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Interaction of Activated Ras with Raf-1 Alone May Be Sufficient for Transformation of rat2 Cells

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v-H-*ras* effector mutants have been assessed for transforming activity and for the ability of the encoded proteins to interact with *Raf-1*-, *B-Raf*-, *byr2*-, *ral*GDS-, and *CDC25*-encoded proteins in the yeast two-hybrid system. Transformation was assessed in rat2 cells as well as in a mutant cell line, rv68BUR, that affords a more sensitive transformation assay. Selected mutant Ras proteins were also examined for their ability to interact with an amino-terminal fragment of Raf-1 in vitro. Finally, possible cooperation between different v-H-*ras* effector mutants and between effector mutants and overexpressed Raf-1 was assessed. *Ras* transforming activity was shown to correlate best with the ability of the encoded protein to interact with Raf-1. No evidence for cooperation between v-H-*ras* effector mutants was found. Signaling through the Raf1–MEK–mitogen-activated protein kinase cascade may be the only effector pathway contributing to *RAS* transformation in these cells.

A number of cellular proteins bind Ras and are candidates for downstream effectors that function in cell transformation by the *Ras* oncogene. The Raf protein kinases (38, 54, 72, 75, 76, 82), phosphatidylinositol-3-OH kinase (63), P120 GAP (71, 74), neurofibromin (4, 6), *ral*GDS (35), zeta PKC (18), and a number of other less well-defined species isolated in two hybrid screens (28, 73) all have the property of binding Ras. In each case, the interaction depends on Ras being in the GTP-dependent "on" state, and in many cases the interaction has been documented with purified components in vitro. Each interaction also depends on the integrity of the effector region; severely impaired effector mutants generally block the interaction. In contrast, the interaction of Ras with proteins related to the CDC25 class of guanyl nucleotide exchange protein bind Ras in a complex with either GTP or GDP, and this interaction is not generally sensitive to mutations that remodel the effector loop.

There is copious evidence that Ras regulation of the Raf family of protein kinases is important for growth and cell transformation. Raf members phosphorylate and activate MEK, a dual-specificity kinase that phosphorylates and activates mitogen-activated protein kinase (MAPK), a serine kinase that has multiple targets (14, 15, 30, 40). Growth factors that activate MAPK do so largely through activation of Ras (64). Both *Raf-1* and *MEK1* can be transforming (8, 13, 46). Ras signaling can largely be blocked by dominant negative *Raf-1* and *MEK1* mutants (13, 39) and by the drug PD098059, a specific inhibitor of MEK (20).

Also, there is growing evidence that Ras can function by Raf-independent mechanisms to bring about oncogenic transformation in some situations. Transformation-impaired mutant Ras species with different binding specificity toward putative effectors show strong synergy in transformation assays, suggesting that multiple Ras-regulated pathways contribute to cell transformation (34, 78). Different *Ras* effector mutants were also reported to cooperate to bring about stimulation of DNA synthesis in fibroblasts (33). Furthermore, whereas activated versions of Ras and Raf each behave similarly in fibroblasts and can each individually activate MAPK in epithelial cells, Ras alone can elicit morphologic transformation in the latter situation, apparently by an autocrine mechanism (61). It has also been suggested that the interaction of Ras with *ral*-GDS facilitates the regulation of the ral GTPase and phospholipase D (32).

The likelihood that Raf-independent mechanisms must be important in mammalian systems is highlighted by a consideration of Ras function in *Saccharomyces cerevisiae*, where Raf proteins have not been found and evidence for multiple Ras-regulated pathways has been garnered (56, 79). Similarly, there is evidence that in *Schizosaccharomyces pombe*, Ras regulates the *byr2* protein kinase as well as some other pathway associated with cell morphology (11). The regulation of the *byr2* protein kinase and downstream protein kinases might be particularly relevant to Ras signaling in mammalian cells. A Byr2 homolog in mammalian cells, MEK kinase (MEKK), has been described (43). Although there is no evidence that Ras directly interacts with MEKK, there is evidence that Ras can signal through the MEKK-MKK4 (SEK)-JNK (SAPK) cascade in some cell types (17, 42, 51, 78). Thus, an attractive hypothesis is that Ras signals through multiple effectors, regulates distinct pathways, and thereby brings about the pleiotropic changes associated with oncogenic transformation.

On the other hand, we have found that in rat2 cells, transformation-defective v-H-*ras* effector mutants, the activated version of H-*Ras* found in the Harvey sarcoma virus, can be suppressed by somatic mutations in *MEK1* (8, 68). The mutant Ras species all interact very weakly with Raf-1, while the selected mutant MEK species act as coupled amplifiers that increase Ras signals above the threshold needed for transformation. These mutant cell clones are hypersensitive to transformation by a wide variety of v-H-*ras* effector mutants. Furthermore, the mutant *MEK1* cDNAs are highly transforming on their own when modestly overexpressed in parental rat2 cells. Thus, our data support the thesis that the stimulation of MEK and MAPK, probably mediated by Raf-1, is the only significant effector pathway contributing to the transformation of rat2 cells by v-H-*ras*. Non-Raf cellular proteins that interact with Ras, then, might represent Ras effectors that have some normal function but do not participate in transformation. Alternatively, they may be effectors of other GTPases with Ras-like effector regions.

Using a large collection of defined mutations in v-H-*ras*, we

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have now studied the relationship between transforming activity in wild-type and mutant rat2 fibroblasts and the ability of the encoded proteins to interact with putative effectors in the yeast two-hybrid system. *Ras* effector mutant libraries have been screened for Ras species that interact with Raf-1 with greater than normal affinity. Some of these discriminate effectively between different putative effectors. We have also studied the interaction of selected Ras proteins with the Ras-binding domain of Raf-1 in vitro. Finally, to help distinguish between hypotheses of single versus multiple signaling pathways in *Ras* transformation, we have looked for synergistic transforming activity in cells that express either two v-H-*ras* effector mutants or that express one v-H-*ras* effector mutant and overexpress *Raf-1*.

MATERIALS AND METHODS

RAS mutations. Most of our studies involve v-H-*ras* which includes two activating substitutions, Gly12Arg and Ala59Thr, that contribute to transforming potential. Included in this study were the v-H-*ras* effector domain substitutions described previously (68, 70). Also studied were new v-H-*ras* effector domain mutations, including a complete set of codon 32 mutations constructed by the cassette mutagenesis technique (70). We have studied three linker substitution mutations that affect loop 4 and helix 2 (switch region 2) in v-H-*ras* (80). Finally we have studied two mutations that activate the transforming potential of c-H-*ras*, Pro34Arg (69) and Ala59Thr (41) in the proto-oncogene background.

Yeast two-hybrid studies. The two hybrid system (24) was used to study protein interactions. pGBT10–v-H-*ras* plasmids and pGADGH-*RAF*$^+$ (wild-type, full-length rat *Raf-1*) have been characterized previously (8). Plasmid pRIP51 expresses residues 48 to 178 of human Raf-1 (75). Plasmid pRIP35 expresses the carboxyl-terminal region of *ral*GDS fused to VP16 (75). B-Raf–VP16 contains the entire B-Raf sequence fused in frame to VP16. *byr*2 and CDC25 fusion plasmids were the same as described previously (72).

To identify v-H-*ras* alleles that enhanced the Ras–Raf-1 interaction, we screened *Ras* effector domain libraries (16, 69) by using the two-hybrid system. Mutant *Ras* sequences in pGBT10 were introduced into strain YPB2 along with pGADGH-*RAF*$^+$. Double-transformant colonies were selected with Trp-Leu-dropout medium, and about 600 such colonies were individually tested by the β-galactosidase spot test. About 300 colonies that tested positive were individually tested in a quantitative assay to determine whether they had increased activity. In selected cases, DNA was isolated from the yeast and plasmid *Ras* sequences were then recovered by PCR and subjected to DNA sequence analysis. Each of the three *Ras* mutations selected for further study was reconstructed in v-H-*ras* by the cassette mutagenesis technique and transferred to a *neo* retrovirus vector for transformation studies (68). The three possible double-mutation alleles and the triple mutation were similarly constructed and studied.

Retrovirus vectors, cells and transformation assays. Helper-free retrovirus stocks were used to engineer drug-resistant rat2 clones that expressed mutant Ras species, and transforming activity was scored by examining the morphology of cells in drug-resistant (Neo$^+$) colonies as previously described (8, 68). The somatic mutant rv68BUR (68) was used as a more sensitive assay of *Ras* transforming activity. This clone is heterozygous for an apparently silent mutation, Ala226Val, in *Raf-1* and an activating mutation, Gln56Pro, in *MEK1* (8). Extensive studies, including two-hybrid studies with nearly every Ras species listed in Table 1, have failed to find any phenotype associated with the Ala226Val *Raf-1* mutation.

To obtain rat2 cells expressing two mutant protein species, pools of clones were first obtained after infection with virus expressing one selectable marker. This pool was then infected with a second virus, and doubly drug-resistant clones were selected. *Raf-1* (rat) was overexpressed with the pBabePuro retrovirus vector (55).

Biochemical assays. The expression of all new species of v-H-*ras* was documented by immune precipitation of [^{35}S]methionine-labeled protein with monoclonal antibody Y13-238 followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (68). v-H-*ras* products expressed in *E. coli* were purified as described previously (69), except that anion-exchange chromatography involved fast protein liquid chromatography on Mono-Q matrix. After first loading 10 pmol of purified Ras with [α-^{32}P]GTP as described previously (69), binding of Ras-GTP was separately conducted with recombinant glutathione *S*-transferase (GST) and GST–Raf-1(1–149) fusion proteins that were attached to glutathione-Sepharose. As a control, Ras-GTP species were bound to Y13-259 anti-Ras antibody on Sepharose beads coated with rabbit anti-rat immunoglobulin G. Binding and washing conditions were as described previously (76). After scintillation counting of the washed beads, the background counts obtained with GST protein were subtracted and the remainder obtained with GST–Raf-1 was expressed as a percentage of the value obtained with the Y13-259 antibody.

RESULTS

Binding of Ras to Raf-1 correlates with morphologic transformation of rat2 cells. We have compared the transforming activity of mutant forms of *Ras* to the ability of the encoded GTPase to bind full-length Raf-1 in the yeast two-hybrid system. As a more sensitive test, we have used the truncated Raf-1 encoded by the RIP51 clone (75). Overall, there is an excellent correlation between binding of Ras and Raf-1 and the ability of the *Ras* gene to cause morphological transformation (Table 1). Considering v-H-*ras* effector domain mutants that transform rat2 cells, all give strong two-hybrid signals with Raf-1. Some v-H-*ras* mutations that give weak or modestly strong two-hybrid signals do not transform rat2 cells but do transform the mutant cell line rv68BUR described above. These v-H-*ras* mutants interact more strongly with the truncated RAF species encoded by the RIP51 clone than with full-length Raf-1. Mutants that do not transform either wild-type or mutant rat fibroblasts encode proteins that interact very weakly or not at all with Raf-1 proteins.

We noted that a GBT10–v-H-*ras* plasmid which we refer to as Tyr32Ala* provided an apparent exception to the correlation of Ras–Raf-1 interaction and transforming activities; the encoded Ras species interacted well with Raf-1, but the Tyr32Ala v-H-*ras* vector transformed only the rv68BUR mutant. Sequence analysis revealed that the pGBT10-Tyr32Ala* plasmid contained two substitutions, Val45Ile and Thr59Ala, not expected of a v-H-*ras* gene. The codon 45 mutation must be a PCR-induced error, while the Thr59Ala change might be a PCR-induced error or a case of a contaminating c-H-*ras* sequence somehow recombining during the PCR. We constructed a second pGBT10-Tyr32Ala (v-H-*ras*) plasmid and found that the encoded protein interacted very weakly with the RIP51 fusion protein and not at all with full-length Raf-1, in line with the poor transforming activity of this allele. Reciprocally, we transferred the Tyr32Ala* sequence (Gly12Arg Tyr32Ala Val45Ile Thr59Ala) to a retrovirus vector and showed that it has readily detectable transforming activity in rat2 cells. Thus, the ability of Tyr32Ala v-H-*ras* protein to interact with Raf-1 and transform rat2 cells depends on whether the Val45Ile and Thr59Ala mutations are also present (Table 1; Fig. 1, spots 5 and 6).

The Tyr32Met v-H-*ras* mutation is an exception to the correlation. This encoded protein interacted strongly with Raf-1 in the two-hybrid system (Table 1; Fig. 1, spot 8), but transforming activity is not observed in wild-type rat2 cells. In this case, no additional mutations are present.

We previously reported that Tyr32Phe v-H-*ras* is nontransforming in rat2 cells (8). This result is surprising because the less conservative changes Tyr32His and Tyr32Trp are both moderately transforming. This result is also important, because it has been reported that Tyr32Phe is capable of binding Raf-1 in vitro as well as in the yeast two-hybrid system (2, 65). We have constructed an independent Tyr32Phe allele and virus stock and tested a larger number of Neo$^+$ colonies. In this study, we found that Tyr32Phe was weakly transforming in rv68BUR, although no sign of transformation was observed in rat2 cells. Also, we found that the Tyr32Phe protein gave a weak signal in the pRip51 interaction test but was negative with pGADGH-*RAF*$^+$ (Table 1). Thus, the Raf-1 interaction is in line with transforming activity for this v-H-*ras* mutant, although both activities are lower than observed for some other structurally more radical codon 32 substitutions.

Evidence has been presented that a second region of Ras plays a role in effector interaction. Linker insertion mutations that perturb this "switch region 2" (loop 4/helix 2 of the crystal

TABLE 1. Transformation and two-hybrid properties of Ras effector mutants

Ras mutant[a]	Transformation properties[b]		Two-hybrid properties[b]					
	rat2	68BUR	c-Raf-1	Rip 51	B-Raf	Ral-GDS	Byr2	CDC25
v-*ras*	+++	+++	+++	+++	+++	+++	+++	+++
Tyr32Trp	++	+++	+++	+++	+++	−	+++	++
Tyr32His	+	+++	+++	+++	++	−	+++	+++
Tyr32Phe	−	+	−	++	−	−	−	++++
Tyr32Cys	−	+++	−	++	−	−	−	+++
Tyr32Met	−	+++	+++	+++	++	−	−	+++
Tyr32Val	−	+++	−	+	−	−	−	+++
Tyr32Leu	−	+++	++	+++	++	−	−	+++
Tyr32Ile	−	+++	++	+++	++	−	−	+++
Tyr32Ala	−	+++	−	±	−	−	−	+++
Tyr32Ala*	++	ND	+++	+++	++	−	++	++
Tyr32Gly	−	++	−	±	−	−	−	+++
Tyr32Pro	−	+++	−	±	−	−	−	+++
Tyr32Asn	−	+	−	−	−	−	−	++
Tyr32Arg	−	+++	−	±	−	−	−	+++
Tyr32Thr	−	+++	−	++	−	−	−	+++
Tyr32Ser	−	++	−	−	−	−	−	++
Tyr32Glu	−	−	−	−	−	−	−	+++
Tyr32Asp	−	−	−	−	−	−	−	+++
Tyr32Gln	−	−	−	−	−	−	−	+++
Tyr32Lys	−	−	−	−	−	−	−	+++
Asp33Glu	+++	+++	+++	+++	+++	−	+++	+++
Asp33Gln	++	+++	+++	+++	+++	−	+++	+++
Asp33Asn	++	+++	+++	+++	+++	−	−	++
Pro34Ser	−	+++	++	+++	−	−	++	+++
Pro34Gly	−	++	−	++	−	−	−	+++
Pro34His	−	−	−	−	−	−	−	+++
Pro34Arg	−	−	−	±	−	−	−	+++
Thr35Ser	−	+++	+	+	−	−	−	+++
Thr35Met	−	+++	−	−	−	−	−	+++
Ile36Leu	++	+++	+++	+++	+++	−	+++	+++
Ile36Val	++	+++	+++	+++	+	−	+++	+++
Ile36Met	±	+++	++	+++	−	−	+	+++
Ile36Ala	−	−	−	−	−	−	−	+++
Glu37Asp	+++	+++	+++	+++	+++	−	−	+++
Glu37Ala	−	+++	++	++	++	++	+++	++
Glu37Gly	−	−	−	−	−	−	++	++
Asp38Glu	−	+++	−	+	−	−	−	+++
Asp38Ala	−	−	−	−	−	−	−	++
Ser39Cys	++	+++	+++	+++	+++	+++	+++	+++
Ser39Thr	+++	+++	+++	+++	+++	−	+++	+++
Tyr40Phe	+	+++	+++	+++	++	−	++	++++
Tyr40Leu	−	++	−	−	−	−	−	+++
Tyr40Ile	−	−	−	−	−	−	−	++
Tyr40Cys	−	−	−	−	−	−	−	++
Tyr40Gly	−	++	−	−	−	−	−	+++
Tyr40Arg	−	−	−	−	−	−	−	+++
Tyr40Ser	−	−	−	−	−	−	−	+++
Tyr40Val	−	−	−	−	−	−	−	−
31 LIR 38	−	−	−	−	−	−	−	+++
DM1	+++	+++	+++	+++	+++	−	−	+++
DM2	+++	+++	+++	+++	+++	−	+++	+++
DM3	+++	+++	+++	+++	+++	−	−	+++
TM1	+++	+++	+++	+++	+++	−	−	+++
63 SDQ 73	++	+++	+++	+++	−	−	−	−
68 ADQ 77	+	+++	+++	+++	−	−	−	−
71 TDQ 77	+	+++	+++	+++	−	−	++	−
c-*ras*	−	−	+++	+++	+++	+++	+++	++
Pro34Arg	+++	+++	+++	+++	+++	+++	+++	++
Ala59Thr	ND	ND	+++	+++	+++	+++	+++	+++

[a] v-H-*ras* effector mutants are arranged with amino-terminal substitutions near the top. Other notes are as follows: Tyr32Ala* is a complex v-H-*ras* derivative described in the text; mutations Asp33Glu, Glu37Asp, and Ser39Thr were selected as tight-binding mutants in the two-hybrid system; DM1 to DM3 are double mutations Asp33Glu Glu37Asp, Asp33Glu Ser39Thr, and Glu37Asp Ser39Thr, respectively; TM1 is triple mutation Asp33Glu Glu37Asp Ser39Thr; 31 LIR 38 is the loop 2 linker insertion mutation, and 63 SDQ 73, 68 ADQ 77, and 71 TDQ 77 are loop 4/helix 2 linker insertion mutations (linker-encoded residues are shown with the single-letter code between the numbers of the unaffected residues); Pro34Arg and Ala59Thr (last two rows of table) are mutations in the c-H-*ras* background.

[b] Transformation in rat2 and rv68BUR was scored according to the morphology of drug-resistant colonies (68); two-hybrid results were scored on the basis of the colorimetric spot test after overnight incubation. −, negative result; ±, very weakly positive result; +, weakly positive result; ++, moderately positive result; +++, strongly positive result; ++++, strongly positive result that developed after a few minutes of incubation; ND, not done.

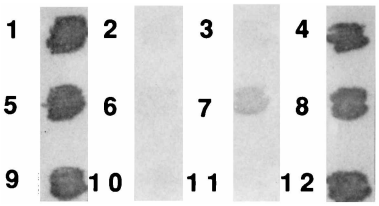

FIG. 1. Interactions of Ras fusion proteins with putative effectors. Double transformants were selected on medium lacking Trp and Leu, spotted on filters and then analyzed for β-galactosidase expression by the spot test. The v-H-*ras* genes, indicated on the left of each construct below, were all expressed in pGBT10. 31LIR38 is a null v-H-*ras* linker mutation (Table 1). Spots 1 and 4 are duplicates. The data are representative of six filters. Spots: 1, v-H-*ras* × pGADGH-*RAF*$^+$; 2, 31LIR38 × pGADGH-*RAF*$^+$; 3, v-H-*ras* × pGADGH (no insert); 4, v-H-*ras* × pGADGH-*RAF*$^+$; 5, Tyr32Ala* × pGADGH-*RAF*$^+$; 6, Tyr32Ala × pGADGH-*RAF*$^+$; 7, Thr35Ser × pGADGH-*RAF*$^+$; 8, Tyr32Met × pGADGH-*RAF*$^+$; 9, v-H-*ras* × *ral*GDS; 10, 31LIR38 × *ral*GDS; 11, Glu37Asp Ser39Thr × *ral*GDS; 12, Glu37Asp Ser39Thr × pGADGH-*RAF*$^+$.

structure) were therefore included in this study. All three mutant versions of v-H-*ras* transformed rat2 cells, although the level of transformation is less obvious than with wild-type v-H-*ras*. The encoded proteins interacted well with Raf-1 (Table 1).

The Pro34Arg mutation is intriguing because its behavior depends upon the genetic background. This mutation was selected in the c-H-*ras* background as an activating mutation, and the encoded protein does not interact significantly with P120GAP (69) or neurofibromin (unpublished data). Pro34Arg c-H-*ras* binds Raf-1 well in the two-hybrid system (Table 1), and this species has also been shown to bind Raf-1 in vitro (18a). These findings confirm our previous conclusions that the structural requirements for Ras-effector interaction and Ras-GAP interaction are quite different (69). The Pro34Arg mutation is a null in the v-H-*ras* background, probably because the encoded protein is unstable in rat2 cells (8). We find that the Pro34Arg species in the v-H-*ras* background gave a very weak signal with pRIP51 and no signal with full-length Raf-1 (Table 1), suggesting that this protein might be somewhat unstable in yeast as well.

It has been reported that the Ala59Thr substitution in Ras blocks the Ras–Raf-1 interaction in vitro (65). This finding is extraordinary because the substitution is found, along with activating codon 12 substitutions, in both v-H-*ras* and v-K-*ras*. Furthermore, it has been reported that the Ala59Thr alone is capable of activating the transforming activity of c-H-*ras* (41). We found, however, that Ala59Thr Ras interacted well with Raf-1 in the two-hybrid system (Table 1).

In its interaction with Ras mutants, B-Raf was similar to Raf-1 with two general exceptions. First, Ile36Met and Ile36Val interacted significantly better with Raf-1 than with B-Raf (Table 1), and second, the loop 4/helix 2 mutations did not interact with B-Raf.

Novel v-H-*ras* effector mutations dissociate Raf-1 and *ral*GDS interactions. To find v-H-*ras* proteins that interacted more strongly than wild-type with Raf-1, and to find Ras mutations that dissociate transformation activity and binding of Ras to potential effectors, we screened v-H-*ras* effector domain mutation libraries using the two hybrid method. From the approximately 300 clones that were scored as positive in the Ras–Raf-1 interaction, 7 isolates that appeared to exhibit enhanced interaction were identified. DNA sequence analysis revealed that the seven clones defined three v-H-*ras* substitutions at the amino acid level. Asp33Glu was recovered once, Glu37Asp was recovered three times, and Ser39Thr was recovered three times. To find stronger tight-binding v-H-*ras* mutations, we used a cassette mutagenesis procedure to construct all three double mutations and the triple mutation. These mutations, as well as the three single mutations, were all constructed in the v-H-*ras* background and were initially carried in a retrovirus vector so that their biological activities could be studied in mammalian cells. All were then transferred into pGBT10 for qualitative and quantitative testing in the two-hybrid system, as described previously (8). In these tests, the single mutations gave only marginally higher activity than the wild type while some of the multiple mutations (e.g., Glu37Asp Ser39Thr) exhibited about twofold-higher Raf-1 interaction activity than did wild-type v-H-*ras*. The qualitative result obtained with the Glu37Asp Ser39Thr form of v-H-*ras* is shown in Fig. 1 (spot 12). Each of these single and multiple v-H-*ras* mutations was shown to be highly transforming. While not remarkable in their transforming or Raf-1-interacting properties, they have proven useful in studies of proposed alternate effectors.

Interaction of Ras with alternate effectors. In general, Ras effector mutants interacted poorly or not at all with *ral*GDS (Table 1). In no case was *ral*GDS a better partner than Raf-1. This weaker interaction with *ral*GDS was particularly striking for the v-H-*ras* mutants that were selected for increased affinity for Raf-1 such as the v-H-*ras* double effector mutant Glu37Asp Ser39Thr; no interaction was detected (Table 1; Fig. 1, spot 11).

byr2 encodes a primary Ras effector in *S. pombe*. Although a homologous kinase, MEKK, is present in mammalian cells and appears to be Ras regulated in certain circumstances, *byr2* serves only as a model for two-hybrid studies here. In general, mutant Ras proteins gave weaker interaction signals with the *byr2* product than with Raf-1. The Glu37Gly v-H-*ras* protein was exceptional in that it interacted (weakly) with Byr2 but not with full-length Raf-1. This effector substitution in v-H-*ras* behaves as a null mutation in transformation assays (Table 1).

Interaction of Ras with CDC25. The binding of Ras to the GAL-CDC25 fusion protein served as a positive control in these studies (Table 1). We found that most of the Ras proteins that scored as null in the Raf-1- interaction assay were positive in the CDC25 test, confirming that these GAL4-Ras fusion proteins were stable in yeast. The failure of the loop 4/helix 2 mutations to interact physically with CDC25 has been observed by others (52, 57). We were intrigued by the observation that relatively strong signals were obtained when CDC25 was tested against the v-H-*ras* effector mutants Tyr32Phe and Tyr40Phe. The increased interaction with Tyr32Phe and CDC25 has been noted previously by another investigator (12a). Also of interest, we found that the Glu37Gly substitution in v-H-*ras* was positive in the CDC25 test. This protein in the wild-type and Gly12Val backgrounds was reported to be the first case of an effector domain mutation that does not bind CDC25 (78).

In vitro binding studies. The conditions under which Ras interacts with Raf-1 in the yeast system might not always be relevant to the situation in rat2 cells. The yeast growth temperature is 30°C. The interaction might involve unknown yeast components, and negative results are difficult to interpret. Because of these uncertainties, we sought to confirm some of our results with an in vitro binding assay that employs purified components. Selected Ras species expressed in *E. coli* were purified and then bound to [α-^{32}P]GTP. The precipitation of labeled Ras by GST–Raf-1 bound to glutathione beads was then compared to precipitation by an anti-Ras monoclonal antibody. In contrast to our finding with the yeast system, we found that Tyr32Met interacted poorly with GST–Raf-1 (Fig. 2). As expected, the transforming v-H-*ras* protein Glu37Asp binds GST–Raf-1 well while the null v-H-*ras* proteins Ile36Ala and Glu37Gly bind very poorly. Importantly, Thr35Ser in the

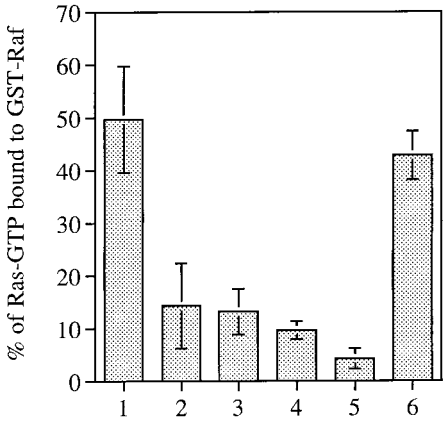

FIG. 2. Binding of v-H-*ras* proteins to GST–Raf-1. Ras proteins were bound to [α-^{32}P]GTP and then precipitated with GST–Raf-1 bound to beads. The radioactivity associated with each species is expressed as a percentage of that obtained with an anti-Ras antibody. 1, v-H-*ras*; 2, Tyr32Met; 3, Thr35Ser; 4, Ile36Ala; 5, Glu37Gly; 6, Glu37Asp. Values shown are the mean of three determinations with the standard deviation of the mean.

v-H-*ras* background greatly diminishes the interaction of Ras with GST–Raf-1 (Fig. 2), consistent with our two-hybrid results (Table 1; Fig. 1, spot 7) (8).

Cooperation between nontransforming forms of Ras and Raf. We coexpressed various nontransforming forms of v-H-*ras* in rat2 cells to test the notion that different effector mutants with distinct binding properties might modulate alternative signaling pathways and cooperate in cell transformation. The effector mutants studied, the Thr35Ser, Glu37Gly, and Tyr40Cys mutants, have previously been used in the Gly12Val background in cooperation studies (33, 34, 78). Pools of rat2 cells expressing two v-H-*ras* alleles were selected sequentially with different drug-resistant markers. In each case, the cells had a flat nontransformed morphology (Fig. 3A to C).

To verify that these pools of doubly drug-resistant cells expressed the expected Ras species, a biochemical analysis was performed. We have found that most mutant *Ras* products display distinct electrophoretic mobility patterns when immunoprecipitated proteins are analyzed on polyacrylamide gels. Parental rat2 cells, as well as cells expressing the various single and double v-H-*ras* species, were labelled with [^{35}S]methionine, and Ras proteins were immunoprecipitated with the Y13-238 monoclonal antibody. Each of the proteins was expressed as expected (Fig. 4).

In contrast to the negative complementation results above, we found that the Tyr32Met form of v-H-*ras* was complemented by coexpression of wild-type Raf-1 with a retrovirus vector. Between 5 and 10% of the doubly drug-resistant cells exhibited a fully transformed morphology (Fig. 3D). Raf-1 overexpression alone is nontransforming. Furthermore, cooperation between overexpressed Raf-1 and other alleles of v-H-*ras*, such as Thr35Ser and Glu37Gly, was not observed.

To demonstrate that this complementation involves a functional interaction between the mutant Tyr32Met v-H-*ras* protein and Raf-1, rat2 cells expressing Tyr32Met v-H-*ras* were

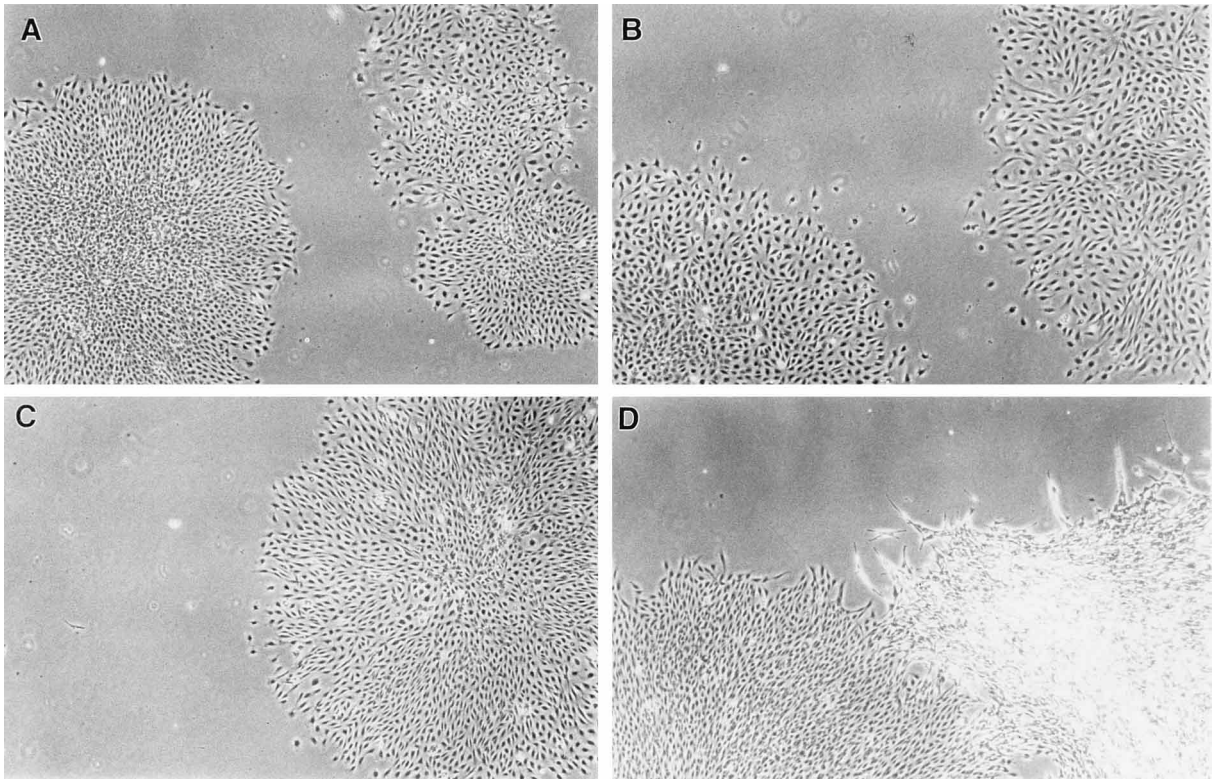

FIG. 3. Morphology of cells expressing two alleles of v-H-*ras* or expressing one allele of v-H-*ras* and overexpressing Raf-1. Rat2 cells were infected with retrovirus vectors, and drug-resistant colonies were selected. Pooled colonies were then infected with a second retrovirus vector, and colonies resistant to two drugs were selected. Shown are typical morphologies for rat2 cells expressing Thr35Ser plus Glu37Gly v-H-*ras* (A), Glu37Gly plus Tyr40Cys v-H-*ras*, (B), Thr35Ser plus Tyr40Cys v-H-*ras* (C), Tyr32Met v-H-*ras* plus overexpressed *Raf-1* (D). Transformed colonies similar to that shown on the right in panel D amounted to 5 to 10% of the total in the last case.

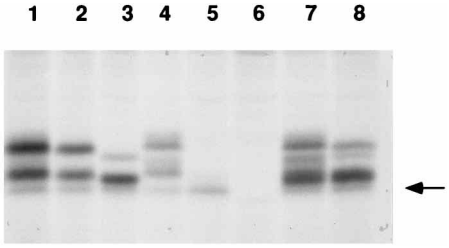

FIG. 4. Expression of v-H-*ras* proteins in rat2 cells. v-H-*ras* species expressed in rat2 cells were labelled with [^{35}S]methionine, immunoprecipitated, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to fluorography and PhosphorImager analysis. Cells expressing two v-H-*ras* species were those shown in Fig. 3A to C. Ras species were (with the amount of radioactivity relative to uninfected rat2 shown in parentheses) Tyr40Cys plus Thr35Ser (23.5) (lane 1), Thr35Ser (13.0) (lane 2), Glu37Gly (9.5) (lane 3), Tyr40Cys (8.2) (lane 4), uninfected rat2 (1.0) (lane 5), uninfected rat2, no primary antibody (0.0) (lane 6), Glu37Gly plus Tyr40Cys (20.8) (lane 7), and Thr35Ser plus Glu37Gly (14.6) (lane 8). The arrow indicates the position of the endogenous Ras.

separately infected with viruses expressing the *Raf-1* Arg89Lys and Arg89Asp mutant alleles. Genetic studies have shown that substitutions of Arg89 interfere with RAS signaling to Raf-1 and structural studies have shown that Arg89 forms a salt bridge with Asp38 of the RAS effector domain (22, 58). The two mutations in *Raf-1* studies here each abolish the Ras–Raf-1 interaction as assessed in the yeast two-hybrid system, and they encode stable, nontransforming versions of Raf-1 in rat2 cells (data not shown). Doubly drug-resistant cells expressing Tyr32Met v-H-*ras*, and either one of these two mutant *Raf-1* species were nontransformed.

DISCUSSION

That Raf proteins are important Ras effectors in cell transformation seems beyond dispute. Ras may merely bind Raf proteins and thereby recruit them to the plasma membrane. At the membrane, these kinases are probably activated by mechanisms that involve phosphorylation by tyrosine kinases and by Thr/Ser kinases, including Raf kinases (22, 23, 44, 45, 47, 67). Alternatively, Ras may play a more active role in Raf activation, as suggested by the demonstration that a membrane-free complex of B-Raf and 14-3-3 proteins is activated in vitro by Ras (60). Another point of uncertainty is whether Ras signaling through alternative effectors contributes to transformation. If binding of Ras to Raf-1 at the plasma membrane were the only significant function of Ras in transformation of rat2 cells, one might expect a direct correlation between the transforming and Raf-1-binding properties among *Ras* effector mutants.

Several approaches to documenting the biological effector function of Ras including focus induction in NIH 3T3 cells, maturation of *Xenopus* oocytes, and neurite induction in PC12 cells, have been widely used. Likewise, a number of approaches to documenting the interaction between Ras effector mutants and either truncated or full-length Raf proteins are available. These include a variety of equilibrium (27, 29, 50) and nonequilibrium binding studies, the yeast interaction system, and functional protein kinase assays based on Ras and Raf overexpression. A given *Ras* effector mutant could give quite dissimilar results in these different biological and biochemical assays depending on the sensitivity of the method and effective protein concentrations studied. The characterization of *Ras* effector mutants is further complicated by the facts that Raf proteins exist as multimeric protein complexes in vivo consisting of heat shock proteins and 14-3-3 proteins (26, 77) and that posttranslational processing of both Ras and Raf proteins in vivo affects their interactions (12, 31, 81).

Considering all these points, the correlation found in the present study between the ability of v-H-*ras* effector mutants to elicit morphological transformation in rat2 fibroblasts and the ability of the encoded species to interact with Raf-1 in the two-hybrid system is remarkable. We have studied only morphological transformation to date. In other studies with *Ras*-, *Raf-1*-, and *MEK1*-transformed rat2 cells, however, we have found that this phenotype is invariably proportional to other transformation phenotypes such as focus formation, anchorage-independent growth, and tumorigenicity in immune-deficient mice. Our method of analysis allows one to assess the lack of activity of nontransforming *Ras* alleles based on a positive observation (i.e., morphologically flat cells). By contrast, negative results in a focus formation assay might derive from gene toxicity or ineffective gene transfer. Furthermore, the analysis of pooled drug-resistant colonies has allowed us to document the stability of all the encoded Ras proteins, with one notable exception (Pro34Arg in v-H-*ras*). The use of the mutant cell line rv68BUR in the colony morphology assay permits a more sensitive test of Ras effector function. We have proposed that the phenotype of rv68BUR results from a weak Ras–Raf-1 interaction being amplified by the point mutation in *MEK1*. Using rv68BUR in the colony morphology assay has allowed us to discriminate between very weak and null alleles of *Ras* and to strengthen the correlation.

The detailed analysis of an apparent exception, v-H-*ras* Tyr32Ala, uncovered a complex interaction between the codon 32 substitution and two unanticipated substitutions at codons 45 and 59. Taking the effects of these mutations into account, we extended the correlation and demonstrated that the strength of a weak Ras–Raf-1 interaction can be increased by forces involving the substitutions at codons 45 and/or 59. We suspect that the codon 45 change is responsible for this phenomenon, since the codon 59 mutation alone has no obvious effect on the Ras-Raf interaction.

The Tyr32Met allele of v-H-*ras* is the only exception to the correlation between interaction in the yeast system and transformation in rat2 cells. This allele is exceptional in other respects, however. The encoded protein fails to interact with GST–Raf-1 in vitro, in line with its lack of transforming activity in rat2 cells. On the other hand, this form of v-H-*ras* exhibits substantial transforming activity when Raf-1 is overexpressed, in line with its ability to interact with Raf-1 in the yeast system. We propose that in the yeast system and in rat2 cells overexpressing Raf-1, the concentration of the two species is high enough for the Ras–Raf-1 interaction to be productive. In this latter situation, Ras and Raf-1 must physically interact, as shown by the failure of Raf-1 codon 89 mutations to cooperate with v-H-*ras* Tyr32Met. In the in vitro binding assay and in rat2 cells expressing the normal amount of Raf-1, the concentration of Ras and Raf-1 is too low for productive interaction to be registered. In support of this threshold model, Tyr32Met has readily demonstrable transforming activity in the somatic mutant rv68BUR, which harbors a mutation in *MEK1* that acts as an amplifier of Ras signaling (8).

The finding that no transforming version of *Ras* encodes a protein that fails to bind Raf-1 argues that Raf-1 interaction is necessary for transformation. If wild-type v-H-*ras* normally interacted with a second, non-Raf-1 effector in cell transformation, one might have expected to find mutants, in such a large collection, that interact with Raf-1 but failed to stimulate this hypothetical second effector and failed to transform. The finding that Ras interaction with Raf-1 does correlate with transforming effector function would tend to support the hy-

pothesis that Raf-1 interaction is sufficient for transformation. This single-pathway hypothesis is in line with our previous finding that rv68BUR is sensitive to transformation by many v-H-*ras* effector mutants and that this *MEK1* mutant gene is a powerful transforming sequence when modestly overexpressed (8). It is possible, however, that the hypothetical second effector recognizes all Ras species that are recognized by Raf-1. Binding studies with PI3 kinase and some of our mutant Ras species might prove interesting.

Other investigators studying *RAS* effector mutations have used the correlation between Raf-1 interaction and transforming activity to argue that signaling through Raf-1 is important for *Ras* oncogene activity. In one case, substitutions of Ras residues flanking the core effector region with those normally found in Rap were studied (82). The rationale for studying these mutations is unclear since Rap also binds Raf-1. Although under exceptional circumstances Rap can antagonize Ras effector function (37), the normal function of this protein may be quite unrelated to Ras (36). In any case, it was reported that the two moderately transforming *Ras* double mutants Asn26Gly His27Ile and Asp30Glu Glu31Lys interacted moderately well with Raf-1. The exceptional Val45Glu allele also interacted with Raf-1, although it had been previously classified as null in a focus formation assay (48). We have found, however, that all three forms of *Ras* in the Gly12Val background are moderately transforming in rat2 and strongly transforming in rv68BUR using the colony morphology assay (data not shown), calling into question this apparent lack of correlation.

In the other studies that established the links between Ras and putative downstream signaling molecules, one or a few severe *Ras* effector mutants that abolished both Raf-1 interaction and biological function were examined. Extrapolating from small collections of effector mutants might be misleading. In earlier studies, excellent correlations were obtained for P120GAP interaction and Ras transforming function with modest collections of effector mutants, and these earlier studies were held in support of the hypothesis that P120GAP was the Ras effector (1, 10).

The decreased interaction of B-Raf, relative to Raf-1, with the v-H-*ras* substitutions Ile36Val and Ile36Met might be explicable in terms of the structure of the complex formed between Rap and a protein fragment corresponding to the Ras-binding domain of Raf-1, residues 51 to 131 (58). Ile36 of Rap shares a water molecule with Val69 of Raf-1. Although this latter residue is conserved in both Raf-1 proteins, its exact conformation and ability to interact with Ras may be different in the two situations, since Val is followed by Pro in B-Raf but Asn in Raf-1. The failure of B-Raf to interact with the loop 4/helix 2 linker mutation species cannot be interpreted in terms of the currently available structural information. These two-hybrid results and the observation that loop 4 mutations have obviously attenuated transforming activity in rat2 cells suggest a role for this region of Ras as a secondary effector region. Other investigators have argued that Ras loop 4 has an effector function (59, 66) and participates in the binding of putative effector proteins (53). Indeed, several studies have provided evidence that in addition to the interaction between the Ras effector region and the Ras-binding domain of Raf-1, Ras independently interacts with the Cys-rich domain of Raf-1 (9, 19, 31). A role for loop 4 of Ras in this interaction was suggested by one of these studies (19). Our results may not bear on the subject of transformation of rat2 cells, however. Using an antibody that readily detects B-Raf in other cell types, we have so far been unable to detect B-Raf in rat2 cells.

The use of Ras effector mutants to dissociate different Ras effector interactions from each other and from biological phenotypes potentially represents a powerful approach to defining effector pathways. The approach has hitherto been greatly compromised by the lack of availability of mutants that cleanly affect one set of interactions without influencing another. Most mutants affect all the interactions but to different degrees. Our strategy for finding v-H-*ras* mutants that had increased interaction with Raf-1 yielded mutants that cleanly dissociate the ability of Ras to interact with Raf-1 and to transform from the ability of Ras to interact with *ral*GDS.

Ras binds through its effector region to *ral*GDS, which has been shown to interact with and activate the ral GTPase in a manner analogous to the activation of Ras by Ras-specific guanyl nucleotide exchange factors (3). Furthermore, ral has been implicated in the regulation of phospholipase D (32). The level of phospholipase D is only modestly elevated in v-H-*ras* transformed rat2 cells, however (49). The strong transforming activity of v-H-*ras* alleles that fail to interact with *ral*GDS would argue that this putative Ras effector does not play an important role in transformation of rat2 cells. Further studies with purified components in vitro are warranted, however.

The interaction of most of our Ras species with CDC25 provides an important positive control for our two-hybrid methodology. Several Ras species that fail to interact with Raf-1 do interact with CDC25, demonstrating that stable Ras species are expressed in yeast. Binding of CDC25 to a distinct surface of Ras probably promotes a conformational change in loop 2 that permits the escape of guanyl nucleotide. Increased CDC25-interacting activities observed with Tyr32Phe and Tyr40Phe are potentially explicable in terms of some sort of molecular reciprocity within Ras, whereby Ras effector domain substitutions might affect guanyl nucleotide exchange and thereby influence the strength of the Ras-CDC25 interaction.

The behavior of the Ras species Thr35Ser, Glu37Gly, and Tyr40Cys in our experiments bears careful consideration, since other studies with these substitutions have previously yielded so different a picture of Ras signaling than emerges here. We found that the Thr35Ser v-H-*ras* product binds very poorly to Raf-1 and does not activate MAPK when expressed in rat2 cells (8). We found that Glu37Gly v-H-*ras* protein does not interact with a truncated Raf-1 but does interact with CDC25 in our yeast experiments. Most importantly, we find that the Thr35Ser and Glu37Gly v-H-*ras* alleles do not cooperate in transformation of rat2 cells. In these respects, our results differ from those published (34, 78). We did confirm that Glu37Gly interacted with *byr2* protein but not full-length Raf-1. Furthermore, we confirmed that although Glu37Gly v-H-*ras* protein does not transform rat2 cells at all, it does cooperate with the Ser257Leu mutation in *Raf-1* in a cell transformation assay (unpublished results). Furthermore, these two species do interact strongly in the yeast interaction system, as described by White et al. (78). The Ser257Leu *Raf-1* mutant allele has substantial constitutive transforming activity when expressed alone in rat2 cells with a retrovirus vector, however (10a).

Tyr40Cys fails to interact with Raf-1 and it is nontransforming in our studies. In microinjection experiments, Gly12Val Ras exhibits the ability to reorganize actin filaments and induce membrane ruffling (5, 62). The Tyr40Cys Ras mutation in the Gly12Val background leaves this property intact (33). Microinjected Tyr40Cys Ras cooperates with Thr35Ser Ras in a DNA synthesis induction assay in REF52 cells (33). These results have been interpreted to mean that Ras can utilize two signaling pathways, one that involves the cell morphology and one that involves the kinase cascade. While these results are not directly comparable to the results reported here, we find no pairwise cooperation between the Tyr40Cys and either of the Thr35Ser or Glu37Gly RAS alleles in rat2 cell transformation.

Furthermore, in rat2 cells expressing Tyr40Cys alone at modest levels (Fig. 4, lane 4), we find that actin filaments are normal, as assessed with rhodamine-labelled phalloidin in an immunofluorescence method (data not shown).

The results of our studies so far support the thesis that Ras signals through Raf-1 to MEK and MAPK and that this is the only significant Ras effector pathway in the transformation of rat2 cells. Several explanations can be offered to explain the differences obtained here and those reported previously (34, 78), notably the Ras genetic background (v-H-*ras* versus Gly12 and Gly12Val H-*RAS*), the cells used (rat2 cells versus rat4 and NIH 3T3 cells), the gene transfer protocols, the protein expression levels, and the transformation assay methods.

In our experiments, we are always careful to document the expression of the mutant Ras species. We generally observe modest overexpression levels. The issue of the "correct" level of Ras expression to use in transformation studies is moot, however, since the dominance of Ras is clear only when expression levels are in excess of the normal haploid expression level (25). Our studies with the Pro34Arg species (69; see above), although not critical to the arguments presented here, do show that some effector mutants are unstable. Furthermore, they show that the behavior of an effector mutant in the proto-oncogene form does not necessarily reflect its behavior in the context of activating mutations.

In the final analysis, the task of defining the Ras effector pathways that function in human cancers will be more important than sorting out the details of fibroblast transformation. Activated versions of Ras are not generally found in fibrosarcomas but, rather, contribute to epithelial tumors, as well as certain forms of leukemia (7). Since Ras activates non-Raf effectors in certain model systems, it seems not unlikely that Ras exploits multiple pathways in human cancer. This proposal might help explain why Ras oncogenes are common in human tumors while Raf oncogenes are not. Defining the relevant contribution of different Ras-regulated biochemical pathways to tumorigenesis will be critical for the rational design of drugs that seek to perturb Ras signaling at downstream points. Hopefully, some of the genetic reagents, experimental strategies, data, and arguments we have presented here and previously will facilitate these future studies.

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