Cell, Vol. 80, 533–541, February 24, 1995, Copyright © 1995 by Cell Press

Multiple Ras Functions Can Contribute to Mammalian Cell Transformation

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Summary

We have developed a generalized approach, using two hybrid interactions, to isolate Ha-Ras effector loop mutations that separate the ability of Ha-Ras to interact with different downstream effectors. These mutations attenuate or eliminate Ha-ras(G12V) transformation of mammalian cells, but retain complementary activity, as demonstrated by synergistic induction of foci of growth-transformed cells, and by the ability to activate different downstream components. The transformation defect of Ha-ras(G12V, E37G) is rescued by a mutant, raf1, that restores interaction. These results indicate that multiple cellular components, including Raf1, are activated by Ha-Ras and contribute to Ha-Ras-induced mammalian cell transformation.

Introduction

Ras is an essential component in the transduction of extracellular signals that induce proliferation and differentiation. It is a membrane-localized guanine nucleotide–binding protein that is active in the GTP-bound state. Activating mutations in Ras result in constitutive signaling to downstream elements and are found at a high frequency in a wide variety of tumors (Barbacid, 1987; Bollag and McCormick, 1991). The serine/threonine kinase Raf is a leading candidate for a downstream target of Ras. Mammalian *ras* is a proto-oncogene (Bonner et al., 1986), and the activated form induces cellular responses similar to those of activated Ras (Morrison, 1990). Raf acts downstream of Ras, at least in some cell types, as inferred by the ability of activated Raf to bypass dominant interfering ras mutants or anti-Ras antibodies (Smith et al., 1986; Cai et al., 1990), and by the ability of dominant-negative mutants of Raf to interfere with Ras signaling (Kolch et al., 1991). Furthermore, Ras and Raf can be coimmunoprecipitated from activated mammalian cells (Finney et al., 1993; Koide et al., 1993; Hallberg et al., 1994), and immobilized purified GTP-bound Ras can bind Raf from cell extracts (Moodie et al., 1993). A Ras–Raf interaction is also observed between Saccharomyces cerevisiae–expressed fusion proteins in the yeast two-hybrid system (Van Aelst et al., 1993; Vojtek et al., 1993), and purified Ras can bind a purified truncated form of Raf in vitro (Warne and Viciana, 1993; Zhang et al., 1993). Raf activates the serine/threonine protein kinase MAPK (for mitogen-activated protein kinase) through an activation of MEK (for MAPK or extracellular signal–regulated kinase [ERK] kinase) (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992; Macdonald et al., 1993). These kinases are believed to convey growth and differentiation signals to the nucleus, resulting in the induction of appropriate gene expression (Hill et al., 1993; Marais et al., 1993; Cowley et al., 1994). These results have led to a linear model of Ras signaling in which Ras activation leads to the sequential activation of Raf, MEK, and MAPK.

Ras is conserved in every eukaryote examined thus far (reviewed by Bollag and McCormick, 1991). All known *ras* genes encode an identical stretch of nine amino acids in the amino-terminal half (amino acids 32–40 in Ha-Ras) that undergoes a major conformational shift when Ras binds GTP. Certain mutations in this region eliminate the ability of Ras to bind and activate known downstream components without affecting binding and hydrolysis of GTP. For these reasons, this region of Ras has been termed the effector loop (reviewed by Polakis and McCormick, 1993). Despite a conserved effector loop, several different downstream targets have been identified that interact with Ras in diverse cell types. These include Raf, S. cerevisiae adenylyl cyclase (CYR1) (Vojtek et al., 1993), and the putative serine/threonine protein kinase byr2 in Schizosaccharomyces pombe (Van Aelst et al., 1993). S. cerevisiae CYR1 is necessary for the regulation of cell growth and is activated by RAS in vitro (Wigler et al., 1988). S. pombe byr2 is required for pheromone-induced sexual differentiation (Wang et al., 1991). It acts downstream of ras (Wang et al., 1991) and binds ras in the two-hybrid system (Van Aelst et al., 1993). Byr2 has structural homology to mammalian MEK kinase (MEKK) in its catalytic domain (Lange-Carter et al., 1993). Other proteins that interact with the effector loop of Ras are the GAP/NF-1 family of GTPase-activating proteins (reviewed by Bollag and McCormick, 1991), the scd1 protein of S. pombe (Chang et al., 1994), and phosphatidylinositol-3-OH kinase (PI(3)K) (Rodriguez-Viciana et al., 1994). Aside from the kinase domains of Raf1 and byr2, which are not required for interaction with RAS, there is no known homology among the Ras effector loop–binding proteins.

Ras not only interacts with diverse targets in evolutionarily divergent organisms, it also interacts with diverse targets within the same cell. Genetic evidence from both S. pombe and S. cerevisiae indicates the existence of multiple Ras effectors in each organism (Wigler et al., 1988; Wang et al., 1991; Chang et al., 1994). There is also evidence of multiple Ras targets in vertebrate systems. Activated Ras induces the expression in PC12 cells of certain neurite-specific genes that are not induced by activated Raf (D'Arcangelo and Halegoua, 1993), possibly through the activation of MEK by a mechanism independent of Raf (Lange-Carter and Johnson, 1994; Lange-Carter et al., 1993; Minden et al., 1994a). In 3T3 L1 cells, oncogenic

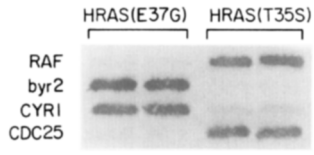

Figure 1. E37G and T35S Mutations Separate the Ability of Ha-Ras to Interact with Targets in the Yeast Two-Hybrid Assay

β-Galactosidase activity was determined by filter assay. Ha-ras mutants were expressed as LexA-binding domain (LBD) fusions, and targets were expressed as GAD fusions (RAF, byr2) or VP16-activating domain (VAD) fusions (CYR1, CDC25) in the yeast reporter strain L40 (Vojtek et al., 1993). Yeast patches containing interacting protein pairs are dark, owing to β-galactosidase activity producing an X-Gal cleavage product. Yeast patches containing noninteracting protein pairs remain white, owing to the absence of detectable β-galactosidase activity.

ras activated MAPK, but oncogenic *raf* did not (Porras et al., 1994). Ha-Ras is required for multiple signals induced by v-Src in fibroblasts, some of which may be independent of Raf (Qureshi et al., 1992), and the induction of human T cell receptor β gene expression by v-Raf is blocked by a dominant interfering ras mutant (Wotton et al., 1993).

In this report, we have explored whether Ha-Ras can transform mammalian cells via multiple interactions. We have used the yeast two-hybrid assay developed by Fields and Song (1989) to isolate Ha-ras mutants defective in their effector interactions and have assayed their function in yeast and mammalian cells.

Results

Ha-ras(T35S) and Ha-ras(E37G) Have Complementary Two-Hybrid Interactions

We used the yeast two-hybrid protein interaction detection system (Fields and Song, 1989) to identify mutations in Ha-Ras that separate its ability to interact with Raf1 from its ability to interact with byr2. Ha-*ras* was randomly mutagenized along its entire length by polymerase chain reaction (PCR), taking advantage of the inherent nucleotide misincorporation frequency of Taq polymerase as described by Zhou et al. (1991). A library of mutated Ha-*ras* genes was fused to the GAL4 DNA-binding domain (GBD). This library was separately screened with both a byr2–GAD (for GAL4-activating domain) fusion and a Raf1–GAD fusion to identify clones that failed to interact with these targets in the yeast two-hybrid reporter strain YPB2. In this fashion, 150 Ha-ras clones that failed to interact with byr2 and 150 Ha-ras clones that failed to interact with Raf1 were isolated. Clones that failed to interact with byr2 were in turn screened for those that still interacted with Raf1; and clones that failed to interact with Raf1 were screened for those that still interacted with byr2. Among these, Ha-ras mutations that passed the second round of screening were isolated and sequenced. One of these, Ha-ras (T35S), contains a conservative substitution of serine for threonine at position 35 and was found to interact with Raf1 but not byr2. Another, Ha-ras(E37G), contains a single amino acid substitution of glutamic acid for glycine at position 37, which disrupts the interaction of Ha-Ras with Raf1 but not with byr2. Ha-ras(T35S) and Ha-ras(E37G) were also tested for the ability to interact with the S. cerevisiae RAS effector CYR1 (for adenylyl cyclase) and the S. cerevisiae RAS GDP/GTP exchange factor CDC25. Both of these proteins have previously been shown to interact with Ha-Ras in the two-hybrid system (Vojtek et al., 1993). Ha-ras(T35S) retains the ability to interact with CDC25, but is defective in CYR1 interaction. Ha-ras(E37G) retains the ability to interact with CYR1, but is defective in CDC25 binding (Figure 1, Table 1). Both Ha-ras mutants are within the effector loop. The E37G mutation is to our knowledge the first effector loop mutation shown to have an effect on physical interaction with a GDP/GTP exchange factor.

Table 1. Two-Hybrid Interactions between Ha-ras Mutants and Ha-Ras Targets

Mutant	STE11	Raf1	byr2	CYR1	CDC25	raf1(S257L)
STE11	+	−	−	−	−	−
Ha-Ras	−	+	+	+	+	+
Ha-ras(G12V)	−	+	+	+	+	+
Ha-ras(E37G)	−	−	+	+	−	+
Ha-ras(G12V, E37G)	−	−	+	+	−	+
Ha-ras(T35S)	−	+	−	−	+	+
Ha-ras(G12V, T35S)	−	+	−	−	+	+
Ha-ras(G15A)	−	−	−	−	+	−
Ha-ras(T35A)	−	−	−	−	+	−

Ha-*ras* alleles and *STE11* were expressed as LBD fusions. Ha-Ras targets were expressed as GAD or VAD fusions (see Figure 1). Pairs were coexpressed in the yeast reporter strain L40. STE11 is an S. cerevisiae kinase that interacts with itself. Plus represents a positive indication of β-galactosidase activity using filter assays. At least four independent transformants were tested for each pair.

In Yeast, Activity of Ha-ras(T35S) and Ha-ras(E37G) Is Consistent with Effector Interactions

The ability of Ha-ras(T35S) and Ha-ras(E37G) to interact with byr2 in vivo was tested by expression in *ras1*^{null} S. pombe haploid and diploid strains. S. pombe *ras1* is required for sexual differentiation and normal cell shape (Nadin-Davis et al., 1986). A *ras1*^{null} strain is defective in conjugation and sporulation and has a rounded morphology rather than the elongated morphology of wild-type cells. Byr2 acts immediately downstream of ras1, and overexpression of *byr2* rescues the sporulation defect but not the conjugation or morphology defect of *ras1*^{null} cells (Wang et al., 1991). Ha-Ras fully substitutes for ras1, including induction of wild-type levels of sporulation in a *ras1*^{null}/*ras1*^{null} diploid (Nadin-Davis et al., 1986). Ha-ras(T35S) was defective in the ability to induce sporulation, possibly owing to a defect in interaction with byr2. Ha-ras(E37G) induced wild-type levels of sporulation in *ras1*^{null}/*ras1*^{null} cells, but cell shape remained aberrant (Figure 2A). Conjugation was not rescued in haploid *ras1*^{null} S. pombe expressing either Ha-ras(E37G) or Ha-ras(T35S) (data not shown).

Ha-ras(T35S) and Ha-ras(E37G) were also tested for function in S. cerevisiae. An S. cerevisiae *ras1*^{null}, *ras2*^{ts} strain cannot grow at 37°C, presumably because of reduced CYR1 activity (Powers et al., 1989). Wild-type Ha-

Table 2. Focus Formation Frequencies in NIH 3T3 and RAT4 Cells

	NIH 3T3		RAT4	
Variant	pCEP4	Ha-ras(G12V, E37G)	pCEP4	Ha-ras(G12V, E37G)
Ha-ras(G12V)	1.00		1.000	
Ha-ras(G12V, T35S)	0.05	0.40	0.034	0.37
pDCR	<0.01	<0.01	<0.010	<0.02

	NIH 3T3			RAT4		
Variant	pCEP4	Raf1	raf1(S257L)	pCEP4	Raf1	raf1(S257L)
Ha-ras(G12V)	1.000	1.500	0.330	1.000	1.500	1.300
Ha-Ras	0.080	0.200	0.070	<0.003	<0.003	<0.002
Ha-ras(G12V, E37G)	<0.003	0.020	0.620	<0.001	0.010	0.150
pDCR	<0.002	<0.003	<0.001	<0.002	<0.003	<0.003

Frequencies were determined as number of morphologically altered foci per number of G418-resistant colonies, then normalized to the focus formation frequency of Ha-ras(G12V), which was set at 1. Values are representative of at least two independent transfections for each pair, performed in duplicate or triplicate. For those pairs in which no foci were observed in any experiment, frequencies are reported as less than one focus per experiment, normalized to the Ha-ras(G12V) focus formation frequency. Ha-Ras variants in the column were introduced (5–250 ng/transfection) in pDCR, which contains the G418-resistance gene. Ha-Ras and Raf1 variants in the rows were introduced (500 ng/transfection) in pCEP4 without selection.

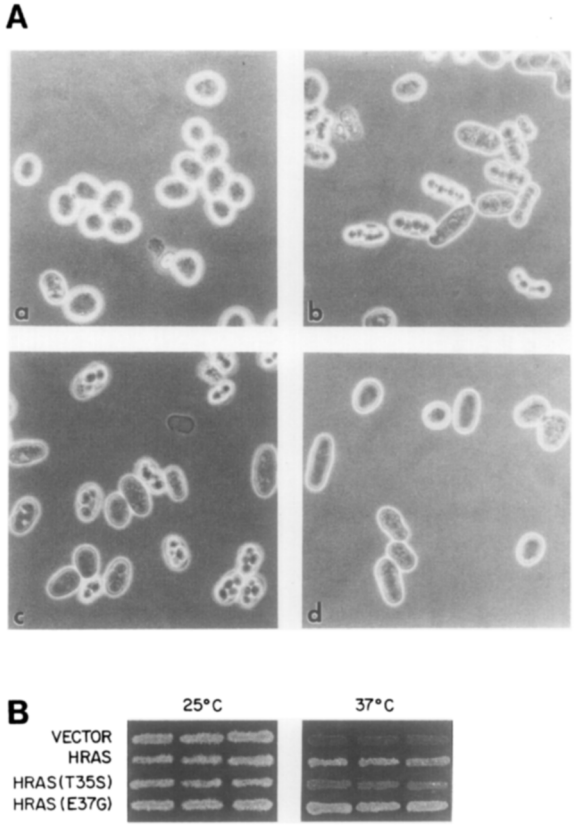

Figure 2. Complementation of *ras1*^{null} Defects in S. pombe and *ras1*^{null} *ras2*^{ts} in S. cerevisiae

(A) Ha-Ras and Ha-ras mutants, under control of the S. pombe *adh1* promoter, were expressed in the *ras1*^{null}/*ras1*^{null} diploid SPRN1D. (a), vector alone; (b), Ha-ras; (c), Ha-ras(E37G); (d), Ha-ras(T35S).

(B) Ha-Ras and Ha-ras mutants, under control of the S. cerevisiae *ADH1* promoter, were expressed in the S. cerevisiae *ras1*^{null} *ras2*^{ts} strain STS8. Three independent transformants were analyzed.

Ras can activate CYR1 (Kataoka et al., 1985) and rescues this temperature sensitivity. Ha-ras(T35S) does not restore growth at 37°C, reflecting its inability to interact with CYR1. On the other hand, Ha-ras(E37G) fully restores the temperature-sensitive growth of the *ras1*^{null}, *ras2*^{ts} strain. The results of in vivo function are thus consistent with the two-hybrid results.

Ha-ras(G12V, T35S) and Ha-ras(G12V, E37G) Have Complementary Transforming Activity in Mammalian Cells

To explore the activity of the mutant Ha-ras proteins in mammalian cells, the activating mutation, G12V, was introduced in each. Ha-ras(G12V, T35S) and Ha-ras(G12V, E37G) have the same profile of two-hybrid interactions as those observed with Ha-ras(T35S) and Ha-ras(E37G), respectively (Table 1). Ha-ras(G12V, T35S) and Ha-ras (G12V, E37G), under control of the cytomegalovirus (CMV) promoter, were transfected into NIH 3T3 and RAT4 cells. Ha-ras(G12V, E37G) was completely defective in focus formation, displaying levels of activity >300-fold lower than Ha-ras(G12V). Ha-ras(G12V, T35S) was attenuated in the ability to induce foci, producing approximately 20-fold fewer foci per microgram of DNA than Ha-ras(G12V) (Table 2). When Ha-ras(G12V, E37G) and Ha-ras(G12V, T35S) were cotransfected, there was an 8- to 10-fold increase in focus formation above the level observed with Ha-ras(G12V, T35S) alone (Table 2). This result suggests that Ras-induced transformation requires at least two separate Ras activities, each mutant providing a complementary activity, resulting in a strong transformation signal.

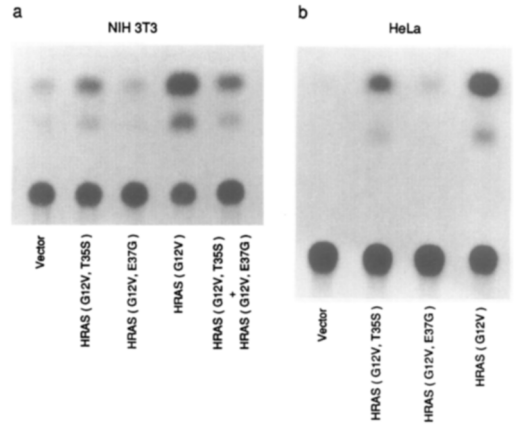

Figure 3. Activation of SRE-Dependent CAT Expression by Ha-ras Mutants

(a) The indicated pDCR-Ha-Ras plasmids were transfected in NIH 3T3 cells at 3 μg/transfection. Empty vector (6 μg) was used as the negative control. Cell extract volumes used in CAT assays were normalized to expression of a cotransfected β-galactosidase reporter plasmid, pCH110.

(b) The indicated pDCR-Ha-Ras plasmids or empty vector were transfected in HeLa cells at 5 μg per transfection. Extract volumes used were normalized as in (a). All experiments were repeated at least twice with similar results.

Ha-ras(G12V, E37G) and Ha-ras(G12V, T35S) Have Different Profiles of Activation in Vertebrate Cells

Induction of serum response element (SRE)-dependent transcription is a consequence of Ras and Raf activation (Yamauchi et al., 1993; Hipskind et al., 1994). We measured the ability of Ha-ras(G12V, E37G) and Ha-ras(G12V, T35S) to induce SRE-dependent transcription during transient expression in NIH 3T3 cells by using a chloramphenicol acetyltransferase (CAT) reporter construct under control of a 5X-SRE promoter (Lee and Gilman, 1994). Ha-ras(G12V, E37G) did not induce expression of the CAT reporter, which was highly induced by Ha-ras(G12V). Ha-ras(G12V, T35S) retains the ability to induce this reporter, although to a lesser extent than that observed with Ha-ras(G12V). Cotransfection of Ha-ras(G12V, T35S) and Ha-ras(G12V, E37G) did not induce expression of the CAT reporter above the level observed with Ha-ras(G12V, T35S) alone (Figure 3a). Therefore, these mutants do not act cooperatively on this pathway.

Another class of serine/threonine protein kinases that is responsive to activated Ras in mammalian cells consists of the c-Jun N-terminal kinases (JNKs) (Hibi et al., 1993;

Table 3. JNK and ERK Activation in HeLa Cells

Ha-Ras Variant	JNK	ERK
Ha-ras(G12V)	100	100
Ha-ras(G12V, E37G)	60 ± 8	32 ± 3
Ha-ras(G12V, T35S)	70 ± 11	84 ± 12
Control	19 ± 5	3 ± 1

Ha-ras mutants were expressed in HeLa cells in the vector pSRα. Control is HeLa cells with the vector alone. JNK and ERK activity induced by Ha-ras(G12V) was set at 100. Activities induced by the mutants and control were normalized to Ha-ras(G12V). Values are expressed as the average of two independent experiments.

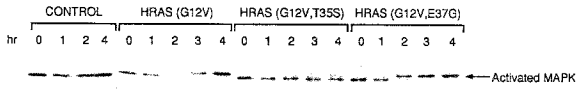

Figure 4. Activation of p42 MAPK in Xenopus Oocyte Extracts

Extracts were incubated for the indicated times with 150 μg/ml purified recombinant His_6–Ha-Ras proteins attached to ProBond resin (Invitrogen Corporation). The control extract was incubated in buffer with ProBond resin alone. The phosphorylated (activated) form of p42 MAPK migrates with a slower mobility on polyacrylamide gels and is indicated by the arrow.

Dérijard et al., 1994). We examined the ability of Ha-ras(G12V, E37G) and Ha-ras(G12V, T35S) to activate JNK in HeLa cells as assayed by c-Jun phosphorylation by anti-JNK1 immune complexes (Minden et al., 1994b). In contrast with SRE induction, the E37G mutation does not disrupt JNK1 activation by activated Ha-Ras (Table 3). Ha-ras(G12V, T35S) also retains the ability to activate JNK.

We compared JNK activation by the mutant Ha-ras proteins with their ability to activate extracellular signal–regulated kinase 2 (ERK2), a MAPK downstream of Raf (Dent et al., 1992; Kyriakis et al., 1992), as assayed by phosphorylation of myelin basic protein (MBP) by anti-ERK2 immune complexes from HeLa cells. Ha-ras(G12V, E37G) reproducibly activated MBP kinase activity in the ERK2 immune complex. However, the level of activation was lower than that observed with Ha-ras(G12V) and Ha-ras(G12V, T35S) (Table 3). Interestingly, while both Ha-ras(G12V) and Ha-ras(G12V, T35S) induced SRE-dependent transcription in HeLa cells, Ha-ras(G12V, E37G) did not (Figure 3b), suggesting either that ERK2 activation by Ha-ras(G12V, E37G) was too weak to induce this response, or that it does not mediate the response.

Xenopus p42 MAPK is responsive to activated Ha-Ras in cell-free oocyte extracts (Shibuya et al., 1992). A component present in oocytes called REKS (for Ras-dependent ERK/MAP kinase stimulator) has been identified that activates a MAPK through a MEK in an activated Ras-dependent manner (Itoh et al., 1993). We examined the ability of the Ha-ras mutants to activate p42 MAPK in Xenopus oocyte extracts by use of purified recombinant oligohistidine (His_6)-tagged Ha-Ras proteins. In contrast with the results obtained in HeLa and NIH 3T3 cells, Ha-ras(G12V, E37G) is a strong activator of this kinase, whereas Ha-ras(G12V, T35S) has a detectable but attenuated activity (Figure 4).

Complementation of Ha-ras(G12V, E37G) by an Interacting Mutant, raf1

The Ha-ras(G12V, E37G) transformation defect may be due to its inability to interact with Raf1. To test this, we used the two-hybrid assay to identify Raf1 mutations that suppress the Raf1-binding defect of Ha-ras(E37G). The N-terminal half of Raf1 interacts with Ras, while the C-terminal half contains the catalytic domain that interacts with MEK (Van Aelst et al., 1993; Vojtek et al., 1993). A library of mutations in the N-terminal half of Raf1 (amino acids 1–281) was created by PCR using the same protocol used to create the mutated Ha-*ras* library. This library was fused to the wild-type C-terminal half of Raf1 in pGADGH to produce full-length genes fused to GAD. The Ha-ras(E37G)–GBD fusion was used to screen this library for mutated Raf1 proteins that had acquired the ability to interact with the mutant Ha-ras. To ensure that full-length raf1 proteins were expressed, clones that interacted with Ha-ras(E37G) were further tested for their ability to interact with MEK. Three different raf1 mutations were isolated that interacted with Ha-ras(E37G) and retained the ability to interact with MEK. All three mutations were located in conserved region 2 (CR2), the second of the three regions highly conserved among all Raf homologs (Morrison, 1990). CR2 is 20 amino acids in length and is rich in serine and threonine residues. The mutations found were single amino acid substitutions consisting of a change from arginine to glycine at position 256, from serine to leucine at position 257, and from serine to proline at the same position. These mutations did not diminish interaction with wild-type Ha-Ras or Ha-ras(G12V), or restore interaction with Ha-ras(G15A) or Ha-ras(T35A), mutations previously found to disrupt Raf1 interaction (Van Aelst et al., 1993; Vojtek et al., 1993) (see Table 1).

Raf1(S257L), under control of a CMV promoter, was tested for its capacity to restore transformation potential to Ha-ras(G12V, E37G). NIH 3T3 and RAT4 cells were transfected with wild-type Ha-Ras, Ha-ras(G12V), Ha-ras(G12V, E37G), Raf1, and raf1(S257L), alone or in combination. The results are summarized in Table 2. As noted above, Ha-ras(G12V, E37G) does not induce foci. Both wild-type Raf1 and raf1(S257L) also failed to induce foci. Wild-type Ha-Ras weakly induces foci in NIH 3T3 cells. This induction is slightly increased by cotransfection with wild-type Raf1, as has been previously reported (Cuadrado et al., 1993). Ras(G12V) is a potent inducer of focus formation, but also cooperates with wild-type Raf1. Raf1(S257L) does not cooperate with either Ha-Ras or Ha-ras(G12V) and appears to be interfering when cotransfected with Ha-ras(G12V) in NIH 3T3 cells. The transforming potential of Ha-ras(G12V, E37G) is partly rescued by cotransfection with wild-type Raf1 in both cell types. Cotransfection with raf1(S257L), however, results in a >30-fold increase in focus formation in NIH 3T3 cells and a >10-fold increase in focus formation in RAT4 cells above the level observed with a cotransfection of Ha-ras(G12V,

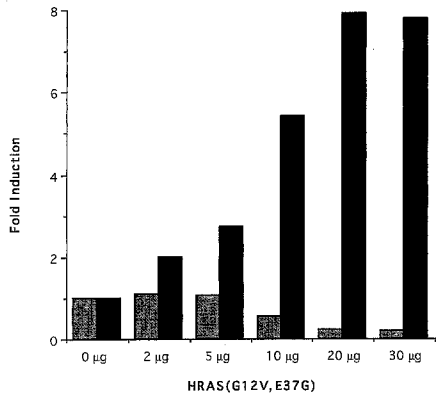

Figure 5. Ha-ras(G12V, E37G) and raf1(S257L) Synergize to Induce SRE-Dependent CAT Expression

NIH 3T3 cells were transfected with 5 μg of pCEP4-Raf1 (gray bars) or pCEP4-raf1(S257L) (black bars) along with the indicated concentration of pDCR-Ha-ras(G12V, E37G). CAT activity is expressed as the fold induction over the basal level (set at 1) observed with Raf1 alone or raf1(S257L) alone. In repeated experiments, basal CAT activity induced by raf1(S257L) was similar to or less than that induced by Raf1. pDCR-Ha-ras(G12V, E37G) was transfected alone at the highest concentration used in the cotransfections (30 μg) and did not induce CAT activity above the level observed with empty vector. These experiments were repeated twice with similar results.

E37G) and wild-type Raf1. Similar results were obtained with the other two raf1 mutants (data not shown).

The ability of Ha-ras(G12V, E37G) to induce SRE-dependent transcription when coexpressed with Raf1 or raf1(S257L) was also examined. A constant concentration of Raf1 or raf1(S257L) DNA was transfected into NIH 3T3 cells with increasing concentrations of DNA encoding Ha-ras (G12V, E37G). Both Raf1 and raf1(S257L) induce expression of the SRE–CAT reporter when transfected alone. The level of raf1(S257L) induction is similar to or less than the level of Raf1 induction (data not shown). Cotransfection of Ha-ras(G12V, E37G) with raf1(S257L) resulted in increased levels of SRE induction relative to raf1(S257L) alone, whereas cotransfection of Ha-ras (G12V, E37G) with wild-type Raf1 resulted in decreased levels of SRE induction relative to Raf1 alone (Figure 5).

Discussion

We have used the yeast two-hybrid system to identify complementary Ha-ras mutants that have different downstream activities in mammalian cells. Again using the two-hybrid system, we have identified mutations in Ha-Ras and Raf1 that were used to demonstrate that interaction between these proteins can mediate transformation of mammalian cells. The approaches we have taken can be used more generally to dissect the roles played by proteins that have multiple functions and to evaluate the biological significance of the interaction between two proteins.

Ha-ras Effector Loop Mutants That Distinguish Downstream Targets Have Altered Profiles of Activity

Ha-ras(T35S) and Ha-ras(E37G) contain mutations that were selected by using the two-hybrid system to separate the ability of Ha-Ras to interact with known downstream targets. These mutations both map to the effector loop, yet the profiles of activity of Ha-Ras proteins containing these mutations differ greatly. While yeasts are responsive to wild-type Ha-Ras, the T35S mutant is inactive in both S. cerevisiae and S. pombe, whereas the E37G mutant retains some Ras functions in both yeasts. Ha-ras(G12V) is a strong activator of p42 MAPK in Xenopus oocytes, a response that is probably mediated by a protein kinase called REKS (Itoh et al., 1993). The Ha-ras(G12V, E37G) fully retains this activity, whereas Ha-ras(G12V, T35S) appears significantly attenuated. In contrast with the results with yeasts and Xenopus oocytes, Ha-ras(G12V, E37G) is completely defective in focus formation in NIH 3T3 and RAT4 cells, while Ha-ras(G12V, T35S) retains partial activity. Consonant with this, in transient expression studies, the former does not induce SRE-dependent transcription of a reporter gene in NIH 3T3 or HeLa cells, while the latter does. We also examined the ability of these mutants to activate ERK2 and JNK1 protein kinases in transient expression studies. While both mutants were able to activate these kinases, Ha-ras(G12V, E37G) was clearly attenuated in ERK2 activation. This is consistent with the observation that oncogenic *ras* activates c-Jun and JNK via a separate pathway from the activation of ERK (Westwick et al., 1994; Minden et al., 1994a, 1994b).

raf1 Mutants That Restore Interaction with Ha-ras(E37G) in Yeast Restore Its Activity in Mammalian Cells

We could detect absolutely no focus-forming activity of the Ha-ras(G12V, E37G) mutant in either NIH 3T3 or RAT4 cells. Since the Ha-ras(E37G) mutant was selected by its inability to interact with Raf1 in the yeast two-hybrid system, this defect might be the result of the failure of the mutant to interact properly with Raf1 in mammalian cells. In strong support of this idea, all three raf1 mutants with which Ha-ras(G12V, E37G) can interact in the yeast two-hybrid system rescued focus formation by Ha-ras(G12V, E37G). These mutants are not activated forms of Raf1. First, they are unable to induce foci alone, and second, raf1(S257L) did not induce SRE-dependent expression above the level observed with wild-type Raf1. Furthermore, the raf1(S257L) mutant did not cooperate with either Ha-ras(G12V) or wild-type Ha-Ras in focus induction. We found that Ha-ras(G12V, E37G) acted synergistically with raf1(S257L) but not with wild-type Raf1 to induce an SRE reporter gene. These results constitute a formal genetic demonstration that one path by which Ha-Ras can act upon mammalian cells is through its physical interaction with Raf1.

The three raf1 mutants that can interact with Ha-ras(G12V, E37G) all have single mutations located in the CR2 of Raf1 (Morrison, 1990). This implies that a common mechanism is at play to restore interaction. Previous studies have not found a role for CR2 in binding Ha-Ras. Both two-hybrid interactions (Vojtek et al., 1993) and in vitro protein binding assays (Warne and Viciana, 1993; Zhang et al., 1993) indicate that the CR1 region of Raf1 is suffi-

cient for interaction with Ha-Ras. In keeping with this, we have found that Ha-ras(E37G) retains the ability to bind a truncated form of Raf1 that lacks the CR2 region (data not shown). Thus, the CR2 region might contribute a so-called proofreading function that ensures specificity in Raf1 interactions. CR2 may block binding to Ha-ras (E37G), and the CR2 mutations may relieve this inhibition. More complex models are also possible. For example, R256G, S257L, and S257P may disrupt binding between the CR2 region of Raf1 and a third protein, X, that is conserved between S. cerevisiae and mammals. The E37G mutation may prevent Ha-Ras from binding to a Raf1–X complex but not affect binding to Raf1 alone. Possible candidate proteins for X are 14-3-3 proteins, which have been shown to bind CR2 of Raf1 (Freed et al., 1994) and are conserved in S. cerevisiae (van Heusden et al., 1992). Such models predict the formation of nonproductive complexes between some normal cellular components and our mutant proteins.

Multiple Ha-Ras Activities Can Contribute to Transformation

Ha-ras(G12V, T35S) is severly impaired in its ability to induce foci in NIH 3T3 and RAT4 cells. This mutant still interacts with Raf1 in yeast, retains the ability to induce SRE-dependent transcription, and can activate ERK2. Its interaction with Raf1 in mammalian cells may nevertheless be defective (Shirouzu et al., 1994), or it may also be defective in other respects. Ha-ras(G12V, E37G) is clearly defective in its interaction with Raf1 and is incapable of focus induction yet retains an ability to enhance focus formation by Ha-ras(G12V, T35S) greatly. Thus, Ha-ras (G12V, E37G) supplies an activity that is lacking in Ha-ras(G12V, T35S).

These observations suggest that Ha-Ras has at least two functions in mammalian cells, both of which can contribute to transformation, and which act synergistically. While it is conceivable that both mutants act solely upon Raf1, this seems unlikely to us. First, the synergy of the mutants in focus induction cannot be explained as a simple additive effect on Raf1, as the Ha-ras(G12V, E37G) mutant is completely defective for focus formation. Second, Ha-ras(G12V, E37G) does not cooperate with wild-type Raf1 or Ha-ras(G12V, T35S) in SRE induction. To rule out Raf1 formally as the sole target, however, would require monitoring all its biological functions in stably transformed and cotransfected cells.

High levels of activated Raf can transform mammalian cells (Morrison, 1990). This does not conflict with our conclusion that Ha-Ras can activate at least one other pathway that contributes to transformation. In S. pombe, ras acts on at least two highly divergent pathways that converge upon sexual differentiation (Wang et al., 1991; Chang et al., 1994). In S. cerevisiae, RAS also acts on at least two effectors, one of which is cryptic and becomes apparent when the major effector pathway is blocked (Wigler et al., 1988). It is unclear whether activation of all the endogenous Raf1 present in mammalian cells would be sufficient to account for full transformation by Ha-ras(G12V).

We do not know all of the proteins that interact with Ha-Ras in mammalian cells and which of these still interact with Ha-ras(E37G). Among these may be another protein kinase, perhaps homologous to S. pombe byr2; PI(3) kinase (Rodriguez-Viciana et al., 1994); and proteins involved in morphogenesis (Chang et al., 1994). We are currently using wild-type Ha-Ras and the Ha-Ras mutants to screen cDNA libraries for interacting proteins. Our ras mutants, and candidate effectors, and mutants derived from them, can facilitate dissection of the physiological roles of Ras in such processes as tumorigenicity, oncogene cooperation, apoptosis, morphogenesis, and differentiation.

Experimental Procedures

Yeast Strains and Media

The genotype of the S. cerevisiae reporter strain YPB2, used to test interactions between GBD and GAD fusions, is *MATa ura3–52 his3–200 ade2–101 lys2–801 trp1–901 leu2–3,112 can^r gal4–542 gal80–538 LYS2::GAL1$_{uas}$–LEU2$_{TATA}$–HIS3 URA3::GAL1$_{17\text{-}mers(3X)}$–CYC1$_{tata}$–lacZ* (provided by P. Bartel and S. Fields). The genotype of L40, used to detect interactions between LBD and GAD or VAD fusions, is *MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ* (Vojtek et al., 1993). STS8 is *MATa his3 leu2 ura3 trp1 ade8 can1 ras1:URA3 ras2^{ts}* (Powers et al., 1989). Strains were grown according to standard conditions in rich or synthetic media with appropriate supplements (Sherman et al., 1986) at 30°C, except for STS8, which was incubated at 25°C.

The S. pombe strains SPRN (*h^{90} leu1–32 ade6–210 ura4-D18 ras1::ura4*) and SPRND (*h^{90} leu1–32 ade6–210 ura4-D18 ras1::ura4/h^{90} leu1–32 ade6–210 ura4-D18 ras1::ura4*) (Chang et al., 1994) were grown in rich or nutrient-limited synthetic media with the appropriate auxotrophic supplements (Nadin-Davis et al., 1986).

Library Construction and Screening

Full-length human Ha-*ras* encoding a protein containing the C186S mutation was randomly mutagenized by PCR (Zhou et al., 1991) and ligated into the vector pHP5 (kindly provided by H.-P. Xu) to create in-frame fusions with the GAL4 DNA-binding domain (amino acids 1–147). Screening for mutations that affected binding of Ha-Ras–GBD fusions to Raf1–GAD or byr2–GAD was performed in YPB2. Approximately 3 × 10^4 transformants (selected on synthetic media lacking leucine and tryptophan) from each screen were assayed for β-galactosidase activity on filters (Bartel and Fields, 1994). A mutant human *raf1* library was constructed as described for the mutant Ha-*ras* library, except that only nucleotides 1–843 were subjected to mutagenesis. These fragments were then ligated into pGADGH (described by Van Aelst et al., 1993) already containing wild-type *raf1* sequences from nucleotides 844–1946 to create full-length genes in-frame with the GAL4-activating domain. Screening for *raf1* mutations that interacted with Ha-ras(E37G)-GBD was performed in YPB2.

Plasmids

pHP5-Ha-ras(E37G) and pHP5-Ha-ras(T35S) were isolated from the mutant Ha-*ras* library and express Ha-Ras–GBD fusions. pHP5 is a variant of pGBT10 with a 4 bp insertion in the EcoRI site. pLexA-Ha-Ras, pLexA-Ha-ras(G12V), and pLexA-Ha-ras(G15A), which express Ha-Ras–LBD fusions, were provided by A. Vojtek (Vojtek et al., 1993). pBTM116-Ha-ras(E37G), pBTM116-Ha-ras(T35S), pBTM116-Ha-ras (T35A), pBTM116-Ha-ras(G12V, E37G), and pBTM116-Ha-ras (G12V, T35S) express Ha-Ras–LBD fusions. Full-length Ha-*ras* genes were inserted as BamHI–SalI fragments into the BamHI–SalI site of pBTM116 (Vojtek et al., 1993). pBTM116-STE11 is described by Marcus et al. (1994). pVP16-CYR1 and pVP16-CDC25 were kindly provided by A. Vojtek (Vojtek et al., 1993). pGAD-Raf1, pGAD-byr2, and pGAD-STE11 are as described (Van Aelst et al., 1993; Marcus et al., 1994). pGAD-raf1(R256G), pGAD-raf1(S257L), and pGAD-raf1(S257P) express Raf1–GAD fusions and were isolated from the mutant *raf1* library. pAAU-Ha-Ras, pAAU-Ha-ras(E37G), and pAAU-Ha-ras(T35S) have BamHI fragments of full-length genes inserted into the BamHI site

of the S. pombe *adh1* expression vector pAAU (Wang et al., 1991). pAD4-Ha-Ras, pAD4-Ha-ras(E37G), and pAD4-Ha-ras(T35S) have SalI–SacI fragments of full-length genes in the SalI–SacI site of the S. cerevisiae *ADH1* expression vector pAD4 (Ballester et al., 1989). pDCR is a mammalian expression vector containing the cytomegalovirus promoter followed by unique SalI and BamHI sites and the rabbit β-globin terminator and splice sequence. This region and the *neo*^{r} gene under control of an SV40 promoter are flanked by Moloney murine leukemia retrovirus 5′ and 3′ long terminal repeats. pDCR-Ha-Ras and pDCR-Ha-ras(G12V) contain full-length Ha-*ras* inserted as SalI–BglII fragments in the SalI–BamHI site of pDCR. pDCR-Ha-ras(G12V, E37G) and pDCR-Ha-ras(G12V, T35S) contain full-length genes inserted as SalI–BamHI fragments in the SalI–BamHI site of pDCR. pCEP4-Raf1 and pCEP4-raf1(S257L) contain full-length genes inserted as XhoI–BamHI fragments in the XhoI–BamHI site of pCEP4 (Invitrogen Corporation). pCEP4-Ha-ras(G12V, E37G) contains the full-length Ha-*ras* mutant inserted as a SalI–BamHI fragment in the XhoI–BamHI site of pCEP4. pSRα-Ha-ras(G12V), pSRα-Ha-ras(G12V, E37G), and pSRα-Ha-ras(G12V, T35S) contain full-length genes under control of the SV40 promoter inserted as EcoRI–PstI fragments in the EcoRI–PstI site of pSRα (Minden et al., 1994a). pTrcHisB-Ha-ras(G12V, E37G) and pTrcHisB-Ha-ras(G12V, T35S) contain full-length genes inserted as SalI–EcoRI fragments in the SalI–XhoI site of pTrcHisB (Invitrogen Corporation) to produce in-frame fusions with six histidine residues at the N-terminus. pTrcHis-Ha-ras(G12V) is as described (Shibuya et al., 1992).

Mammalian Cell Transfections

NIH 3T3 and RAT4 cells were transfected by the calcium phosphate precipitation method as described (Wigler et al., 1979). NIH 3T3 cells were passaged in Dulbecco's modified Eagle's medium (DMEM) (ICN Biomedicals, Incorporated) plus 10% calf serum (GIBCO BRL Life Technologies, Incorporated); 24 hr after transfection, cells were split into DMEM plus 5% calf serum for the focus assay and DMEM plus 10% calf serum supplemented with 0.5 mg/ml G-418 sulfate (GIBCO BRL Life Technologies, Incorporated) to determine the transfection efficiency. Foci were scored under magnification after 14 days. RAT4 cells were treated in an identical fashion, except that the focus assay was performed in DMEM plus 10% calf serum, and foci were scored under magnification after 21 days.

CAT Assays

NIH 3T3 or HeLa cells were transfected (Wigler et al., 1979) with 5X-SRE-CAT (Lee and Gilman, 1994), pCH110 (Pharmacia Biotech, Incorporated), and tester plasmid(s). After transfection (12–16 hr), the cells were incubated in DMEM plus 0.5% calf serum for 24 hr. Cell extracts and CAT assays were performed as described (Sambrook et al., 1989). Transfection efficiencies were normalized by β-galactosidase assays (Sambrook et al., 1989). CAT reactions were incubated for 2 hr at 37°C and separated on chromatography plates in one dimension. Reactions were visualized by autoradiography, or radioactivity was quantitated using an Ambios β-scope plate reader.

Protein Kinase Assays

HeLa cells were cotransfected with expression vectors encoding hemagglutinin epitope–tagged ERK2 or JNK1 together with tester plasmids. Protein kinase activity in cell extracts was measured by immune complex kinase assays using either MBP or glutathione S-transferase (GST)–cJun(1–79) as substrate as described previously (Minden et al., 1994a). Reactions were separated by SDS–polyacrylamide gel electrophoresis, and phosphorylation of substrates was quantitated by phosphorimager.

Xenopus oocyte extracts were prepared as described (Shibuya et al., 1992). Expression and purification of His_6–Ha-Ras proteins was as described (Shibuya et al., 1992). Extracts were incubated with 150 μg/ml of protein for 0–4 hr. Reactions were separated on a polyacrylamide gel, followed by Western blotting as described (Shibuya et al., 1992).

Acknowledgments

Correspondence should be addressed to M. H. W. We thank Anne Vojtek, Stevan Marcus, and Stanley Fields for supplying some of the plasmids and yeast strains used in this study. We thank Linda Rodgers and Michael Riggs for valuable technical assistance. A. M. was supported by a postdoctoral fellowship from the Tobacco-Related Disease Research Program. L. V. A. is supported by the Belgian National Fund for Scientific Research. M. H. W. is an American Cancer Society Research Professor. This study is supported by grants from the American Cancer Society and the National Cancer Institute.

Received October 17, 1994; revised December 9, 1994.

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