of action are not fully understood. In this work, we have undertaken the structural and biochemical characterization of a dominant negative Ras mutant, RasG60A. Previously, the G60A mutation was shown to inhibit the ability of v-H-Ras to transform NIH 3T3 cells and to activate the downstream effector Raf-I. Moreover, v-H-RasG60A completely abolished the ability of v-H-Ras to induce germlinal vesicle breakdown in Xenopus oocytes (12–14). Because the RasG60A mutant we describe here is the first structure of a dominant negative Ras protein, it represents a unique opportunity to understand the structure/function relationship of this class of mutants. Two key features of RasG60A-GppNp explain its inhibitory effects.

First, the switch regions of RasG60A adopt open conformations that result in uncharacteristic coordination of the Mg\(^{2+}\) ion and reduced binding to RafRBD (Fig. 7 and Ref. 12) and GAP. Thus, the primary consequence of the G60A mutation is to prevent GTP from inducing an active signaling conformation of Ras such as the one seen in effector or GAP complexes (25–28). As a consequence, RasG60A is unable to bind and activate Ras effectors. The unique open conformation seen in the RasG60A-GppNp structure might thus be responsible for the dominant negative effect observed for this mutant. Whether the observed open conformation of the switch regions is conserved in other dominant interfering mutants of Ras including RasS17N remains to be seen.

Second, the reduced ability of RasG60A to bind RafRBD (Fig. 7) or RalGDS (13) combined with its ability to bind Sos (Figs. 6 and 7) suggest that in cells, RasG60A does not bind and activate Ras effectors but instead, sequesters RasGEFs into non-productive complexes. The inability of Sos even at 100-fold excess to accelerate the release of mant-GTP from RasG60A despite the relatively high concentrations of competing GTP (Fig. 5) supports the idea that the RasG60A-GppNp:Sos ternary complex is stable. Thus, unlike WT-Ras, which binds transiently to the GEF, RasG60A sequesters Sos into stable complexes. This sequestration should deplete the intracellular pool of available GEF necessary to activate endogenous Ras and contribute to the dominant negative effect. The sequestration of the GEFs is likely the reason why v-H-RasG60A blocks the transformation ability of v-H-Ras (13). v-H-Ras, which has a slow GTPase activity, requires the exchange factor to keep it active after one round of GTP hydrolysis. The sequestering of RasGEFs by v-H-RasG60A ensures that v-H-Ras is not activated and consequently blocks its transforming capability.

In addition to being the first structure of a dominant negative form of Ras, the RasG60A-GppNp structure presented here is the first structure of an open conformation of Ras that stabilizes a
such flexibility could be essential to induce Ala59 into other reason. Alternatively, the need of flexibility or absence of a /H18528 Ras complexes were precipitated with glutathione-Sepharose (untagged Ras proteins loaded with GppNp as indicated.)) or G60A incubated with increasing amounts of wild-type (untagged Ras with Gly60 may be sufficient to unfold because altering flexibility at this position is sufficient to unfold the switch regions and prevent the release of Sos. Second, the interaction between the nucleotide and the Mg2+ ion. Thus, flexibility at position 60 is necessary for nucleotide exchange, because altering flexibility at this position is sufficient to unfold the switch regions and prevent the release of Sos. Second, the nucleotide exchange results demonstrate that Sos is unable to dislodge the guanine nucleotide from RasG60A; instead, its binding to RasG60A stabilizes a ternary complex (Fig. 6). Several arguments can be put forward to explain the inability of Sos to accelerate the release of the nucleotide from RasG60A. The difference in the protein interface in Sos binding to WT-Ras and RasG60A might be one reason. The inability of Sos to dislodge Lys117 and Lys147 from stabilizing the nucleotide could be another reason. Alternatively, the need of flexibility or absence of a bulky side chain at position 60 or both is required to expel the nucleotide. Such flexibility could be essential to induce Ala95 into a Mg2+-binding inhibitory conformation as seen in the Sos/Ras structure (15).

The RasG60A-GppNp structure described in this study shares common features with WT- and NF-Ras, two states of the GTPase on the reaction path for nucleotide exchange. The interactions in RasG60A made by the P-loop and the NKD119 motif with the nucleotide are present in WT-Ras, whereas restructuring of the switch regions to pull Phe98, Thr35, and Gly80 away from the GTP and the Mg2+ ion are reminiscent of NF-Ras. Based on these structural similarities and its ability to capture a ternary complex in the presence of Sos, which was shown by Lenzen and colleagues (29) to be an intermediate for Ras-catalyzed nucleotide exchange, it is tempting to ascribe the RasG60A-GppNp structure to that of a transient intermediate for nucleotide exchange after GTP binding to NF-Ras but before the final GTP-bound conformation is reached. Whether the RasG60A structure represents such an intermediate requires further investigation.

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