Identification and Characterization of Mutations in Ha-Ras That Selectively Decrease Binding to cRaf-1*

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The oncprotein Ras transforms cells by binding to one or more effector proteins. Effector proteins have been identified by their ability to bind to Ras in the GTP but not GDP form, and by their requirement for the Ras effector domain for binding. The best understood Ras effectors are serine/threonine kinases of the Raf family, but other candidate Ras effectors, including a Ral guanine nucleotide dissociation stimulator and phosphatidylinositol 3-kinase (PI3 kinase) have also been identified. To investigate the mechanism of binding of cRaf-1 to Ras, and to investigate the roles of other candidate Ras effectors in transformation, we have isolated and characterized mutants of activated Ras with decreased binding to cRaf-1 relative to other candidate effectors. Examination of these mutants indicates that surface-exposed residues of Ras outside the minimal effector domain interact differentially with cRaf-1 and other Ras-binding proteins, and that fibroblast transformation correlates with cRaf-1 binding and mitogen-activated protein (MAP) kinase activation. Furthermore, activation of PI3 kinase can occur in the absence of significant MAP kinase activation, suggesting that PI3 kinase activation is a primary effect of Ras.

The Ras proteins Ha-Ras, K-rasA, K-rasB and N-Ras are membrane-associated proteins that cycle between GDP and GTP-occupied states. Mutations in Ras that increase the amount of bound GTP activate the protein and are oncogenic, causing morphological and mitogenic transformation. The features of the Ras molecule that are needed for Ras transformation have been revealed by studying mutations that reduce the oncogenicity of activated Ras (1). Such loss of function mutations include those that inhibit GTP binding, promote GTP release, or prevent membrane localization. In addition, many substitutions of residues 32–38 inactivate Ras without reducing GTP binding or membrane localization. Therefore this region is postulated to be needed for Ras to transmit a signal to cell proteins that induce malignant transformation. These proteins are known as Ras effectors and the region of Ras that they contact is the effector domain. Because a nononcogenic relative of Ras, Rap1A, contains an identical effector domain, Ras residues separate from the effector domain must also needed for binding or activation of Ras effectors (2). Analysis of chimeras between Ha-Ras and Rap1A suggests that these additional residues flank the effector domain in the Ras sequence (residues 21–45) (3). The solution of a three-dimensional structure of Ras has shown that the effector domain between residues 32 and 40 (Switch I) changes conformation between the GDP and GTP states, together with residues 60–72 (Switch II) (see Wittgenhofer and Nassar (4)). The GTP-induced rearrangement of the backbone and side chains of Switch I is consistent with this portion of the molecule directly contacting effectors dependent on GTP.

A growing number of cell proteins have the biochemical properties expected of Ras effectors, binding to Ras dependent on GTP and the effector domain, but it seems unlikely that all are important for Ras signaling (1, 4, 5). These proteins include Raf-family serine/threonine kinases (cRaf-1, A-Raf, and B-Raf), GTPase-activating proteins (GAPs),1 proteins related to the Raf guanine nucleotide dissociation stimulator (RalGDS (6–8), RLF (9), and RGL (10)), the p110 (catalytic) subunit of heterodimeric PI3 kinase (specifically p110α), Rin-1 (11), AF6/ canoe (12), and Ras-1 (13). Of these putative effectors, Raf and PI3 kinase have been shown to have effector functions. Genetic analyses in Drosophila and Caenorhabditis show that Raf is required for Ras to induce specific cellular differentiation events in those organisms. In mammalian systems, artificial activation of Raf causes the same effects as activated Ras independently of Ras, suggesting that Raf activation is sufficient for some Ras responses. In addition, expression of full-length kinase-defective Raf or fragments containing the Raf Ras-binding domain (RBD) block many Ras responses. Unfortunately, such experiments are difficult to interpret because such “dominant-negative” mutants are in competition with all Ras effectors. One additional effector likely to be regulated by Ras in the cell is PI3 kinase. Downward and co-workers have shown that the stimulation of PI3 kinase activity by Ras does not require Raf and is likely due to direct association of Ras with PI3 kinase (14–16).

To investigate the role of cRaf-1 relative to other candidate Ras effectors, we have identified mutations in activated Ha-Ras that decrease interaction with cRaf-1 while retaining interaction with RalGDS and a PI3 kinase. The locations of the mutations outside the minimal effector domain suggests that effectors make specific contacts with other parts of Ras. The properties of the mutants suggest that Raf activation is essential for Ras to transform NIH3T3 cells.

1 The abbreviations used are: GAP, GTPase-activating protein; GDS, guanine nucleotide dissociation stimulator; PI3 kinase, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein; RBD, Ras-binding domain; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PtdInsP2, phosphatidylinositol-(4,5)-bisphosphate; GTPyS, guanosine 5'-3-O-(thio) triphosphate; PtdIns(4,5)P2, phosphatidylinositol-(4,5)-bisphosphate.

* This work was supported in part by United States Public Health Service, National Institutes of Health Grant CA54786 and National Institutes of Health NIGSA CA66281 (to D. G. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Experimental Procedures

Two-hybrid Screens and Mutant Selection—Two-hybrid analysis using LexA-Ras and VP16-RBDs and dual lacZ and HIS3 reporters has been described before (17). Because Ras-Raf interactions gave low and variable signals in solution β-galactosidase assays (17), interaction strength was determined by filter assays for β-galactosidase, using 0.75 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside and incubations from 4 h to overnight at 30 °C. Activation of the HIS3 reporter was assessed by plating serial dilutions of cultures onto plates containing different concentrations of 3-amino-triazole and calculating plating efficiency. Negative and positive controls were included in every assay.

Ras Switch I mutants were the kind gift of J. Stone, University of Alberta (18, 19) and were subcloned into LexA vector BTM116 (17) using a polymerase chain reaction.

The Ras G12V mutant open reading frame was mutagenized by polymerase chain reaction amplification using T7 polymerase in the presence of MnCl2 (20) and ligated into BTM116. DNA was electroporated into Escherichia coli and approximately 45,000 transformants pooled for DNA extraction. The library DNA was transformed into L40 strain yeast containing VP16-Caf1, and 2,500 transformants plated out at approximately 250 colonies per plate. Filter assays were performed to identify colonies that were not expressing J25 and J31. J25A and J25B were derived similarly from J31. J25A and J25B were derived from J25 making use of the BOMBI site at codon 47. J1A was derived from J1 using the Ncol site at codon 110, and J31A was derived similarly from J31. J32A and J32B were derived from J25 making use of the BomBI site at codon 47.

Bacterial Expression and Binding Assays—Ras G12V and mutants were moved into vector pGEX-2T (21), and glutathione-S-transferase (GST) fusion proteins purified as described previously (17, 21). Binding assays were performed as described elsewhere (17). GST-Ras fusion proteins were incubated with GTP or GDP in the presence of EDTA. Mg2+ was added, and the solutions were added to amylose resin carrying maltose binding protein fused to codons 51–210 of cRaf-1. After 1 h at 4 °C, the resin was washed, and proteins were eluted with maltose and analyzed by SDS-PAGE, immunoblotting with antibodies to GST. Equal recovery of maltose binding protein fusion proteins was confirmed by Coomassie Blue staining. The binding reactions contained approximately 700 nM Caf1-RBD and 330 nM GST-Ras.

Expression in and Transformation of Mammalian Cells—Activated Ras mutants were transferred from the BTM116 vector to mammalian expression vectors CS2 (22) and DOJ (23) by using polymerase chain reaction to add appropriate restriction sites for subcloning. The inserts were sequenced after subcloning. The ability of the activated Ras mutants to transform NIH3T3 cells (from C. Der, University of North Carolina (24)) and Rat1 cells was assessed as described elsewhere (25). Equal transfection of DOJ constructs was confirmed by selection for the drug selection markers present on the DOJ vector. For production of NIH3T3 and Swiss 3T3 cell lines, retroviral stocks were prepared from the DOI vector constructs (26–27). 60-mm plates of 293T cells were transfected with 5 μg of DOJ construct and 10 μg of egcocret retroviral packaging vector (26). The media were changed the next morning, and the virus collected 60 h later. The virus stock was supplemented with 8% polybrene, and 50% confluent NIH3T3 cells or Swiss 3T3 cells infected for 3 h. Cells resistant for 3 days were selected, and protein expression was determined by immunoblotting with monoclonal antibody specific for Ha-Ras (LA-669, Quality Biotech). Growth Curve—NIH3T3 cells stably transfected with the DOI vector or with DOI vector containing the Ras mutants were plated at 3 × 104/well into 24-well plates. The cells were fed every 3rd day. Each day, cell number was determined. Cell density was maximal on the 6th day.

Microscopy—Ras-expressing Swiss 3T3 cells were plated onto glass coverslips that had been washed with ethanol, coated with type III collagen (W. Carter, FHCR, 64 μg/ml, 30 min), and blocked with bovine serum albumin (1 mg/ml, 20 min). Following 2 h for cell attachment, coverslips were washed twice with Dulbecco′ modified Eagle’s medium containing 0.5% serum and incubated overnight in the same medium. The cells were stained with bodipy phalloloid (Molecular Probes) essentially as recommended by the manufacturer. The coverslips were washed three times with warm PBS and treated with the following solutions at room temperature: (i) 3.7% formaldehyde in PBS, 10 min; (ii) PBS (two washes); (iii) 0.1% Triton X-100 in PBS; (iv) PBS (three washes); (v) 5% calf serum in PBS, 1 h; (vi) bodipy phalloloid (1.5 units/200 μl) and 5% calf serum in PBS, 40 min; (vii) PBS (two washes); (viii) water (two washes). Coverslips were mounted in Vectamount (Vector Laboratories).

To examine the rate of cell spreading, 104 cells were plated as above on collagen-coated coverslips and allowed to attach for 10 min. Unattached cells were then removed with washing with medium containing 10% fetal bovine serum, and at various times the cells were fixed, permeabilized, and blocked as above. Coverslips were then incubated with anti-phosphotyrosine (antibody 4G10, Upstate Biotechnology, 1:200 dilution) for 2 h. The cells were washed three times with PBS containing 0.1% Triton X-100 and incubated in 5% calf serum in PBS for 30 min. The cells were then incubated with fluorescein isothiocyanate (FITC)–anti-mouse secondary antibody (Jackson ImmunoResearch, 1:500) for 2 h, washed 3 times in PBS 0.1% Triton X-100, 3 times in PBS, and stained with phallolidin as described above.

MAP Kinase Activation—To examine the phosphorylation state of MAP kinases, lysates of Swiss 3T3 cells were analyzed by 15% SDS-PAGE. The gel was transferred to polyvinylidene difluoride membrane and probed with an rabbit anti MAP kinase antibody (antisum 1913) (28). For transient assays, Myc epitope-tagged Xenopus MAP kinase (28) in the CS2 vector was transfected along with Ras mutants (also in the CS2 vector) into 293T cells (26) by the BBS/CaCl2 precipitation method. The media were changed the next day and incubated for a further 24 h. Forty hours after transfection the medium was changed to Dulbecco’s modified Eagle’s medium containing 0.5% serum, and 72 h after transfection the cells were washed with PBS and lysed in 0.4 ml of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.05% SDS, 0.5 mM EDTA, 0.5% EGTA, 10% glycerol, 0.1% mercaptoethanol, 5 mM NaF, 0.02 mM NaVO3, 0.1 mM phenylmethylsulfonyl fluoride, 1% protein). Following brief sonication, the lysates were clarified at 20,000 × g. Forty μl of the clarified lysate were reserved for SDS-PAGE analysis, and the remainder was immunoprecipitated with 9E10 anti-Myc epitope antibody (29) for 30 min. Fixed Staphylococcus aureus, coated with goat anti-mouse antibodies, was added for a further 2 h, and immune complexes were collected by centrifugation. Immunoprecipitates were washed four times with lysis buffer containing 500 mM NaCl and suspended in 150 μl of 50 mM HEPS, pH 7.4 and split into aliquots for Western blot and for immune complex kinase assays. For immune complex assays, immune precipitates were recovered by centrifugation and resuspended in 30 μl of a kinase mix (50 mM HEPES, pH 7.4, 100 mM NaCl, 15 mM MgSO4, 15 mM MnCl2, 15 μM ATP, 0.02 mM Na3VO4, 0.17 μM of γ-32PATP/m, 0.17 μg of myelin basic protein/ml). The reactions were mixed and immediately placed at 30 °C. After 10 min the reactions were stopped by the addition of an equal amount of 2 × SDS-PAGE loading buffer. The immunoprecipitates were analyzed by 7.5% SDS-PAGE for Western blot and by 15% SDS-PAGE for kinase assays.

P3 Kinase Assays—Levels of phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P3) were determined as described previously (15). Briefly, 5-cm plates of 293T cells were transfected with 5 μg of pSG5–110α DNA and 1 μg Ras G12V or mutant DNA in the CS2 vector and incubated for 24 h. Cells were then treated with 0.1% trypsin (0.17 mg of myelin basic protein/ml). The reactions were mixed and immediately placed at 30 °C. After 10 min the reactions were stopped by the addition of an equal amount of 2 × SDS-PAGE loading buffer. The immunoprecipitates were analyzed by 7.5% SDS-PAGE for Western blot and by 15% SDS-PAGE for kinase assays.

Molecular Graphics—Structure 6Q21 (c-Ha-Ras p21 protein catalytic domain complexed with β3-methylene-GTP) was retrieved from the Brookhaven Protein Data base, and selected residues were colored using Quanta.
Characterization of Mutations in Ha-Ras

Table I

| Ha-ras interacting protein | Two-hybrid library isolates (number recovered) | Interaction domain | Reference |
|---------------------------|-----------------------------------------------|-------------------|-----------|
| cRaf-1                    | Rip3 (1)                                      | 26-175            | 17        |
| A-Raf                     | Rip54 (1)                                     | 1-91              | 17        |
| RalGDS                    | Rip56 (1)                                     | 104-140           | 17        |
| p110δ                     | Rip65 (1)                                     | 735-822           | 17        |

Table II

| Mutant | cRaf-1 RalGDS RalGDS RBD p110δ RBD RGL RBD Raf RBD CDC25 |
|--------|----------------------------------------------------------|
| G12V   | + + + + + + + + + + + + + + + + + + + + + + + + +       |
| P34R   | + + + + + + + + + + + + + + + + + + + + + + + + +       |
| E37A   | + + + + + + + + + + + + + + + + + + + + + + + + +       |
| E37D   | + + + + + + + + + + + + + + + + + + + + + + + + +       |
| P34G   | + + + + + + + + + + + + + + + + + + + + + + + + +       |
| Y32F   | + + + + + + + + + + + + + + + + + + + + + + + + +       |
| D35A   | + + + + + + + + + + + + + + + + + + + + + + + + +       |
| T35L/E37R | + + + + + + + + + + + + + + + + + + + + + + + + +   |

RESULTS

Fragments of candidate Ha-Ras effectors were identified in a yeast two-hybrid screen of a mouse embryo cDNA library (17). Clones that interacted with wild-type or G12V (activated mutant) but not with T35L/E37R (effector domain double mutant) or G15A (dominant-negative mutant) forms of human Ha-Ras were sequenced and found to contain fragments of five different proteins: cRaf-1 (17), A-Raf (17), RalGDS (6), RGL (10), and a novel P13 kinase p110 subunit (p110δ) (30) (Table I). Direct binding studies using recombinant proteins showed that the fragments of cRaf-1, RalGDS, and RGL contained RBDs that bound to GDP- but not GTP-Ha-Ras (17) (data not shown). These RBDs and corresponding full-length clones were used to identify features in Ras needed for binding different candidate effectors.

We first examined the ability of the nontransforming Ras relative, Rap1a, to interact with the Ras effector clones, to determine if residues outside the conserved core effector domain (Switch I) affect the interaction between Ras and a particular effector. Rap1a interacts with Rip51, containing the Raf RBD, the RBD of RalGDS, and with full-length RapGDS. In striking contrast with Ha-Ras, Rap1a does not interact with full-length cRaf-1, full-length A-Raf or its RBD, full-length B-Raf, or the RBD of p110δ (data not shown). These results indicate that residues outside the core effector domain are required for interaction of Ha-Ras with cRaf1, A-Raf, B-Raf, and the P13 kinase RBD, but that residues within the core effector domain may be sufficient for Ha-Ras and Rap1a to interact with RapGDS, the RalGDS RBD, and the Raf RBD.

To identify features in Switch I needed for binding each candidate effector, seven effector domain mutants of Ras were chosen (Table II). Five are point mutants that are nontransforming in rodent fibroblasts (18), and one (P34R) is transforming but fails to interact with RasGAP (19). The seventh mutant is a nontransforming double mutant (31). These mutants were tested for interaction with the RBDs of cRaf-1, p110δ, RapGDS, or RGL, as well as with full-length cRaf-1 and RapGDS (Table II). All Ras mutants interacted with CDC25, a Ras exchange factor, indicating that they were all expressed.

Mutant D38A was unable to interact with any fragment or full-length effector tested (Table II). Mutants Y32F and P34R did not interact detectably with full-length cRaf-1 or RapGDS, or with the RBDs of RapGDS, p110δ, or RGL, but they did interact with the Raf RBD. Mutant P34R interacted detectably with the RapGDS RBD and more strongly with the Raf RBD. Mutant E37A did not interact with full-length cRaf-1, but interacted weakly with RapGDS and strongly with the RBDs of RapGDS, p110δ, RGL, and Raf. Mutant P34R, which alone of the effector domain mutants tested has transforming activity in mammalian cells (19), interacted with all effectors tested. Most of the effector domain mutants can be ranked in a hierarchy (Table II). The data are consistent with the hypothesis that the effector mutants tested reduce interactions with all effectors proportionately, and that effectors differ in their strength of interaction with the wild-type effector domain, full-length cRaf-1 being weakest and the Raf RBD most robust.

Mutations that clearly discriminate between effectors were not evident in this small sampling of effector domain mutants.

To identify mutations that might affect the specificity of interaction of Ras and discriminate between effectors, we screened a collection of random mutants of activated (G12V) Ha-Ras. The library was first screened for mutations that reduced interaction with cRaf-1. Approximately 20% of the mutagenized population had greatly decreased interaction with cRaf-1. These mutants were picked and tested individually for interaction with the p110δ RBD and RapGDS. One percent of the Ras mutants that did not interact with cRaf-1 retained the ability to interact with either RapGDS or the p110δ RBD, or both. Four mutant DNA fragments were isolated and retested by transformation into yeast together with various effector protein plasmids (Table III). All four mutants interact with RapGDS, the p110δ RBD and the Raf RBD, but not detectably with cRaf-1. Sequencing of the isolates showed that mutant J10 contains a single residue substitution, E31K. Mutants J1, J25, and J31 all contain more than one substitution. J1 and J31 are double mutants, J25 is a triple mutant. All the mutations lie outside Switch I. This suggests that mutations outside Switch I can alter specificity.

To investigate the contributions of the individual residue substitutions in the double and triple mutant Ras molecules, single mutants were constructed. The D34G mutation present in double mutant J1 is sufficient to strongly inhibit interaction with cRaf1 (J31A, Table III). The R41Q mutation in double mutant J31 greatly reduces interaction with cRaf1 (J31A, Table III), although the Y157N mutation also contributes to the phenotype (J31B, Table III). The triple mutant J25 is complicated. The Q43E mutation alone (J25A, Table III) does not reduce interaction with cRaf1, and the combined mutations at D54N and E63K (J25B, Table III) reduce interaction only partially. Other double and single mutants were not tested, but it remains possible that all three mutations contained in J25 are needed to fully inhibit interaction with cRaf1.

To test whether the Ras mutants bound to the Raf RBD in a biochemical assay, selected Ras mutants were prepared in E. coli, loaded with GTP or GDP, and incubated with a Raf RBD fusion protein (Fig. 1). J10, J25, and J31 showed little binding above background. J1 protein was unstable in E. coli and was not tested. We conclude that mutants J10, J25, and J31 have reduced ability to bind full-length Raf in yeast and to the Raf RBD in vitro, while retaining interactions with RapGDS and P13 kinase in yeast. Thus these mutants have altered specificity.

To determine whether the mutations had altered the ability
TABLE III
Mutations that affect binding to cRaf-1 more than to other effectors

| Designation | Mutations            | cRaf-1 | Raf RBD | Ras RBD | p110α RBD |
|-------------|----------------------|--------|---------|----------|-----------|
| G12V        | (G12V)               | +      | +       | +        | ++        |
| J1          | D54G/I139T           | -      | NTb     | +        | +         |
| J10         | E31K                 | +      | +       | +        | +         |
| J25         | Q43E/D54N/E63K       | +      | +       | +        | +         |
| J31         | R41Q/Y157N           | +      | +       | +        | +         |
| J1A         | D54G                 | -      | NTb     | +        | +         |
| J31A        | R41Q                 | +      | +       | +        | +         |
| J25A        | Q43E                 | +      | +       | +        | +         |
| J25B        | D54N/E63K            | +      | +       | +        | +         |

a All mutants also contain the G12V mutation.
b Not tested.

![Fig. 2](image-url)

**Fig. 2.** A, activation of MAP kinase by transient overexpression of activated Ras mutants in 293T cells. Cells were transfected with Ras mutants and epitope-tagged MAP kinase. 72 h later MAP kinase was recovered and assayed by incubation with myelin basic protein (MBP) and radioactive ATP. Reaction products were analyzed by SDS-PAGE and autoradiography (upper). Expression of the Myc-tagged MAP kinase (middle) and Ras (lower) was assayed by Western blotting. B, phosphorylation-mediated MAP kinase electrophoretic mobility shifts in Swiss 3T3 cells stably expressing Ras mutants (upper). Expression of Ras mutants in the cells was assayed by Western blotting (lower).

![Fig. 3](image-url)

**Fig. 3.** PtdInsP3 production in cells overexpressing Ras G12V and mutants. 293 cells were transfected with vectors expressing p110α and Ras mutants and labeled with [32P] orthophosphate, and the phospholipids were extracted and analyzed by thin layer chromatography. An autoradiograph (90 min exposure) of the lower part of the thin layer plate is shown. PtdInsP3 was identified by comigration with standards, and quantified using a PhosphorImager. PtdInsP3 content was normalized relative to total recovered radioactivity, and then expressed relative to the PtdInsP3 content of cells expressing Ras G12V as 100%. PtdInsP3 represented 1.92% of total recovered radioactivity in the Ras G12V sample.

especially when expressed from a powerful promoter (simian cytomegalovirus) and when tested in Rat1 rather than NIH3T3 cells. Mutants J10 and J25 showed no (NIH3T3) or low (Rat1) focus forming activity. We conclude that mutations in Ras that decrease interaction with cRaf-1 also decrease transforming activity in fibroblasts.

Ras transformation is accompanied by alterations in cell morphology, actin cytoskeleton and adhesion. To determine whether the Ras mutations affected the transformed phenotype, polyclonal cell lines were established in Swiss 3T3 cells. Mutants J10 and J25 caused little increase in saturation density, compared with parental Ras G12V, and mutants J1 and J31 were intermediate (Fig. 4). Swiss 3T3 cells expressing mutants J10 and J25 were also relatively normal when examined by immunofluorescence microscopy (Fig. 5). In low serum medium, cells expressing Ras G12V were relatively flat but crossed over their neighbors and lacked stress fibers (Fig. 5B). Control cells and cells expressing mutant J25 did not cross and had strong stress fibers (Fig. 5, A and C). An additional aspect of the transformed phenotype is a reduction in cell-substrate focal contacts (34). Assembly of focal contacts can be detected by immunofluorescence with antibodies to phosphotyrosine (35, 36). Cells were plated onto a collagen matrix and examined at various times. Focal contacts containing tyrosine-phosphorylated proteins started to form within 30 min of plating control or J25-expressing Swiss 3T3 cells (Fig. 5, D and F). At this
time Ras G12V cells had attached but were not spread and showed only tiny points of phosphorytrosine immunoreactivity (Fig. 5E). By these criteria, Swiss 3T3 cells expressing the nontransforming mutant J25 could not be distinguished from control cells.

**DISCUSSION**

We have identified four mutant forms of Ha-Ras that exhibit reduced interaction with cRaf-1 but maintain normal interactions with RapGDS and PI3 kinase. These mutants have reduced or absent focus forming activity in NIH3T3 and Rat1 cells. One mutant in particular (J25) does not activate Erk2 even when greatly overexpressed. Swiss 3T3 cells expressing this mutant are contact inhibited, morphologically normal, and do so indirectly, for example, by altering the orientation of the actin cytoskeleton, cell attachment, spreading, or saturation density of Swiss 3T3 cells. The results further indicate that PI3 kinase activity in vivo when overexpressed. These results suggest that cRaf-1 may be necessary for fibroblast transformation, although we cannot distinguish between a requirement for cRaf-1 and a requirement for other effectors, such as B-Raf and A-Raf, that may have the same binding requirements as cRaf-1. We also found that stimulation of PI3 kinase in the absence of Erk2 stimulates the actin cytoskeleton, cell attachment, spreading, or saturation density of Swiss 3T3 cells. The results further indicate that PI3 kinase can be activated independently of Raf and Erk2, most likely as a direct consequence of binding to Ras.

A small survey of mutants in the Switch I region of the effector domain did not identify mutants with an altered specificity for different effectors. This suggested that different effectors might recognize common features of the effector domain. Random mutagenesis of activated Ha-Ras (G12V), followed by screening for decreased cRaf-1 interaction with maintenance of p110δ RBD and RapGDS interaction, yielded four mutants. Of the total of 8 residues substituted in these mutants, none are in Switch I. However, mutations were found in residues 31, 41, and 43, which flank Switch I and are in the broader region where differences between Ha-Ras and Rap1a are responsible for the transforming activity of Ha-Ras and the anti-transformation activity of Rap1a (1). All the residues affected (except for Ile129, which appears to be unimportant for the adhered binding of the D54G/I139T double mutant J1) are exposed on the same surface of Ras as the effector domain, and thus could contact effectors (Fig. 6). The phenotypes of two of the Ras mutants, J10 and J31, are likely explained by changes in Ras residues that contact cRaf-1.

Ras mutant J10 contains a single substitution of E31K. This mutation has the effect of replacing Ras residue Glu31 with the corresponding Lys residue from nontransforming Rap1a. Rap1a is known to bind the cRaf-1 RBD with decreased affinity relative to Ras (37). The double mutant D30E/E31K of Ha-Ras is reportedly nontransforming, lacks the ability to induce differentiation of PC12 cells, and does not bind GAP (38–40), and we have shown that the single mutant E31K is nontransforming, with the caveat that this mutant is poorly expressed. The E31K mutant has been tested previously for binding to cRaf-1, with inconclusive results. Activated (G12V) E31K mutant Ha-Ras loaded with GTP binds to an N-terminal Raf fragment (41, 42), whereas full-length cRaf-1 does not bind to E31K Ha-Ras loaded with GTPyS (43). In another assay, cRaf-1 was found to associate with but not be activated by G12V/E31K Ha-Ras when co-overexpressed (44). Experimental differences could influence whether or not binding is detected. A recent structural study supports the hypothesis that Glu31 directly contacts the cRaf-1 RBD (45). Introducing Glu31 into Rap1a in place of Lys31 increases the binding affinity for the cRaf-1 RBD 20-fold. In a co-crystal of E30D/K31E Rap1a complexed with the RBD, the Glu31 side chain salt bridges to Lys41 of cRaf-1, suggesting that Glu31 of Ras normally makes the same contact (4, 45). Our finding that the RapGDS and p110δ RBDS interact with E31K Ras suggests that these RBDS will not have a basic residue in a position equivalent to Lys31 of Rap1a.

The R41Q mutation in J31 (R41Q/Y157N) may also interfere with a direct contact to the cRaf-1 RBD. Arg41 is conserved in Rap1a, and the side chain of Rap1a Arg41 contacts the side chain and peptide carbonyl of Asn44 of the RBD (45, 46). The importance of Arg41 in Ha-Ras has been shown previously (18, 43, 47). An R41A mutant of v-Ha-Ras has reduced cRaf-1 association, even though it still induces PC12 cell differentiation (43). An R41L mutant of v-Ha-Ras is nontransforming at elevated temperature, but transforms at reduced temperature (18, 47).

Other mutations we identified that reduce Raf binding may do so indirectly, for example, by altering the orientation of residues in the effector domain. Asp52, which is mutated to G1y in the double mutant J1 (D54G/I139T) and to Asn in the triple mutant J25 (Q43E/D54N/E63K), lies close to residue 41 at the end of the effector loop and may affect its orientation (48, 49). Residues 42–55 have been called the “constitutive effector domain” (50), in that certain mutations in this region inhibit effector function but the conformation of the region is not regulated by GTP. However, a role for residue 54 has not been noted previously. The conservative C52M/D54E double mutant has been considered functional since it induces PC12 cell differentiation (50).
The roles of residues Glu63 and Gln43, mutated together with Asp54 in the triple mutant J25, are less clear. Glu 63 is most distant from the effector domain of Ras, but lies in Switch II, whose conformation is regulated by GTP. An E63K mutant has not been investigated before, but an E63Q mutant of Ha-Ras binds cRaf-1 normally and is active in PC12 cells (40, 43). The Q43E mutation is essential for the reduced cRaf-1 interaction of triple mutant J25. Q43A and Q43H mutants of Ha-Ras have reduced cRaf-1 binding although they are still active in a PC12 assay (43, 50). However, the side chain of Gln43 does not appear to be close to the cRaf-1 RBD in the Rap1a/RBD co-crystal (46). Gln43 is a candidate residue to interact with another region of cRaf-1. In this regard, the cysteine-rich region of cRaf-1 (residues 139–184) has been found to bind to the inactive and active conformations of Ras (51–53). Mutation of Ras residue 26, 45, or 60 reduces binding. It is possible that this second mode of interaction between cRaf-1 and Ras is important for cRaf-1 activation and may be disrupted by the Q43E mutation in nontransforming mutant J25.

Others have sought Ras mutants that alter specificity (54, 55). An E37G mutant loses interaction with cRaf-1 but retains interaction with RalGDS and AF6 (24, 54). The Y40C mutant also loses interaction with cRaf-1 but retains interaction with AF6 (24, 55). These two mutations both lie in Switch I. In our limited sampling of effector domain mutants, E37A was the only one that appeared somewhat selective, losing interaction with cRaf-1 yet retaining strong interaction with the RBDs of p110δ, RGL, RalGDS, and Raf, and weak interaction with p110β, RGL, RalGDS, and Ral.

**Fig. 5.** Swiss 3T3 cells stably expressing the activated Ras mutant J25 are phenotypically normal. A–C, Swiss 3T3 cells expressing vector alone (A), parental Ras G12V (B), or J25 (C), were plated overnight in medium containing 0.5% fetal bovine serum, and processed for immunolocalization of F-actin. Cells expressing activated Ras G12V show decreased stress fibers and cross over each other more than control cells or cells expressing mutant J25. In high serum medium, cells expressing Ras G12V are highly transformed, while cells expressing vector alone or J25 retain normal morphology (not shown). D–F, the formation of focal contacts during cell spreading. Swiss 3T3 cells expressing vector alone (D), parental Ras G12V (E), or J25 (F), were plated onto coverslips coated with collagen III and fixed at various times. The 40-min time point is shown. Fixed cells were permeabilized and stained for phosphotyrosine. Spreading of the cells and initial formation of focal contacts was slowed in cells expressing parental activated Ras G12V and not slowed in cells expressing mutant J25. G, Ras expression levels were assessed by Western blot.

**Fig. 6.** Residues that affect Ras target specificity. Residues 31 (orange, mutant J10), 54 (red, mutants J1 and J25), 43 and 63 (green, mutant J25), and 41 and 157 (magenta, J31) are color-coded. Residues 32–40 are colored blue and labeled switch I. The edge of the GTP molecule can be seen (yellow). Residue 139 (mutant J1) is hidden in this view.
full-length RalGDS. E37A is a classic non-transforming effector mutant (18). However, the other effector mutants tested fell into a continuum with the widely interactive P34R at one extreme and the completely inactive D38A at the other. P34R Ha-Ras does not bind GAP, but is transforming (18). Pro44 of Rap1a does not contact the Raf/RBD directly (46). However, the side chain of Asp38 in Rap1a forms a critical salt bridge to the side chain of Arg29 in the RBD, which is essential for binding (46, 56, 57). The D38A mutant of Ha-Ras is nontransforming, has an unaltered conformation, and has a greatly reduced affinity for the cRaf-1 and RalGDS RBDs (37, 58–60). In moving from the least functional to the most functional Switch I affinity for the cRaf-1 and RalGDS RBDs (37, 58–60). In moving from the least functional to the most functional Switch I affinity for the cRaf-1 and RalGDS RBDs (37), the mutant binding to the Raf RBD is first detected, then the Raf1a/Raf1a/RBD complex before the release date from the Brookhaven data base, S. Campbell, J. Dragan and C. Der for interesting discussions of unpublished data, B. Stoddard for assistance with and W. Carter for advice on adhesion and spreading assays.

Our results support the conventional wisdom that Raf activation is needed for fibroblast transformation by Ras. However, we were surprised that cells expressing Ras mutants that activate PI3 kinase is needed in some instances for vesicle transport and sorting, pinocytosis, membrane ruffling, cell adhesion, cytoskeletal organization and mitogenesis (see Carpenter and Cantley (61, 62) and references therein). Importantly, the activation of endogenous PI3 kinase by injection of stimulatory antibody or thiolipophosphopeptides induces DNA synthesis and glucose transport in the absence of other signals (63, 64). The overexpression of activated mutants of p110 also stimulates DNA synthesis and gene expression (51, 65), possibly via stimulation of Ras-dependent pathways via autocrine loops (66). Why then do Swiss 3T3 cells overexpressing Ras mutant J25 appear so normal? One possibility is that stimulation of PI3 kinase at intracellular sites, as may occur with injected antibodies, phosphopeptides or overexpression constructs, may have more pronounced effects than stimulation at the plasma membrane, where the mutant Ras is presumably located (67).

Seminal work from Downward’s laboratory has shown that PI3 kinase binds directly to Ras GTP in vitro, and has the properties of a Ras effector. Activated Ras and tyrosine phosphorylated synthetic peptides cooperate to stimulate PI3 kinase in vitro (15). Moreover, activated Ras stimulates the accumulation of PtdIns(3,4)P2, the product of PI3 kinase, in cells (14). PI3 kinase is a heterodimer of a p85 noncatalytic, phosphotyrosine-binding subunit and a p110 catalytic subunit, and the RBD is in the p110 subunit (15). A point mutation in the RBD blocks Ras binding and constitutively activates p110a. Furthermore, platelet-derived growth factor-dependent activation of PI3 kinase needs only PI3 kinase binding to the platelet-derived growth factor receptor but also accumulation of Ras GTP (68). The stimulation of PI3 kinase activity in vivo by Ras mutant J25, which does not activate Erk2 or interact with cRaf-1, further suggests that PI3 kinase is activated in vivo independently of cRaf-1 when Ras is constitutively active.

The nontransforming Ras mutant J25 also binds to RalGDS. To date, the biological functions of RalGDS and its close relatives, RGL and RLF, are unclear. RalGDS synergizes with Raf to activate transcription from the fos promoter (69), and activated Ral synergizes with Ras and Raf for transformation (70), suggesting a Ras-RalGDS-Ral signaling pathway (71). In that case, the mutant J25 suggests that RalGDS binding is not sufficient, in the absence of Raf activation, to stimulate morphological changes or increases in saturation density. It is also possible that RalGDS does not normally mediate Ras signals. Rap1a binds more strongly to RalGDS than to cRaf-1 (Table II) (37), suggesting that RalGDS may be an effector for Rap1a.
Characterization of Mutations in Ha-Ras

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