Involvement of the Switch 2 Domain of Ras in Its Interaction with Guanine Nucleotide Exchange Factors*

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Ras proteins are activated by stimulated GDP release, which enables acquisition of the active GTP-bound state, little is known about how guanine nucleotide exchange factors (GEFs) interact with Ras to promote this exchange reaction. Here we report that mutations within the switch 2 domain of Ras (residues 62-69) inhibit activation of Ras by the mammalian GEFs, Sos1, and GRF/CDC25m. While mutations in the 62-69 region blocked upstream activation of Ras, they did not disrupt Ras effector functions, including transcriptional activation and transformation of NIH 3T3 cells. Biochemical analysis indicated that the loss of GEF responsiveness of a Ras(69N) mutant was due to a loss of GEF binding, with no change in intrinsic nucleotide exchange activity. Furthermore, structural analysis of Ras(69N) using NMR spectroscopy indicated that mutation of residue 69 had a very localized effect on Ras structure that was limited to α-helix 2 of the switch 2 domain. Together, these results suggest that the switch 2 domain of Ras forms a direct interaction with GEFs.

Ras proteins are guanine nucleotide-binding proteins that function as molecular switches to mediate downstream signaling from a variety of cellular receptors that promote cell growth and differentiation (1). Switching of Ras proteins between the active GTP-bound and inactive GDP-bound states in response to cellular stimulation is tightly regulated in vivo by two classes of regulatory molecules; guanine nucleotide exchange factors (GEFs); Sos1, GRF/CDC25m that promote the acquisition of GTP and GTPase-activating proteins (GAPs); p120 and NF1-GAP that stimulate its rapid hydrolysis to GDP (1-3). While the Ras-GTP/GDP ratio can be elevated by inhibition or how GEFs interact with Ras. Measurements of Km and Kcat values indicated that GEF interaction with the poorly responsive Ras(35A), Ras(62H), and Ras(63H) mutants were not lost (7). However, no enzymatic or direct binding assays were performed to determine if a reduction in GEF responsiveness of other Ras mutants correlated with decreased GEF association. It is anticipated that identification of the site(s) of GEF interaction will lead to a better understanding of how Ras and other regulatory GTPases, e.g., Rab, Rho, and heterotrimeric G protein family members (15), are activated and will identify a potential site(s) for pharmacological intervention of growth factor-induced mitogenesis.

To determine the site(s) of GEF interaction with Ras, we previously took advantage of the biochemical properties of two Ras dominant inhibitory mutants. Both Ha-Ras(15A) and Ha-Ras(17N) mutants have higher affinity for GEFs than Ras(WT) and function biologically by sequestering these exchange factors, thus preventing GEF activation of the normal endogenous Ras proteins, resulting in inhibition of cell growth (3). By coupling random mutagenesis of the dominant inhibitory Ha-Ras(17N) mutant with biological selection we recently identified Ras residues 75, 76, and 78 to be critical for its growth inhibitory phenotype, presumably through loss of interaction with the mammalian GEFs, Sos1, and GRF/CDC25m (13). In contrast, mutational analysis of Ha-Ras(15A) revertants in Saccharomyces cerevisiae identified distinctly different residues (62, 63, 67, and 69) that impaired Ras interaction with the yeast CDC25 (12). The 63K mutant has been previously reported to transform NIH 3T3 cells, suggesting a gain rather than loss of function (16). Furthermore, a 76K mutant that was found to disrupt Ras-GEF interaction without affecting Ras effector function in NIH 3T3 cells (13) attenuated oncogenic Ras signaling in S. cerevisiae (12). These observations indi-
cated that specific Ras mutants could have divergent effects in these two biological systems. Whether these contrasting results reflected differences between the yeast and mammalian GEFs, the different biological systems, or between the properties of the Ras(15A) and Ras(17N) dominant inhibitory proteins was unclear.

To determine the importance of residues 62–69 in Ras interaction with mammalian GEFs, we characterized the 62K, 63K, 67I, and 69N mutations, previously identified in yeast, in Ha-Ras and expressed them in NIH 3T3 murine fibroblasts to examine their effects on Ras function. Our results establish that, like residues 75–78, residues 62–69 are essential for Ras action with mammalian GEFs, we characterized the 62K, 63K, 67I, and 69N mutations, previously identified in yeast, in Ha-Ras and expressed them in NIH 3T3 murine fibroblasts to examine their effects on Ras function. Our results establish that, like residues 75–78, residues 62–69 are essential for Ras interaction, without disrupting Ras effector functions. Furthermore, in vitro analysis of the Ras(69N) protein by multidimensional NMR spectroscopy indicated that minor structural alterations associated with this mutation were localized to α-helix 2 in the switch 2 domain. Thus we conclude that helix 2 defines a region of Ras that is directly involved in Ras-GEF interaction.

MATERIALS AND METHODS

Molecular Constructs—Bacterial and mammalian expression constructs of normal, oncogenic, and dominant inhibitory Ras containing each of the 62K, 63K, 67I, and 69N mutations were generated by excising PvuII-NcoI fragments containing human Ha-ras codons 22–110 from pAAC-3GFP(+)NcoI and yeast pAD4-ras expression constructs harboring these mutations (12) and swapping these sequences with cDNA sequences that encoded full-length Ras(WT), Ras(17N), Ras(12R), and with Ras(WT) residues 1–166 in the bacterial expression vector pAT-ras (17). The resulting full-length mutant ras cDNAs were then subcloned as BglII-BamHI fragments into the BamHI site of the mammalian expression vector pZIP-NesSV(x)1 (18). pBABE-SOS1 (19) was provided by S. E. Egger, Hospital for Sick Children, Toronto, p41L-GRF (20) was from L. A. Feig (Tufts University, Boston, MA), and pJEX-NF1-GRD (21) was from F. Tamanori (UCCLA). pZIP-P53, p21 p53, and p21pCDC25-CAAX were made as described (22). Recombinant p120 GAP was kindly provided by G. Bolag (Onyx Pharmaceuticals, Richmond, CA).

Ras-CDC25 Binding Assay—Preparation of glutathione S-transferase (GST)-CDC25 fusion protein was carried out as described previously (23). Wild type and mutant ras cDNAs were cloned into the pRSET Xpress System vector (Invitrogen, CA) for expression of (His)6-tagged proteins and transformed into Escherichia coli strain BL21(DE3). Cells were grown to an A600 of 0.5–1.0, induced with isopropyl-1-thio-galactopyranoside (1 mM) and incubated at 28°C overnight. Cells were then pellet, resuspended in 4 ml binding buffer (50 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9) g cell pellet and lysed by sonication. After centrifugation (39,000 × g, 20 min) the supernatant (100 μg of Ras protein) was mixed with 10 μg of GST-agarose bead-bound GST-CDC25, 4°C, 60 min. The beads were then washed four times with phosphate-buffered saline containing 1% Triton X-100. CDC25-bound Ras was then determined by Western blot using the Ha-Ras-specific 146–3E4 monoclonal antibody and the ImmunoLite blot detection kit (Bio-Rad).

GAP Assays—For Fig. 4, 125 μl of Ras WT or (63K) were prepared by incubating 5 pmol of Ras in 25 μl of 50 mM Tris-HCl, pH 7.5, 20 μm KCl, 1 μm dithiothreitol, 25 μm EDTA, and 15 μM 125I-GRF (30°C, 5 min. The binding reaction was stopped by adding MgCl2 to 5 mM and incubating on ice for 1 min. GAPase assays were performed at 30°C in the presence or absence of 0.1 pmol of p120-GAP. Reactions were terminated and GTP hydrolysis determined as described previously (24). For Fig. 6B, GAPase activity was determined using 4 μM 32P-GTP. 0.2 μM NF1 GAP-related (GRD) as described (25). The amount of phosphate released was quantitated by scintillation counting of 32PO4 following an organic extraction in the presence of ammonium molybdate.

Ras Preparation and NMR Spectroscopy—Procedures for the purification and preparation of recombinant Ras(1–166)(69N) for NMR analysis was as described (26–28). All NMR experiments were recorded under identical conditions on ~1.5 μM samples of Ras (1–166)-GDP uniformly labeled (~90%) with 2H, at 30°C on a Bruker AMX500 NMR spectrometer. Two-dimensional 1H, 2H NMR heteronuclear single quantum coherence and three-dimensional 1H-edited NOESY (nuclear Overhauser enhancement spectroscopy) and TOCSY (total correlation spectroscopy) data were collected and processed as described previously (28).

RESULTS

Mutations in the Residues 62–69 Region of Ras Abolish the Dominant Inhibitory Phenotype of Ras(17N) but Not Oncogenic Ras Transforming Ability—To understand the role of the Ras switch 2 domain in mammalian GEF-mediated Ras activation, we set out to determine if missense mutations in the 62–69 region of Ras, originally isolated as intragenic suppressors of the Ha-Ras(15A) dominant negative mutant in S. cerevisiae (12), similarly reverted the growth inhibitory phenotype of a Ras dominant negative mutants in mammalian cells. Therefore, sequences encoding the 62K, 63K, 67I, and 69N mutations were created in Ha-Ras(17N). Transfection and stable selection of NIH 3T3 fibroblasts expressing these cDNAs in G418-containing growth medium showed that these substitutions reversed the growth inhibitory phenotype of Ras(17N) (Fig. 1). In each case the morphology of cells was similar to that of control NIH 3T3 cells, and mutant Ras(17N) proteins could be detected by Western blotting with an Ha-Ras-specific antibody (146–3E4).2 Therefore the observed reversion of growth inhibition was not due to instability of the Ras(17N) double mutants. Furthermore, introduction of second site mutations into the 62–69 region of oncogenic Ras(12R), which functions independent of GEF stimulation (13), did not inhibit its ability to induce morphologic transformation (Fig. 2, lower panels). This indicated that downstream effector functions of Ras were not perturbed by mutation of the 62–69 region.

This effect was further quantitated using a NIH 3T3 focus-formation assay. As seen in Fig. 3, introduction of mutations into Ras residues 62, 67, and 69 greatly impaired (75–100%) the ability of Ras(WT) to induce transforming focus formation, while only partially reducing the activity of the GTPase-defective constitutively GTP-bound oncogenic Ras(12R). Most significantly, the 69N mutation completely inhibited the focus-forming ability of Ras(WT) while having no effect on the transforming ability of Ras(12R). This suggested that the 69N mutation most severely impairs GEF interaction with Ras without disrupting Ras activation of downstream effector protein(s).

2 L. A. Quilliam, unpublished observation.
In contrast to studies in yeast, but in agreement with the observation of Fasano et al. (16), expression of the Ras(63K) mutant in NIH 3T3 cells resulted in an apparent gain of function as evidenced by morphological transformation (Fig. 2, upper panels). This observation again indicates that conflicting results can be obtained in the yeast and mammalian biological assay systems. The transforming potential of Ras(63K), as quantitated by focus forming activity (Fig. 3A) was found to be approximately 25-fold greater than by Ras(WT). The potent transforming activity caused by oncogenic mutations at codons 12, 13, and 61 of Ras is due to decreased intrinsic and GAP-stimulated GTPase activity, resulting in the proteins accumulating in the active GTP-bound state (29). While the GAP-stimulated GTPase activity of a Ras(63Q) mutant was similar to that of Ras(WT) (30), the mild transforming activity of a Ras(63H) mutant has previously been correlated with a reduced sensitivity of its GTPase activity to GAP stimulation (31). As shown in Fig. 4, the transforming activity of the 63K mutation also correlated with a significant decrease in intrinsic and p120 GAP-stimulated GTPase activity.

Mutations at Residues 62, 67, and 69 of Ras Inhibit GEF-mediated Activation of Ras(WT) without Disrupting Its Intrinsic Biochemical Properties—To determine if the mutations in the 62–69 region of Ras were inhibiting cellular transformation by blocking Ras(WT) activation by GEFs, we measured their

**Fig. 2. Morphology of NIH 3T3 cells stably expressing Ras(WT) and Ras(12R) with mutations at residues 62–69.** NIH 3T3 cells were transfected with pZIP constructs encoding the indicated Ras mutants and stable transfectants selected in G418-containing growth medium. Pooled populations were obtained by combining >100 drug-resistant colonies for each Ras mutant and photographed at x 100 magnification to demonstrate morphology.

**Fig. 3. Mutation of residues 62–69 drastically inhibits Ras(WT)-induced, but not Ras(12R)-induced, transformation in NIH 3T3 cells.** A, transformed focus formation of Ras(WT) mutants. Cells were transfected with 2 μg, or 0.2 μg for Ras(63K), of the indicated pZIP-ras constructs per 60-mm dish and incubated in growth medium for 14 days prior to quantitation of transformed foci. B, transformed focus formation of oncogenic Ras(12R) mutants. Cells were transfected with 20 ng of the indicated pZIP-ras constructs and treated as in A. Results are shown as mean ± S.D. for triplicate plates and are representative of at least three independent experiments.

**Fig. 4. Ras(63K) is impaired in GAP-stimulated GTPase activity.** Five pmol of (His)_6 Ras(WT) and Ras(63K) were loaded with [γ-^32^P]GTP as described under "Materials and Methods" and incubated ± 0.1 pmol of p120-GAP, 30 °C. The percentage of GTP hydrolyzed after 2-min incubation, as determined by nitrocellulose filter binding assay (24), is indicated. Results are mean ± S.D. for triplicate samples.
ability to cooperate with the Sos1 and GRF exchange factors to induce transcriptional activation from a chloramphenicol acetyltransferase reporter plasmid. Chloramphenicol acetyltransferase expression was regulated by a promoter containing the Ets/AP-1 Ras-responsive elements of the polyoma virus enhancer sequence (32). As shown in Fig. 5, Ras(62K), Ras(67I), and Ras(69N) were unresponsive to GEF stimulation, and although the 63K mutant had transactivating ability due to its defective GTPase activity, it did not cooperate with Sos or GRF. Since the GTPase-defective Ras(12R), which is considerably more transforming than Ras(63E), can still respond to GEF stimulation, the results suggest that residue 63 of Ras may be critical for both the GAP and GEF responsiveness of Ras.

The lack of responsiveness of the Ras 62K, 67I, and 69N mutants to GEF stimulation and their inability to induce transactivation could possibly be due to reduced intrinsic nucleotide exchange activity, thereby maintaining Ras in its inactive GDP-bound state. To address this possibility, we compared the biochemical properties of the Ras(69N) mutant to those of Ras(WT). As shown in Fig. 6A, the intrinsic off-rate of GDP from Ras(69N) protein was not significantly reduced from that of Ras(WT). Furthermore, in contrast to the Ras(63K) mutant, there was no effect of the 69N mutation on intrinsic or GAP-stimulated GTPase activity (Fig. 6B). Therefore, the biological properties of Ras(69N) are consistent with a defect in GEF stimulation. This hypothesis was confirmed using a GST-CDC25 catalytic domain fusion protein (23) that promoted nucleotide exchange on Ras(WT) but was completely ineffective on the 62K, 63K, 67I, and 69N mutants in vitro.

Switch 2 Mutations Block Physical Association between Ras and GEFs—Due to the nature of the genetic selection of the Ras(15A) revertant double mutants, they were predicted to disrupt interaction between Ras and yeast CDC25. Consistently, it was previously reported that mutations in residues 62–69 of Ras considerably reduced its ability to stimulate β-galactosidase expression when cotransfected with CDC25 using a yeast GAL4 two-hybrid in vivo binding assay. This loss of GEF interaction was confirmed by measuring the binding of Ras(WT) and the 62–69 mutants to the catalytic domain of CDC25 in vitro. All four mutations reduced Ras-CDC25 complex formation, with the 63K and 69N mutations being most effective (Fig. 7). These data rule out any possibility of an intermediate coupling protein between Ras and GEF in the two-hybrid assay, but cannot address the possibility that mutations within the switch 2 domain can induce a conformational change in other regions of the Ras protein that are the true

FIG. 5. Mutation of Ras residues 62–69 blocks Sos1- and GRF-stimulated transcriptional activation of Ras-responsive elements. NIH 3T3 cells were cotransfected with 2 μg of pZIP or pZIP-ras mutant and either 200 ng of empty pBABE vector, pBABE-Sos1 (A), or 100 ng of pJ4-GRF (B) along with 1 μg of pB4X-CAT reporter construct. Chloramphenicol acetyltransferase activity was determined 48 h post-transfection as described previously (13). Similar results were obtained following cotransfection of Ras mutants with the isolated catalytic domain of Sos1 or a membrane-targeted form of the GRF catalytic domain (not shown).

FIG. 6. Mutation of Ras residue 69 does not disrupt its intrinsic nucleotide exchange activity or its intrinsic or GAP-stimulated GTPase activities. A, recombinant Ras(WT) or Ras(69N)(1–166) was loaded with [8,5-3H]GDP and the rate of loss of nucleotide measured in the presence of excess (0.5 mM) cold GTP at room temperature. B, recombinant Ras(WT) or Ras(69N)(1–166) was loaded with [γ-32P]GTP and incubated in the presence or absence of 0.2 mM NF1-GRD for the indicated times. GTP hydrolysis was determined by measuring the accumulation of labeled inorganic phosphate. All results are mean ± range for duplicate samples and are representative of at least two experiments.

3 J. Han, R. D. Mosteller, and D. Broek, unpublished observation.
sites of GEF interaction. To address this possibility, we compared the tertiary structure of Ras(WT) and the 69N mutant using multidimensional NMR spectroscopy. The 69N mutant was chosen for this study, since it had the most significant consequence on normal versus oncogenic Ras-induced transformation, was most impaired in its ability to respond to Sos1 and GRF in the transcriptional activation assays, and was least effective at association with CDC25 in vitro.

Structural Changes Resulting from Mutation of Aspartate 69 Are Restricted to the Switch 2 Domain. To determine the effect of the 69N mutation on the Ras three-dimensional structure, we conducted a series of 1H-15N-edited multidimensional NMR experiments on C-terminally truncated (residues 1–166) Ha-Ras(69N), uniformly enriched with 15N. NMR data were then compared with that of wild type Ras(1–166) whose NMR solution structure has been solved recently (28).

By using 15N, we could remove all NMR signals not attached to the 15N nucleus, thereby simplifying the NMR spectrum of this 18.9-kDa protein. Comparison of 1H-15N correlation maps between Ras(WT) and Ras(69N) showed chemical shift differences in only a few HN resonances, indicating that the mutation at position 69 produced only minor perturbations in the protein. We were able to assign all of the HN residues in the mutant, with the exception of residue 69, by comparing 15N-edited three-dimensional NOEY and TOCSY data acquired on Ras(WT) and Ras(69N). The only residues that showed sizable changes in both 1H and 15N chemical shifts were localized near residue 69 in helix 2, as well as residues 26 and 28 in loop 2.

Although the chemical shift perturbations associated with residues near the site of the mutation were expected, we were surprised by the chemical shift alterations observed for residues N26 and F28 in loop 2. However, we have previously observed that residue 28 is very sensitive to changes in temperature and environment, which could cause significant changes in the chemical shift of this residue.

To more definitively assess structural changes resulting from the 69N mutation, we examined NOE cross-peaks in Ras(WT) and Ras(69N) three-dimensional 15N-edited NOEY data sets. Variations in short range (≤5 Å) inter-residue proton contacts are identifiable through analysis of the NOE cross-peak intensities. NOE differences observed between Ras(WT) and Ras(69N) provide information regarding alterations in the spatial distribution of protons, which can be related to changes in tertiary structure. Close inspection of three-dimensional 15N-edited NOEY data revealed a partial disruption of inter-residue connectivities in residues spanning from 64 to 76 of switch 2 and also between residues 28 and 147. A ribbon diagram of Ras derived from the Ras(WT)-GDP NMR solution structure is shown in Fig. 8 and illustrates the location of residues that exhibit loss of NOEs associated with the 69N mutation.

Residues that make up an α-helix are expected to show consecutive strong amide-amide (dNN) cross-peaks with adjacent amino acids. NOE correlations ranging from weak to medium intensity are also observed for amino acids up to 4 residues away. Although consecutive dNN cross-peaks between residues 70 and 76 were detected in NOE spectra of Ras(69N), which are usually indicative of an α-helix, we also observed a reduction in the number of medium and long range cross-peaks, indicative of some perturbation of the helix.

We also observed a loss in an NOE connectivity between residue Phe28 and Lys47. These results are intriguing as mutation of Phe28 has been shown to drastically increase the nucleotide dissociation rate of Ras, presumably due to disruption of a critical hydrophobic interaction with the guanine nucleotide base (33). Binding of GEFs to Ras facilitates dissociation of the bound guanine nucleotide substrate, but the mechanism by which this occurs is unknown. While we did not observe loss of NOE cross-peaks between Phe28 and the H-8 proton of the guanine base, we did observe a reduction in the number of medium and long range NOEs in residues 28, 69, and 147.

FIG. 7. Mutation of residues 62, 63, 67, and 69 of Ras disrupts interaction of Ras with the catalytic domain of the yeast GEF, CDC25. 100 µg of recombinant Ras(WT), 62K, 63K, 67I, and 69N proteins were incubated (60 min, 4°C) with a GST-CDC25 catalytic domain fusion protein (10 µg) immobilized on glutathione-agarose beads. Following extensive washing, bead associated Ras was quantitated by Western blotting with a Ha-Ras-specific monoclonal antibody, as described under "Materials and Methods." Mutation of each residue significantly reduced association with CDC25 as compared with Ras(WT), with no detectable binding of the Ras(63K) or Ras(69N) mutants. Binding of all four mutant proteins, but not that of Ras(WT), to CDC25 was lost following more stringent washing.

FIG. 8. Structural analysis of 69N 1–166 (compared with WT). A ribbon diagram derived from the Ras(WT)-GDP NMR solution structure (28) is shown. Residues that demonstrate loss of sequential NOEs in the Ras(69N) mutant are shown in black. NOE changes are localized to residues 64–76 in switch 2 and between residues 28 and 147. Side chains for residues 28, 69, and 147 are illustrated in the ribbon diagram.

DISCUSSION

It is now known that in addition to activation by point mutation, Ras is responsible for mediating cellular transformation induced by deregulated upstream GEFs and by tyrosine phosphorylation.
kinase oncogenes that act via GEF stimulation (22, 34–36). While it has also been established that activation of GEFs, rather than inhibition of GAPs, is the major physiological mechanism for elevating the GTP-bound state of Ras (1), much less is known about Ras interaction with GEFs than with GAPs (6). To better understand the GEF-mediated GDP/GTP exchange reaction, we set out to identify sites of Ras interaction with GEF molecules. Our initial studies identified a domain (residues 75–78) as being critical for GEF-mediated activation of Ras, while yeast studies pinpointed a juxtaposed region (residues 62–69) (12, 13). Since these two independent screens did not identify overlapping regions, we have examined the role of Ras residues 62–69 in mammalian GEF stimulation of Ras and addressed the role of this region in mediating GEF interaction.

Analysis of the consequences of 62K, 67I, and 69N mutations in NIH 3T3 cell transformation and transcriptional activation assays revealed that they abolished the dominant negative Ras(15N) phenotype, inhibited focus formation by WT but not oncogenic Ras, and were poorly or unresponsive to mammalian GEF (Sos1, GRF) stimulation. These data all support the involvement of this region of Ras in GEF-mediated activation. Sos1 and GRF were similarly affected by different Ras mutants (the 62 mutant was slightly more sensitive to GRF than Sos1 stimulation). Thus although the catalytic domains of Sos1 and GRF are only ~30% identical, they presumably function similarly to promote nucleotide exchange. A slight stimulation of transcriptional activation was observed with the 67I mutant in the presence of both GEFs and with the 62K mutant in the presence of GRF. This is consistent with the weak interaction of these, but not the 63K or 69N mutants, with CDC25 in vitro (Fig. 7).

In contrast to the 62, 67, and 69 mutants, but in agreement with a previous observation (16), Ras(63K) was found to be transforming and induced transactivation of Ras-responsive promoter elements. We show here that this enhanced biological activity can be attributed to a defect in GAP-stimulated GTPase activity, similar to that of oncogenic Ras mutants with mutations at residues 12 or 61 (29). The stimulatory phenotype was not observed in yeast, presumably due to the relative differences in GAP activity between cell types. Since mammalian Ras(WT) is insensitive to the yeast Gaps, IRA1 and IRA2, and so accumulates in the active GTP-bound state (29), Mosteller et al. (12) used a strain of yeast that expressed the catalytic domain of the mammalian p120 GAP from a strong promoter. High expression of this protein presumably offset the decreased sensitivity of Ras(63K) to GAP stimulation. Since Ras(63K) lacks responsiveness to Sos1 and GRF in NIH 3T3 cell transfectional activation assays, this mutant also appears to be defective in GEF regulation as found in yeast. Loop 4 of Ras contains residues critical for both GEF and GAP regulation (6, 31). Since Ras(63K) is transforming in mammalian cells, it would appear that the GTPase defect is dominant over the exchange defect as was also observed for the 12R double mutants (Fig. 2).

A number of studies have identified regions of Ras whose integrity are required for GEF-stimulated activation. These include the residues 130–140 (8) and 95–110 regions (8, 14), switch 1 (in particular residues 35 and 38 (7)), and multiple residues in the switch 2 domain: residues 66, 75 (11), 62, 63, 78, 81 (7), 73/74 (10), 75/77 (9), 62, 63, 67, and 69 (12) and residues 75, 76, and 78 (13). Although these mutations of Ras can disrupt GEF-mediated activation, there has been little insight into how GEFs interact with Ras to promote the exchange reaction. Only two studies previously addressed whether reduced responsiveness to GEFs was a result of decreased Ras-GEF binding. While measurement of \( K_m \) and \( K_{cat} \) suggested that 35A, 62H, and 63H mutants could still interact with Ras (7), the two-hybrid analysis of Mosteller et al. (12) suggested that 62K, 63K, 67I, and 69N mutants were greatly impaired in their GEF binding ability. This was confirmed by direct in vitro binding in this study. Although the yeast CDC25 was used in these binding experiments, we anticipate that similar results would have been observed using its mammalian counterparts. Indeed, a recent report by Moodie et al. (37) demonstrated the inability of additional switch 2 mutants to bind to mammalian GEFs. The Y13-259 monoclonal antibody that specifically interacts with residues 63–73 in the switch 2 of Ras (38) competed for binding of Ras to both the yeast CDC25 and mammalian SOS1 (12), further indicating the functional similarity of these GEFs and supporting the involvement of the switch 2 domain of Ras in exchange factor interaction. However, none of these data could exclude the possibility that 62–69 mutations had not disrupted the tertiary structure of Ras, so perturbing GEF interaction with a more remote domain. For example, Stouten et al. (39) have proposed that upon Ras switching between its GDP- and GTP-bound states, movement of switch 2 is relayed to \( \alpha \)-helix 3, located within another domain of Ras previously implicated in GEF sensitivity (14).

Limited structural analysis has been performed on Ras mutants, with most work focusing on the effects of the oncogenic mutations that attenuate GTPase activity (40, 41). It is evident upon examination of NMR data that mutation of residue 69 from Asp to Asn, that completely blocked GEF binding and stimulation of Ras, produced only minor structural alterations in Ras that appear to be primarily localized to helix 2 within the switch 2 domain. This consistent with the fact that Asp69 lies on the exterior face of helix 2 in the GDP-bound form of Ras and that residues in helix 2 form few tertiary contacts with other secondary elements in the NMR structure of Ras(WT) (1–166) (28). Furthermore, NMR analysis did not reveal structural changes in Ras(69N) at other sites previously reported to alter GEF sensitivity (i.e. residues 95–110, 130–140, or the switch 1 domain). The loss of a NOE cross-peak between residues 28 and 147 is an intriguing observation, but further study is required to ascertain whether it was loss as a result of slight changes in solvent conditions as opposed to mutation of residue 69.

While the switch 2 domain undergoes significant structural orientation upon Ras exchanging GTP for GDP, both nucleotide-bound states are equally responsive to GEF stimulation (42). Neither the rate of GDP release from Ras nor its downstream effector functions were disrupted by introduction of the 69N mutation, indicating that conformational switching was not impaired. These observations, taken together with the lack of structural alterations outside of helix 2 of Ras(69N), indicate that the inability of Ras(69N) to interact with GEFs is most likely due to localized disruption of a GEF binding site in the switch 2 domain. Consistent with this conclusion, it has been reported that a swap of positive for negative charge on residue 1374 (Arg to Glu) in the catalytic domain of yeast CDC25 rescued the GEF sensitivity of the Glu to Lys (negative for positive) mutation at Ras residue 63, suggesting that these two residues form a functional ion pair interaction (43). The ability of the Y13-259 anti-Ras antibody, which binds to residues in helix 2 (38), to disrupt binding of Ras to CDC25 and Sos1 further support a role for this region of Ras in GEF interaction.

From biochemical studies with the dominant inhibitory Ras(15A) it is clear that GEFs have a much higher affinity for, and act to stabilize, the nucleotide-free state of Ras (44). Therefore, it is possible that interaction of GEFs with the switch 2 region helps stabilize the nucleotide-free intermediate state of Ras. Future studies will be required to determine if other
regions on the surface of Ras interact with GEFs and how GEF interaction with Ras catalyzes the nucleotide exchange reaction.

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