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Complex formation between RAS and RAF and other protein kinases

(signal transduction/cancer/protein–protein interactions/yeast)

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ABSTRACT **We used a *Saccharomyces cerevisiae* genetic system to detect the physical interaction of RAS and RAF oncoproteins. We also observed interaction between RAS and byr2, a protein kinase implicated as a mediator of the *Schizosaccharomyces pombe* ras1 protein. Interaction with RAS required only the N-terminal domains of RAF or byr2 and was disrupted by mutations in either the guanine nucleotide-binding or effector-loop domains of RAS. We observed interaction between MEK (a kinase that phosphorylates mitogen-activated protein kinases) and the catalytic domain of RAF. RAS and MEK also interacted but only when RAF was overexpressed.**

The *ras* genes encode guanine nucleotide-binding proteins and were first identified as oncogenes of acutely transforming RNA tumor viruses (reviewed in ref. 1). Subsequently, mutated *RAS* genes were found in many human tumors, providing the first evidence of a common genetic defect in cancer. RAS proteins participate in signal transduction pathways regulating cell proliferation and differentiation, but their precise biochemical functions are unknown. In mammals a variety of extracellular agonists, such as insulin, platelet-derived growth factor, and nerve growth factor, that act through protein-tyrosine kinase receptors require RAS to exert their effects (2–7). These agonists activate a set of protein-serine/threonine kinases known as mitogen-activated protein kinases (MAP kinases) reviewed in ref. 8. Activation of the MAP kinases by these factors requires RAS (5–7), and RAS can itself activate the MAP kinases in cells (5–7) and in complex cell-free systems (9, 10).

The *RAF* oncogene is a strong candidate to encode a downstream effector for RAS in mammalian cells. (*i*) Activated *RAF* can bypass the cellular requirement for RAS function (3, 4). (*ii*) Dominant negative mutations of *RAF* can block transformation induced by *RAS* (11). (*iii*) Hyperphosphorylated RAF kinase is observed in cells treated with agonists that activate RAS and in cells containing the activated *RAS* oncogene itself (7, 12). (*iv*) The RAF kinase can activate the MAP kinase kinase known as MEK (13, 14).

RAS proteins are widely conserved in eukaryotes. In many respects, the function of ras1, the *Schizosaccharomyces pombe* RAS homolog, resembles the function of RAS in vertebrates. Genetic evidence indicates that ras1 activates byr2, a protein kinase involved in sexual differentiation (15). Overexpression of byr2 can bypass defects resulting from the loss of ras1, and expression of the N-terminal putative regulatory domain of byr2 appears to interfere with ras1 signaling. Genetic studies place byr2 upstream of byr1, a homolog of mammalian MEK and *Saccharomyces cerevisiae* STE7 protein kinases, each of which is implicated in the activation of protein kinases of the MAP kinase family (16–18). byr2 is itself a homolog of *S. cerevisiae* STE11 (18), which is required for phosphorylation and activation of STE7 (19, 20). Thus, byr2 appears to bear the same relationship to byr1 as does RAF to MEK.

We have sought evidence for interaction between RAS and RAF and between RAS and byr2 by employing a genetic method, known as the two-hybrid system, that detects physical interaction between protein domains when expressed as fusion proteins in the yeast *S. cerevisiae* (21). Interacting fusion proteins combine to form a DNA-binding and transcriptional activation dimer that induces synthesis from a β-galactosidase reporter gene in an appropriate host. This approach has been tested and demonstrated for many pairs of interacting proteins, including *S. cerevisiae* SNF1 and SNF4, *S. cerevisiae* SNF1 and SIP1, mammalian Jun and Fos, and *S. cerevisiae* RAS2 and its guanine nucleotide-exchange protein, CDC25 (21–24).

MATERIAL AND METHODS

Yeast and *Escherichia coli* Strains, Media, and Genetic Manipulations. The *S. cerevisiae* strain used as host for β-galactosidase expression studies was YPB2 (*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$_{17\text{-mers}(3\times)}$–CYC1$_{TATA}$–lacZ*) and was kindly provided by P. Bartel and S. Fields (State University of New York, Stony Brook). Yeast cultures were grown in YPD (1% yeast extract/2% peptone/2% glucose) or in synthetic minimal medium (0.67% yeast nitrogen base/2% glucose with appropriate auxotrophic supplements). Standard genetic methods were followed (25).

Nucleic Acid Manipulations and Analysis. Manipulation and sequencing of DNA were carried out by standard procedures (26, 27). Polymerase chain reactions (PCRs) (28) were performed under standard conditions using primers 25–35 nucleotides in length. pMNCcraf (encoding wild-type RAF), pMNC301 (encoding the dominant negative RAF mutant [Trp^{375}]RAF) (11), and pMNCBXB (encoding the activated RAF mutant RAFΔN1) (29) were gifts of Ulf Rapp. Plasmid pAH-RAF was constructed by inserting a *Bam*HI–*Sac* I fragment isolated from pAD5RAF into the vector pRS423, a *HIS3*-based 2-μm vector (30). pAD5RAF was obtained by subcloning an *Xho* I–*Sac* I PCR-generated fragment containing the entire RAF sequence into pAD5 (18). pADH-HRAS is a *LEU2*$^+$ plasmid that expresses the human Ha-*ras* gene from the *ADH1* promoter (31). The MAP kinase kinase MEK was cloned from a rat embryonic cDNA library by PCR. Plasmid pGBT9, provided by P. Bartel and S. Fields, is a derivative of pMA424-2 (21) and contains an *ADH1* promoter expressing the GAL4 DNA-binding domain (amino acids

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Abbreviations: GAD, GAL4 activation domain; GBD, GAL4 DNA-binding domain; MAP kinase, mitogen-activated protein kinase; GAP, GTPase-activating protein.

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1–147) and the *TRP1* gene as a selectable marker. Plasmid pGBT10 is a derivative of pGBT9, with the modified multicloning site 5′-GAATTCGGATCCCATTTAAATGTCGAC-3′. Plasmid pGADGH, a derivative of pGAD1 (32), was provided by G. Hannon (Cold Spring Harbor Laboratory) and contains the *ADH1* promoter expressing the GAL4 transcriptional activation domain (amino acids 768–881). At the 3′ end of this domain, the multicloning site of pGAD1 was replaced by the *Spe* I–*Kpn* I fragment of the multicloning site of plasmid pBluescript SK(−) (Stratagene). All inserts cloned into these plasmids were obtained by PCR using appropriate primers and templates. The various RAS mutants were obtained by PCR or, in the case of [Ala^{35}]HRAS, by the Transformer mutagenesis kit (Clontech). The sequences of all PCR products were verified by dideoxy sequencing.

β-Galactosidase Filter Assays. The filter assay with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Boehringer Mannheim) as substrate was essentially as described (34) except that Whatman 50 paper was used instead of nitrocellulose paper.

RESULTS

To test interactions between two proteins, they were expressed in yeast, fused at their N termini to either the transcriptional activation domain (GAD) or the DNA-binding domain (GBD) of the yeast GAL4 protein. The various proteins tested in this way included the full length, point mutants, or domains of the human HRAS, *S. cerevisiae* RAS2, human RAF, rat MEK, and *Sch. pombe* byr2 proteins (Fig. 1). *S. cerevisiae* SNF1 and SNF4 fusion proteins were used as controls (21, 40). When jointly expressed as GAL4 fusion proteins, SNF1 and SNF4 induced β-galactosidase. When expressed singly or in combination with other unrelated fusion proteins, β-galactosidase was not induced.

Pairwise combinations of GBD and GAD protein fusions were tested for their ability to interact by induction of β-galactosidase activity when coexpressed in the appropriate *S. cerevisiae* host (Table 1). In one series of experiments, a pairwise combination was tested in a host that overexpressed a third interacting protein (Table 2). β-Galactosidase activity was assayed by the development of blue color in transformed colonies cultured in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, a chromogenic substrate. Results of a representative experiment are shown in Fig. 2.

Mammalian and *S. cerevisiae* RAS Proteins Interact with RAF. In the first series of experiments, we explored the interaction of human HRAS with full-length human RAF. Since the assay can detect interactions only when both proteins translocate to the cell nucleus, we utilized [Arg^{186}]HRAS, which has arginine in place of the cysteine residue of the CAAX motif. This motif, where A represents an aliphatic amino acid, is required for the proper plasma membrane localization of RAS (41, 42). Host cells expressing both RAF and [Arg^{186}]HRAS synthesized β-galactosidase when either one of the proteins was fused to GBD and the other to GAD (Table 1 and Fig. 2). Expression of both RAS and RAF fusion proteins was essential. For example, expression of RAF fused to GAD and SNF4 fused to GBD did not induce β-galactosidase synthesis. We did not observe interaction between RAF and the wild-type HRAS, presumably because the wild-type HRAS fusion protein failed to localize to the nucleus. RAF also interacted with the *S. cerevisiae* [Arg^{319}]RAS2 mutant protein, which lacks the CAAX motif.

We also examined the ability of other RAS mutants to interact with RAF. The substitution of alanine for glycine at position 22 of *S. cerevisiae* RAS2 disrupts the guanine nucleotide-binding domain (37, 38). The Gly^{22} → Ala mutation introduced into [Arg^{319}]RAS2 disrupts interaction with RAF. The Thr^{35} → Ala mutation introduced into [Arg^{186}]HRAS

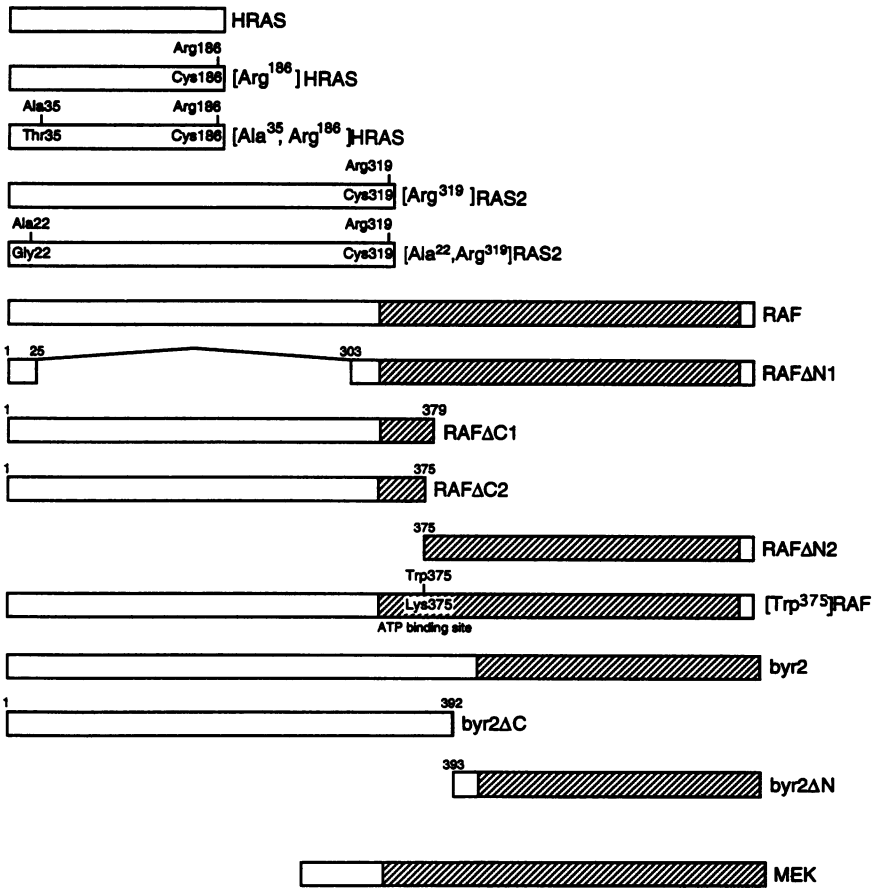

FIG. 1. Schematic representation of various proteins tested in the two-hybrid system. [Arg^{186}]HRAS and *S. cerevisiae* [Arg^{319}]RAS2 contain single amino acid substitutions that prevent their farnesylation and localization to the plasma membrane. [Ala^{35}]HRAS has a mutation in the so-called effector loop (35, 36). The Gly^{22} → Ala mutation disrupts the guanine nucleotide binding of RAS2 (37, 38). RAFΔN1 is a constitutively activated mutant of RAF, and lacks amino acids 26–302 (29). RAFΔN2 contains amino acids 375–648. RAFΔC1 and RAFΔC2 contain amino acids 1–379 and 1–375, respectively. [Trp^{375}]RAF contains a point mutation in the ATP-binding site of the kinase domain and is a dominant interfering form of RAF (11, 39). byr2ΔC and byr2ΔN contain amino acids 1–392 and 393–659, respectively. The hatched boxes represent the catalytic domains of the representative protein kinases.

Table 1. Pairwise combinations of GBD protein fusions (listed at left) and GAD protein fusions (listed across the top)

GBD fusions	β-Galactosidase induction, no. positive/no. scored							
	HRAS	[Arg^{186}]HRAS	[Ala^{35},Arg^{186}]HRAS	RAF	[Trp^{375}]RAF	MEK	byr2	SNF1
HRAS	0/32	—	—	0/32	—	—	—	0/32
[Arg^{186}]HRAS	—	0/48	—	56/56	—	0/56*	32/32	0/32
[Arg^{319}]RAS2	—	—	—	48/48	—	—	24/24	0/24
[Ala^{22},Arg^{319}]RAS2	—	—	—	0/48	—	—	0/24	0/24
RAF	0/24	96/96	0/48	0/48	—	160/160	0/48	0/32
RAFΔN1	—	0/48	—	—	—	96/96	—	0/12
RAFΔC1	—	72/72	—	—	—	0/96	—	0/12
RAFΔC2	—	48/48	—	—	—	0/48	—	0/12
[Trp^{375}]RAF	—	72/72	—	—	—	—	—	0/24
MEK	0/12	0/48*	—	56/56	0/48	—	0/36	0/24
byr2	—	16/16	—	—	—	—	—	0/8
byr2ΔC	—	16/16	—	—	—	—	—	—
byr2ΔN	—	0/24	—	—	—	—	—	—
SNF4	0/24	0/48	0/12	0/12	0/12	0/24	0/12	48/48

The values represent the number of independent transformed colonies that expressed detectable β-galactosidase activity, divided by the total number of independent transformants tested. —, Not tested. The proteins in the vertical column at left were fused to GBD. The proteins at the top were fused with GAD. In most cases, several independently derived plasmids expressing the fusion proteins were tested.
*MEK–RAS interactions were observed when RAF was overexpressed (see text and Table 2).

disrupts the so-called "effector loop" through which RAS is thought to interact with mammalian GTPase-activating protein (GAP) and the *S. cerevisiae* adenylyl cyclase complex (35, 36, 43). The [Ala^{35},Arg^{186}]HRAS mutant failed to interact with RAF.

RAS Interacts with the N Terminus of RAF. To define the region of RAF with which RAS interacts, we constructed fusions of either the C-terminal or N-terminal domains of RAF to GDB. We utilized two N-terminal deletions, RAFΔN1 and RAFΔN2, and two C-terminal deletions, RAFΔC1 and RAFΔC2. RAFΔN1 is a mutant of RAF that transforms cultured animal cells (29). We also utilized [Trp^{375}]RAF, in which the lysine of the ATP binding site of the full-length protein is replaced with tryptophan, to determine whether the catalytic activity of RAF was essential for interaction with HRAS. The [Trp^{375}]RAF protein is known to interfere with wild-type RAS and RAF in mammalian cells (11). [Arg^{186}]HRAS interacts with the N terminus of RAF and with [Trp^{375}]RAF. [Arg^{186}]HRAS did not interact with the C-terminal domain of RAF, yet that domain is capable of interacting with MEK (see next section).

MEK Interacts with the C Terminus of RAF and Forms Complexes with RAS in the Presence of RAF. RAF can activate the MEK protein kinase (13, 14). We therefore examined whether we could detect interaction between RAF and MEK. The MEK gene was fused at its N terminus to either GBD or GAD and then coexpressed with all the RAF constructs depicted in Fig. 1. The results indicated that MEK interacted with wild-type RAF and the RAF C-terminal catalytic domain. MEK did not interact with the N-terminal domain of RAF nor with [Trp^{375}]RAF (Table 1). The interaction between RAF and MEK was weaker than that between RAF and RAS, as judged by the intensity of color development.

Table 2. Interaction between RAS and MEK mediated by RAF

GDB–[Arg^{186}]HRAS	GAD–MEK	RAF	β-Galactosidase induction
+	+	+	16/16
+	+	−	0/16
+	−	+	0/8
−	+	+	0/8
−	−	+	0/8

Host strains were transformed (+) or not (−) with plasmids expressing the GBD–[Arg^{186}]HRAS fusion, the GAD–MEK fusion, or the full-length wild-type RAF protein (pAH-RAF). Individual transformants were scored for β-galactosidase production (no. positive/no. scored) by the filter assay described in *Materials and Methods*.

We did not observe direct interaction between MEK and RAS fusion proteins. However, MEK and RAS interact with different domains of RAF, and we reasoned that if the binding of RAS to RAF did not interfere with the binding of MEK to RAF, the three might form a complex. To test this, we expressed combinations of [Arg^{186}]HRAS and MEK fusion proteins with or without wild-type RAF. Interaction between [Arg^{186}]HRAS and MEK was detected only when RAF was also expressed (Table 2). This interaction was weaker than that observed between RAF and MEK fusion proteins.

***Sch. pombe* byr2 Interacts with RAS.** As we have discussed, byr2 bears a similar relationship to byr1 and RAS as does RAF to MEK and RAS. We therefore examined the ability of byr2 to interact with [Arg^{186}]HRAS. The results indicated that byr2 interacted with [Arg^{186}]HRAS expressed in *S. cerevisiae* (Table 1). As in the case of RAF, the N-terminal putative regulatory domain of byr2 interacted with HRAS. byr2 did not interact with either RAF or MEK.

DISCUSSION

We have found evidence that RAS proteins form complexes with certain protein kinases. Our conclusions depend entirely upon the use of the two-hybrid system. Nevertheless, the interactions we have observed are highly specific. We failed to observe interactions between RAF and RAS proteins with mutations that abolish RAS activity. Moreover, we have tested interactions between 14 pairwise combinations of known fusion proteins and RAF or [Arg^{186}]HRAS and have observed no false positives (data not shown). We have also screened cDNA fusion libraries for genes encoding protein domains capable of interacting with either [Arg^{186}]HRAS or RAF and have found positives at a frequency of <1 in 50,000 for RAF and <1 in 100,000 for [Arg^{186}]HRAS. Even allowing for frameshift and other cloning artifacts, the occurrence of false positives with our RAS and RAF fusion proteins must be quite rare.

Our results strengthen the hypothesis that RAF is a downstream effector of RAS. More significantly, our results suggest that RAS and RAF may interact directly. We cannot conclude this with complete confidence because there may be other factors within the yeast cell that bridge this interaction. A more definitive proof would be the demonstration of the coimmunoprecipitation of RAF and HRAS using purified

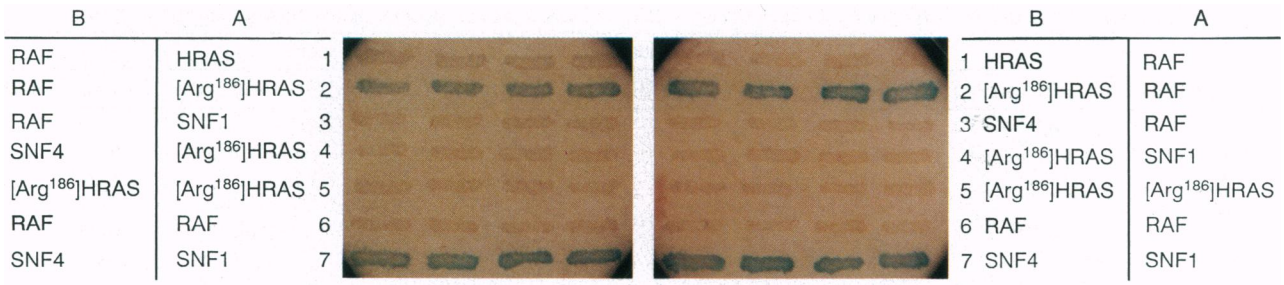

FIG. 2. RAF interacts with unprocessed HRAS. HRAS and RAF fused to either GBD (column B) or GAD (column A) were tested for possible interaction by their induction of β-galactoside expression (blue color). In contrast to wild-type HRAS (row 1), the mutant [Arg^{186}]HRAS (row 2) fused to either GAD (*Left*) or GBD (*Right*) was able to interact with RAF. Rows 3 and 4 represent the corresponding negative controls: no color developed unless both [Arg^{186}]HRAS and RAF were expressed. [Arg^{186}]HRAS (and also RAF) failed to interact with itself (rows 5 and 6). *S. cerevisiae* SNF1 and SNF4 were included as positive controls (row 7). Each patch represents an independent transformant.

components, but we have not as yet observed coprecipitation from cells overexpressing both proteins. In fact, stable physical interactions between RAS and its known partners are exceedingly difficult to demonstrate. We have never observed coprecipitation between RAS and adenylyl cyclase, its effector in *S. cerevisiae* (44, 45), or between RAS and GAPs, proteins that accelerate GTP hydrolysis by RAS (46). Stable interaction between RAS and CDC25 can be demonstrated only with mutant RAS (47, 48). Similarly, crosslinking experiments have been singularly ineffective (49). Thus, the two-hybrid system may be particularly well suited for monitoring weak protein–protein interactions of biological significance.

We have observed an interaction between human HRAS and the *Sch. pombe* byr2 kinase. This interaction requires only the N-terminal domain of byr2. The byr2 kinase resembles mammalian RAF in its overall structure: both kinases are large, with C-terminal catalytic domains. However, we observe essentially no primary structural homology in the N-terminal putative regulatory domains of the two proteins. In particular, byr2 does not contain a cysteine-rich domain similar to the domain of RAF thought to participate in its regulation (29). Nevertheless, our results confirm that the relationship of byr2 to the signal transduction pathways of *Sch. pombe* resembles the relationship of RAF to the signal transduction pathways of vertebrates.

If byr2 is an immediate downstream effector of ras1 in *Sch. pombe*, then *Sch. pombe* ras1 is likely to have another effector, because ras1 is required to maintain normal cell morphology in *Sch. pombe*, whereas byr2 is not (15). Thus, in *Sch. pombe*, as in *S. cerevisiae* (50), there may be multiple RAS effectors. We presume this to be true for metazoans as well. It is possible, for example, that metazoans have homologs of byr2 that participate in RAS signal transduction pathways. Also, RAF is only one member of a family of related protein kinases (reviewed in ref. 51), and members of this family may also be RAS effectors or effectors of other members of the RAS superfamily.

We have confirmed an interaction between RAF and MEK. This interaction requires the intact catalytic domain of RAF but does not require its N-terminal domain. We presume that the interaction between RAF and MEK depends upon the catalytic activity of RAF, since the Lys^{375} → Trp mutation of the putative ATP-binding site disrupts interaction. The interaction of MEK requires the C terminus of RAF, and the interaction of RAS requires the N terminus of RAF. Thus, a trimeric complex may form. Indeed, we observed an interaction between RAS and MEK fusion proteins in yeast, but only when we also coexpressed RAF protein. We conclude that RAF can bridge the interaction of RAS and MEK. Thus the association of RAF with RAS may also directly affect MEK (and even MAP kinase, through its association with MEK) with respect to localization, activation, and access to substrates.

We do not know whether the interaction between RAS and RAF depends upon RAS being in its GTP-bound state. However, we failed to observe interaction when RAS had mutations in its GDP/GTP-binding domain. Moreover, we failed to observe interactions when RAS had mutations in its effector loop. The particular mutation, Thr^{35} → Ala, that disrupts interaction with RAF also disrupts functional interactions between HRAS and other known targets, including mammalian GAP and the *S. cerevisiae* adenylyl cyclase complex (35, 43). These results suggest that RAF forms a complex with RAS when the latter is in its physiologically active state.

Mammalian RAF is capable of interaction with *S. cerevisiae* RAS2. On the one hand this is somewhat surprising, since the two molecules have been separated in evolution at least since the development of metazoans. On the other hand, mammalian HRAS is known to interact with the RAS2 targets in *S. cerevisiae* (44, 52). Moreover, yeast RAS1, when suitably modified at its C terminus, is capable of transforming mammalian cells (53). Thus, evolutionary drift in the RAS pathway seems to result more from the presence or absence of specific targets than from a gradual reshaping of the functional interactions themselves.

We have shown that RAS interacts with RAF and with byr2. It is known that RAS interacts directly with mammalian GAP and with a variety of proteins that are members of the GAP^{RAS} family, such as NF1, IRA1, IRA2, and sar1 (46, 54). These proteins all down-regulate RAS function by accelerating GTP hydrolysis. We do not know whether the GAP^{RAS} proteins bind to the same site on RAS as do byr2 and RAF. However, the mutation at position 35 of HRAS seems to disrupt interactions with both the protein kinases and the GAP^{RAS} proteins. It should not be surprising to find that molecules that down-regulate RAS by accelerating its GTPase activity, on the one hand, and effector molecules, on the other, each bind to the same site on RAS, since all must recognize RAS in its GTP-bound state.

The members of the GAP^{RAS} family have detectable but weak homology to each other, and it is difficult to define a clear region of homology that is responsible for RAS interaction (54, 55). An even more difficult problem arises from analysis of the primary sequences of byr2 and RAF. We observe no homology between byr2 and RAF, outside of their kinase domains, and no homology to the GAP^{RAS} proteins. Moreover, none of these proteins contain significant homology to *S. cerevisiae* CAP or adenylyl cyclase, two potential protein targets of RAS in that organism (33). By using the two-hybrid system it should be straightforward to define the RAS-interacting domains of RAF and byr2, and of GAP as well, by mutagenesis. At that time it might become clearer

whether a RAS-binding protein can be identified by its primary sequence.

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