The EMBO Journal vol.8 no.10 pp.3039 - 3044, 1989

Mutants of H-*ras* that interfere with *RAS* effector function in *Saccharomyces cerevisiae*

T.Michaeli, J.Field, R.Ballester, K.O'Neill and M.Wigler

Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, NY 11724, USA

Communicated by D.Gallwitz

We report a class of interfering mutants of the human H-*ras* gene capable of inhibiting phenotypes arising from the expression of the activated *RAS2* gene, $RAS2^{val19}$, in the yeast *Saccharomyces cerevisiae*. All these mutants encode unprocessed H-*ras* proteins that remain in the cytoplasm. One of the mutants, H-ras^{arg186}, was examined in detail. H-ras^{arg186} protein is a competitive inhibitor of $RAS2^{val19}$ protein. It does not interfere with processing and membrane localization of $RAS2^{val19}$, nor does it appear to compete with *RAS* protein for its proposed regulator, the *CDC25* protein. By several criteria the $RAS2^{val19}$ adenylate cyclase interaction is unaffected by H-ras^{arg186}. We infer from our results that H-ras^{arg186} protein interferes with an alternative function of $RAS2^{val19}$.

Key words: H-*ras*/interfering mutants/*RAS* genes

Introduction

The *RAS* genes are highly conserved in evolution (see Barbacid, 1987, for review). They encode guanine nucleotide binding proteins with weak GTPase activity, and are localized to the inner surface of the plasma membrane. One branch of the *RAS* family in mammals has oncogenic potential, and simple point mutations in either the H-, K- or N-*ras* genes are prevalent in many types of human tumors (Barbacid, 1987). These mutations activate the oncogenic potential of the *RAS* proteins, and, at least in some cases, probably do so by decreasing GTP hydrolysis rates. The function of *RAS* protein in mammals is unknown, although they are thought to be involved in signal transduction pathways.

The yeast *Saccharomyces cerevisiae* contains two genes, *RAS1* and *RAS2*, which are structurally and functionally related to their mammalian counterparts (DeFeo-Jones *et al.*, 1983, 1985; Powers *et al.*, 1984; Kataoka *et al.*, 1985). In yeast, the *RAS* products are primarily required for the activation of adenylate cyclase (Broek *et al.*, 1985; Toda *et al.*, 1985). *RAS* proteins appear to interact directly with an adenylate cyclase complex (Field *et al.*, 1988). More recently, genetic experiments suggest that yeast *RAS* proteins have functions besides the activation of adenylate cyclase (Toda *et al.*, 1987; Wigler *et al.*, 1988; T.Toda and S.Powers, unpublished observations). The mammalian H-*ras* protein is capable of performing all the essential *RAS* functions when expressed in yeasts that lack their own endogenous *RAS* genes (DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985; Wigler *et al.*, 1988).

Like the mammalian *RAS*, the yeast *RAS2* gene can be activated by a simple point mutation, and like the mammalian mutant H-ras^{val12}, the RAS^{val19} gene has a dominant phenotype (Kataoka *et al.*, 1985; Toda *et al.*, 1985). Cells carrying the RAS^{val19} mutation are exquisitely sensitive to heat shock and nitrogen starvation, and fail to accumulate storage carbohydrates (Toda *et al.*, 1985; Sass *et al.*, 1986). The most useful of these defects, for the purpose of genetic screens of *RAS* function, is the heat shock sensitivity induced by RAS^{val19}. The $RAS2^{val19}$ phenotypes are generally ascribed to the activation of the cyclic AMP-dependent protein kinases, since activation of these kinases produces a similar set of phenotypes (Kataoka *et al.*, 1984; Matsumoto *et al.*, 1985; Toda *et al.*, 1985; Cannon and Tatchell, 1987; Marshall *et al.*, 1987; Nikawa *et al.*, 1987a), and since overexpression of cyclic AMP phosphodiesterases can block the $RAS2^{val19}$ phenotype (Sass *et al.*, 1986; Nikawa *et al.*, 1987a).

Wild-type yeast *RAS* proteins require the product of the *CDC25* gene in order to function (Camonis *et al.*, 1986; Broek *et al.*, 1987; Robinson *et al.*, 1987). The *CDC25* product probably catalyzes nucleotide exchange on *RAS* proteins (Broek *et al.*, 1987). Cells containing $RAS2^{val19}$, however, do not require the *CDC25* gene product, presumably because $RAS2^{val19}$ protein has a reduced rate of GTP hydrolysis (Broek *et al.*, 1987; Robinson *et al.*, 1987). Recently, we discovered dominant temperature-sensitive mutants of *RAS2* that appear to interfere with *CDC25* function (Powers *et al.*, 1989). These mutants encode proteins that are altered in a consensus sequence for nucleotide binding, and such mutants have been useful in inferring the functional interaction between *CDC25* and *RAS* proteins. Similar interfering H-*ras* mutants, altered in sequences involved in nucleotide binding, can contribute to the understanding of the elements controlling *RAS* function in mammals (Sigal *et al.*, 1986; Feig and Cooper, 1988).

From the existence of *RAS* mutants that interfere with upstream *RAS* controlling elements such as *CDC25*, we have inferred that there might exist *RAS* mutants that can interfere with the activation of the targets of the *RAS* proteins. We reasoned that such mutants would interfere with the penetrance of the $RAS2^{val19}$ phenotype, and we therefore designed a genetic screen to look for such mutants. We chose to mutate randomly the human H-*ras* gene *in vitro*, and screen pools of mutagenized genes for the ability to render cells containing $RAS2^{val19}$ resistant to heat shock.

Results

An H-ras mutant with interfering properties is isolated

To isolate interfering H-*ras* mutants we used a high copy yeast plasmid that expressed the H-*ras* gene under the control of the strong *S.cerevisiae* alcohol dehydrogenase 1 (*ADH1*)

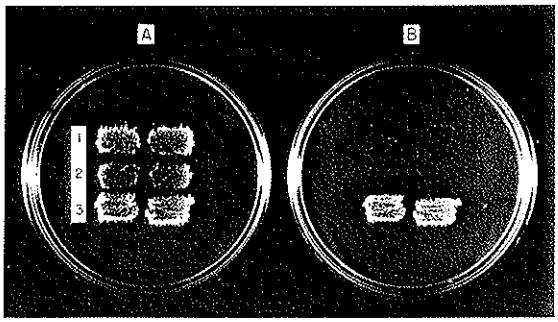

Fig. 1. Isolation of an H-*ras* mutant that suppresses the heat shock sensitive phenotype of *RAS2*^{val19}. Heat shock sensitive *RAS2*^{val19} cells (strain TK161-R2V) were transformed with the following plasmids: (1) pAL1, containing the *LEU2* gene; (2) pADH-H-*ras*, a plasmid derived from pAL1 carrying the H-*ras* cDNA under the control of the *ADH1* promoter; and (3) p*RVS*, a mutated pADH-H-*ras* plasmid encoding H-*ras*^{arg186}. Two independent transformants were patched onto SC-leu plates, incubated at 30°C for 4 days, then replica plated to SC-leu plates and heat shocked (at 55°C) for (A) 0 and (B) 20 min. Following the heat shock plates were incubated at 30°C for 2 days.

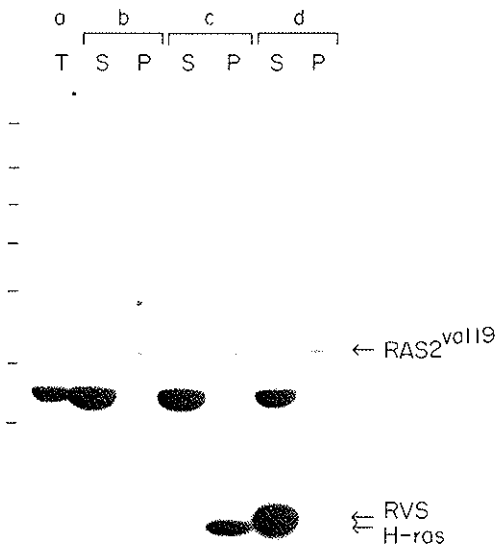

Fig. 2. Subcellular localization of H-*ras*^{arg186} and *RAS2*^{val19}. Cytoplasmic and membrane extracts were prepared from *RAS2*^{val19} cells (strain TK161-R2V) carrying the plasmids (b) pAL1, (c) pADH-H-*ras* and (d) p*RVS* (see legend to Figure 1 for plasmid description). Control extracts from *ras1*$^-$*ras2*$^-$ cells (strain ST100) were included (a). Cytoplasmic and membrane fractions were separated by high-speed centrifugation. Extracts were analyzed by Western blotting using a mixture of anti *RAS2* and anti H-*ras* antisera. *RAS2*^{val19} and H-*ras* proteins were identified by probing blots with each of the individual antisera (data not shown). Anti *RAS2* antiserum also reacts with an abundant soluble protein (of >30 kd) which is found in all strains (see Materials and methods for details). S and P indicate soluble and insoluble fractions respectively and T indicates total extract. The migration of prestained mol. wt markers (from the top: 180, 116, 84, 58, 48.5, 36.5 and 26.6 kd) is depicted on the left-hand side of the figure.

promoter (Powers *et al.*, 1986). The plasmid, pADH-H-*ras*, also contained the *LEU2* gene as a selectable marker. We then created a library of mutagenized H-*ras* expression vectors by propagating the plasmid pADH-H-*ras* in a highly mutagenic strain of *Escherichia coli*. We screened the randomly mutated H-*ras* plasmid population for plasmids that can suppress the heat shock sensitivity induced by *RAS2*^{val19}. Pools of the mutagenized library were transformed into the strain TK161-R2V, which contains the activated *RAS2*^{val19} allele (Toda *et al.*, 1985), and transformants were selected for leucine prototrophy. Six thousand Leu$^+$ transformant colonies were heat shocked and some of the viable survivors were analyzed further. To verify that the resistance to heat shock was due to a mutated plasmid carried by the surviving transformants, the plasmid was segregated from initial survivors by propagating these survivors in non-selective medium. Plasmids were recovered by transforming *E.coli* with DNA from those survivors that appeared dependent upon the H-*ras* expression plasmid for continued resistance to heat shock. These plasmids were then tested for their ability to confer heat shock resistance to TK161-R2V upon transformation. As a result of this screen, we recovered one plasmid capable of efficiently suppressing *RAS2*^{val19}. This plasmid carried a mutant H-*ras*, called *RVS* (*RAS*-valine-*s*uppressor), that was capable of conferring heat shock resistance to TK161-R2V (Figure 1). Other *RAS2*^{val19}-induced phenotypes, such as loss of viability upon starvation and failure to sporulate, were also suppressed by *RVS* (data not shown).

Sequence analysis established that *RVS* contained a single point mutation at codon 186, which normally encodes cysteine. The mutation, TGT to CGT, results in arginine being encoded by *RVS* at position 186. Cysteine 186 is a highly conserved residue among *RAS* proteins and is a part of the C-terminal Cys-A-A-X sequence, where A is an aliphatic amino acid, and X is the C-terminal amino acid (Taparowsky *et al.*, 1983). The Cys-A-A-X sequence is required for the fatty acid acylation that is at least partly responsible for membrane localization of eukaryotic *RAS* proteins (Sefton *et al.*, 1982; Willumsen *et al.*, 1984a,b; Powers *et al.*, 1986; Deschenes and Broach, 1987). We therefore reasoned that, like other C-terminal cysteine mutants, H-*ras*^{arg186} probably encodes an H-*ras* protein that fails to become processed and remains located in the cytoplasm. This was shown as described below.

H-ras^{arg186} *is a cytoplasmic protein that does not interfere with the membrane localization of RAS2*^{val19}

Unprocessed *RAS2*^{val19} is unable to induce its usual phenotypes, including heat shock sensitivity (Powers *et al.*, 1986; Deschenes and Broach, 1987). It is therefore possible that H-*ras*^{arg186} actually inhibits processing and membrane localization of *RAS2*^{val19} and thereby suppresses *RAS2*^{val19} phenotypes. To explore this possibility the cellular localization of *RAS2*^{val19} in the presence of H-*ras*^{arg186} was examined (Figure 2). Cell extracts from *RAS2*^{val19} strains expressing either H-*ras*, H-*ras*^{arg186} or just the selective marker were fractionated into a soluble (or cytoplasmic) fraction and into an insoluble (or membrane) fraction. Western blot analysis with anti H-*ras* and anti *RAS2* polyclonal antisera confirmed that H-*ras*^{arg186} indeed encodes a cytoplasmic protein (lane d). Substantially larger quantities of H-*ras*^{arg186} protein than H-*ras* protein accumulated in cells containing similar expression plasmids, suggesting that the H-*ras*^{arg186} protein is relatively more stable in yeast than is the H-*ras* wild-type protein. Overexpression of either H-*ras* or H-*ras*^{arg186} did not affect the localization of the *RAS2*^{val19} protein. *RAS2*^{val19} protein was found in the membrane fraction in all the strains examined,

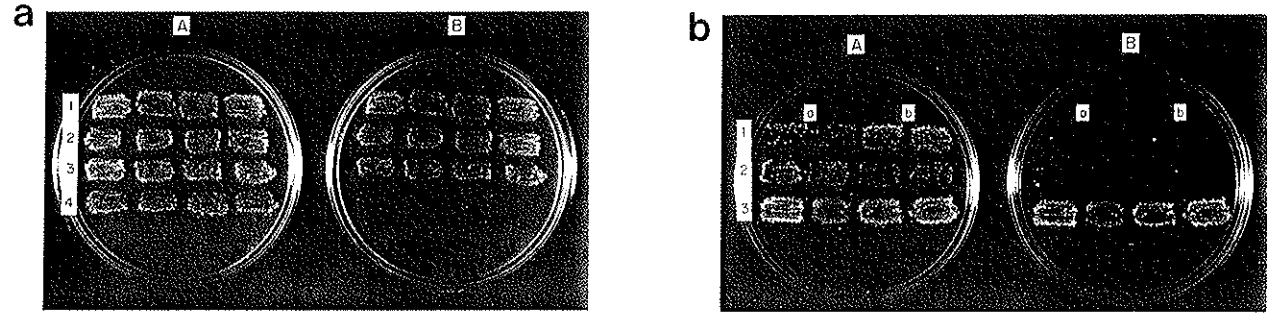

Fig. 3. Sensitivity to heat shock of *RAS2*^{val19} cells containing H-*ras*^{arg186} and overexpressing *CDC25*, *RAS2*, *RAS2*^{val19} or adenylate cyclase genes. (a) *RAS2*^{val19} cells (strain TK161-R2V) containing pURVS, a plasmid carrying the *URA3* gene and H-*ras*^{arg186} under the control of the *ADH1* promoter, were transformed with the following additional plasmids: (1) pAL1, containing the *LEU2* marker; (2) p*ALCDC25*, containing the *LEU2* gene and the *CDC25* gene; (3) p*RAS2*-1, carrying the *LEU2* gene and wild-type *RAS2* gene; and (4) p*RAS2*^{val19}, carrying the *LEU2* gene and the activated *RAS2*^{val19} allele. Four independent transformants were patched onto SC-ura-leu plates and, following 4 days growth at 30°C, were replica plated and heat shocked at 55°C for (**A**) 0 and (**B**) 10 min. (**b**) TK161-R2V cells containing (1) pAL1; (2) pADH-H-*ras* or (3) p*RVS* (see legend to Figure 1 for plasmid description) were transformed with either (**a**) p*TRP*, carrying the *TRP1* gene, or (**b**) pADH-CYR, carrying *TRP1* and the adenylate cyclase gene under the control of the *ADH1* promoter. Two independent transformants were patched onto SC-leu-trp plates and, following 3 days growth at 30°C, were replica plated and heat shocked at 55°C for (**A**) 0 and (**B**) 10 min.

Table II. Adenylate cyclase activity in membranes of *RAS2*^{val19} cells carrying various H-*ras* alleles

Extra-chromosomal plasmid[a]	Experiment[b]	Assay conditions[c]			
		Mn^{2+}	Mg^{2+}	Mg^{2+} + Gpp(NH)p	Mn^{2+}/Mg^{2+}
pAL1	1	58	21.8	23.1	2.7
pADH-H-*ras*	1	81	35.9	38.4	2.2
p*RVS*	1	60	25	27.9	2.4
p*RVS*	2	66	28.3	29.7	2.3
pAL1	3	39	14.2	16.9	2.7
pADH-H-*ras*	3	70	69.0	58.5	1.0
p*RVS*	3	75	34.0	37.4	2.2

[a]The indicated plasmids were introduced into TK161-R2V, a strain containing the activated *RAS2*^{val19} allele. pAL1 carries the *LEU2* gene as a selective marker; pADH-H-*ras* and p*RVS* contain in addition to the *LEU2* gene the H-*ras* and H-*ras*^{arg186} coding regions respectively transcribed from the *ADH1* promoter.
[b]Each experiment was performed on an independent preparation of membranes, assayed in duplicate.
[c]Membranes from the indicated strains were prepared and adenylate cyclase was assayed either in the presence of 2.5 mM Mn^{2+}, 2.5 mM Mg^{2+} or 2.5 mM Mg^{2+} in the presence of 50 μM Gpp(NH)p (see Materials and methods). Gpp(NH)p [guanosine 5′(β,γ-imino) triphosphate] is a non-hydrolyzable analog of GTP. Adenylate cyclase activity is expressed in units of pmol cAMP generated per mg of membrane protein per min. The values indicated are the averages of duplicate samples that deviated < 10% from the average.

of either H-*ras*, H-*ras*^{arg186} or a plasmid carrying only a selective marker. Thus, the interaction between *RAS2*^{val19} protein and adenylate cyclase is not grossly altered by the presence of H-*ras*^{arg186} protein.

Discussion

We have demonstrated the existence of mutant forms of the human H-*ras* gene that interfere with the expression of the phenotype of *RAS2*^{val19} when co-expressed in the yeast, *S.cerevisiae*. The interfering forms of H-*ras*, such as H-*ras*^{arg186}, are mutated in sequences encoding the Cys-A-A-X consensus sequence that is required for proper *RAS* protein processing (Willumsen *et al.*, 1984a; Deschenes and Broach, 1987). Since these interfering H-*ras* mutants do not block the phenotype of *RAS2*^{val19} when *RAS2*^{val19} is overexpressed, we can conclude that the interfering forms of H-*ras* compete for interaction with a yeast protein (or proteins) that normally interacts with the *RAS2*^{val19} protein. In principle, the H-*ras* mutants could interfere with *RAS2*^{val19} function in any of three major ways, as discussed below.

Table III. Intracellular cyclic AMP levels in *RAS2*^{val19} cells carrying various H-*ras* alleles

Extra-chromosomal plasmid[a]	Cyclic AMP level[b] (pmol/mg protein) in experiment				
	1	2	3	4	5
pAL1	3.2	2.6	2.3	1.0	1.4
pADH-H-*ras*	3.2	2.4	2.4	1.1	1.0
p*RVS*	3.5	2.8	2.6	1.3	2.3

[a]See legend to Table II for plasmid descriptions. Plasmids were introduced into TK161-R2V, a *RAS2*^{val19} strain, as described in Table II.
[b]Cyclic AMP levels were determined by a radioimmunoassay as described in Materials and methods. Each experiment was performed on an independent cyclic AMP preparation that was assayed in duplicate. The average value of duplicates is presented.

First, mutant H-*ras* proteins could block the processing of yeast *RAS* proteins. The mutant H-*ras*^{arg186} protein indeed remains in the cytosolic fraction of the cell, and is not processed. However, we have demonstrated directly that cells which express H-*ras*^{arg186} appear to have normal

Table I. The effect of expression of various H-*ras* mutants upon the heat shock sensitivity of TK161-R2V

Mutant[a]	C'-Terminal sequence	Heat shock phenotype[b] in TK161-R2V
H-*ras*	^{185}Lys-Cys-Val-Leu-Ser^{189}	S
H-*ras*^{arg186}	Lys-Arg-Val-Leu-Ser	R
H-*ras*^{ser186}	Lys-Ser-Val-Leu-Ser	R
H-*ras*^{ter186}	Lys	R
H-*ras*^{ter187}	Lys-Cys	R

[a]'ter' designates a termination codon.
[b]The indicated mutant H-*ras* genes were introduced into TK161-R2V, a heat shock sensitive strain which contains the activated *RAS2*^{val19} allele. Transformants were heat shocked and their recovery scored. 'R' indicates transformants are resistant, or 'S' sensitive to heat shock.

and at equivalent levels. Since processing is required for membrane localization, we conclude that the *RAS2*^{val19} protein is processed properly. Thus, H-*ras*^{arg186} protein does not deplete the cell of factors required for the processing of *RAS2* proteins.

The capacity of H-*ras*^{arg186} to interfere with *RAS2*^{val19} may depend only on the unprocessed state of its product. To test this hypothesis, three point mutants of H-*ras* were generated by site-directed mutagenesis: H-*ras*^{ser186}, H-*ras*^{ter186} and H-*ras*^{ter187}, where 'ter' designates a termination codon. All three mutants were as efficient as H-*ras*^{arg186} at suppressing the *RAS2*^{val19}-induced heat shock sensitivity when they were overexpressed using the *ADH1* promoter (Table I). Thus, any mutation that destroys the C-terminal Cys-A-A-X consensus sequence appears sufficient to create an H-*ras* capable of inhibiting *RAS2*^{val19}. Since the H-*ras*^{ter186} protein lacks all four of the C-terminal amino acids most likely required for recognition by processing enzymes, yet possesses interfering properties, we conclude that the ability of these mutant H-*ras* genes to interfere with *RAS2*^{val19} results from their residence in the cytoplasm rather than from any interference in the processing of *RAS2*^{val19}.

Overexpression of RAS2^{val19}, but not of adenylate cyclase or CDC25, can overcome H-ras^{arg186} effects

To gain insight into the mechanism by which H-*ras*^{arg186} protein inhibits *RAS2*^{val19} protein we tested whether H-*ras*^{arg186} depletes known factors that are essential for *RAS2*^{val19} function. To assess whether H-*ras*^{arg186} protein actually competes with *RAS2*^{val19} protein, we increased the relative abundance of *RAS2*^{val19} protein by introducing a high copy plasmid carrying *RAS2*^{val19} into cells expressing H-*ras*^{arg186}. Overexpression of *RAS2*^{val19} protein, estimated on a Western blot to be ~10-fold (data not shown), did not decrease the amount of H-*ras*^{arg186} produced (data not shown) but did render cells sensitive to heat shock (Figure 3a). Thus, H-*ras*^{arg186} protein appears to be a competitive inhibitor of *RAS2*^{val19} protein.

To examine further the competition between H-*ras*^{arg186} and *RAS2*^{val19} protein, we overexpressed gene products that are thought to affect *RAS* or be affected by it. The *CDC25* protein is required to activate *RAS*, and recent genetic studies suggest that the protein interacts with *RAS* proteins (Powers *et al.*, 1989). Introduction of the *CDC25* gene, under the control of the *ADH1* promoter, did not overcome the effects of H-*ras*^{arg186}, and such cells remained resistant to heat shock (Figure 3a). Furthermore, H-*ras*^{arg186} was able to block the *RAS2*^{val19} phenotype in cells in which the *CDC25* gene has been disrupted (data not shown; Broek *et al.*, 1987). Thus, H-*ras*^{arg186} protein cannot be competing with *RAS2*^{val19} protein for binding to *CDC25* protein.

Adenylate cyclase is the one known effector of *RAS* in yeast, and an increasing number of observations indicate that the two proteins are likely to interact directly with each other (Field *et al.*, 1988). To test if elevated expression of adenylate cyclase would restore *RAS2*^{val19}-induced heat shock sensitivity, we introduced into cells a high copy plasmid in which adenylate cyclase gene transcription is driven from the strong *ADH1* promoter. As we previously observed (Field *et al.*, 1988), cells containing this plasmid show a 10- to 20-fold increase in adenylate cyclase catalytic activity as assayed *in vitro* in the presence of Mn^{2+} ions (data not shown). Surprisingly, such cells are still resistant to heat shock (Figure 3b). These observations suggest that while H-*ras*^{arg186} protein does not compete with *RAS2*^{val19} protein, it does not compete for interaction with adenylate cyclase.

H-ras^{arg186} protein does not appear to affect the interaction of RAS2^{val19} protein with adenylate cyclase

The above results are suggestive of the surprising conclusion that H-*ras*^{arg186} does not interfere with *RAS2*^{val19} interaction with adenylate cyclase. To examine this idea further, we tested whether *RAS2*^{val19} protein from strains carrying H-*ras*^{arg186} is capable of stimulating adenylate cyclase. For this purpose membranes from *RAS2*^{val19} containing cells that expressed either H-*ras*, H-*ras*^{arg186} or just the selective marker were prepared and their adenylate cyclase activity assayed (Table II). In the presence of Mn^{2+}, which activates adenylate cyclase independently of *RAS* protein and guanine nucleotides (Broek *et al.*, 1985; Toda *et al.*, 1985), adenylate cyclase activities were comparable whether H-*ras*, H-*ras*^{arg186} or no additional *RAS* was expressed. In *RAS2*^{val19} strains, the *RAS*-dependent adenylate cyclase activity, assayed in the presence of Mg^{2+}, is higher than seen in normal strains and cannot be stimulated further by the presence of non-hydrolyzable guanine nucleotides (Broek *et al.*, 1985; Toda *et al.*, 1985). The *RAS2*^{val19} profile of adenylate cyclase activity was evident even in the presence of H-*ras* or H-*ras*^{arg186} proteins. All three strains assayed exhibited comparably high levels of Mg^{2+}-dependent adenylate cyclase activity which could not be stimulated further by the addition of Gpp(NH)p, a GTP analog. As membrane preparations contain only trace amounts of H-*ras*^{arg186} protein, this profile most likely reflects the activity of the *RAS2*^{val19} protein. Thus, *RAS2*^{val19} protein is apparently functional in membranes prepared from *RAS2*^{val19} H-*ras*^{arg186} cells, confirming that H-*ras*^{arg186} does not interfere with an essential processing step of *RAS2*^{val19} protein, and supporting the idea that H-*ras*^{arg186} protein does not interfere with *RAS2*^{val19} protein interaction with adenylate cyclase.

To test more directly whether H-*ras*^{arg186} protein interferes with *RAS2*^{val19} protein interaction with adenylate cyclase, we measured the amounts of cyclic AMP found in *RAS2*^{val19} cells in the presence and absence of H-*ras*^{arg186} protein. As can be seen in Table III, comparable levels of cyclic AMP accumulated in *RAS2*^{val19} cells in the presence

amounts of *RAS2*^{val19} protein in their membrane and this protein appears to be functional. Moreover, mutant H-*ras* proteins which lack entirely the Cys-A-A-X consensus sequence, and therefore would not be expected to compete for processing enzymes, nevertheless do interfere with *RAS2*^{val19}. Thus, interference appears to result from the cytoplasmic location of H-*ras*, but not from interference with the processing of *RAS2*^{val19}.

Second, the effects of interfering H-*ras* mutants could be explained if such mutants impaired interaction with upstream elements that control *RAS* protein function. This possibility seems unlikely. First, our biochemical analysis suggests that *RAS2*^{val19} protein still interacts normally with adenylate cyclase in the presence of H-*ras*^{arg186} protein. Second, the only upstream element currently known to affect *RAS2* is encoded by *CDC25*. However, the mutationally activated *RAS2*^{val19} protein does not require the *CDC25* product to exert its phenotype and H-*ras*^{arg186} protein can block the *RAS2*^{val19}-induced heat shock sensitivity even in the absence of the *CDC25* gene (Broek *et al.*, 1987; Robinson *et al.*, 1987). In addition, high-copy plasmids carrying the *CDC25* gene do not reverse the blockade of the *RAS2*^{val19} phenotype that results from expression of H-*ras*^{arg186}.

There remains the possibility that H-*ras*^{arg186} could block *RAS2*^{val19} function by competing for one of its effectors. The one known effector for *RAS* is adenylate cyclase (Toda *et al.*, 1985). However, three lines of evidence suggest that this effector function is not impaired by H-*ras*^{arg186}. First, the adenylate cyclase activity in membranes from cells containing *RAS2*^{val19} and expressing H-*ras*^{arg186} is indistinguishable from the activity in membranes from cells containing *RAS2*^{val19} only. Second, expression of H-*ras*^{arg186} does not appear to affect cyclic AMP levels in cells containing *RAS2*^{val19}. Third, overexpression of adenylate cyclase does not reverse the blockade of the *RAS2*^{val19} phenotype caused by overexpression of H-*ras*^{arg186}. Nevertheless, recent experiments (data not presented) strongly suggest that H-*ras*^{arg186} does compete with *RAS2*^{val19} for one of its effectors. The GTPase activating protein (GAP) accelerates the hydrolysis rate of the GTP bound to wild-type mammalian *RAS* proteins (Trahey and McCormick, 1987; Cales *et al.*, 1988; Trahey *et al.*, 1988; Vogel *et al.*, 1988). However, GAP does not effect the GTP hydrolysis of some mutant *RAS* proteins such as H-*ras*^{val12} protein. Recently, we have expressed the human GAP cDNA in *S.cerevisiae* (R.Ballester *et al.*, in preparation). The human GAP protein reverses the effects of H-*ras*^{ser186} protein in *RAS2*^{val19} cells and restores the heat shock sensitive phenotype. To determine whether GAP protein reverses the effects of H-*ras*^{ser186} protein by competing for binding to a yeast protein or by accelerating GTP hydrolysis, we used the mutant H-*ras*^{val12ser186}. This mutant *RAS* protein also inhibits the *RAS2*^{val19}-induced heat shock sensitivity. However, GAP protein cannot block the effects of H-*ras*^{val12ser186} protein on the *RAS2*^{val19} phenotype. These results suggest that H-*ras*^{ser186} protein must be in its active GTP-bound state to exert its inhibitory effects on the *RAS2*^{val19} protein.

We are left with a rather unexpected conclusion: H-*ras*^{arg186} interferes with an effector function of *RAS2*^{val19} that is required for the manifestation of its heat shock sensitive phenotype, but this function does not appear to be the activation of adenylate cyclase. We have recently presented evidence based upon genetic analysis that yeast *RAS* proteins have at least one other essential function besides stimulating adenylate cyclase (Toda *et al.*, 1987; Wigler *et al.*, 1988; T.Toda and S.Powers, unpublished observations). We can thus propose that H-*ras*^{arg186} impairs this second function which, together with the activation of adenylate cyclase, may be required for *RAS2*^{val19} to have its pronounced phenotypic effects. Further work is required to test this hypothesis, but our results clearly demonstrate that the interactions of *RAS* proteins even in the simple eukaryote *S.cerevisiae* now appear more complicated than we previously thought.

Materials and methods

Yeast strains, medial and genetic manipulation

The strain TK161-R2V (*Mata leu2 ura3 his3 trp1 ade8 can1 RAS2*^{val19}) was used in most experiments. ST100 (*Mata leu2 ura3 his3 trp1 ade8 can1 ras1::TRP1 ras2::ADE8* p*SCH9*) was generated by S.Powers (unpublished). p*SCH9* is a plasmid capable of suppressing loss of *RAS* function (Toda *et al.*, 1988). Yeast were grown in YPD (2% peptone, 1% yeast extract and 2% glucose) or, to maintain selective pressure for plasmids, in synthetic medium (0.67 g/l yeast nitrogen base, 2% glucose and appropriate auxotrophic supplements). Transformation into yeast cells was performed as described by Ito *et al.* (1983).

Plasmids

pADH-H-*ras* is a *LEU2*$^{+}$ plasmid that expresses H-*ras* from the *ADH1* promoter (Powers *et al.*, 1986). p*RVS* is a mutated pADH-H-*ras* encoding H-*ras*^{arg186}. pURVS is similar to p*RVS* but contains the *URA3* gene instead of the *LEU2* gene. Sequencing and site-directed mutagenesis were performed in phagemid vectors (Vieira and Messing, 1987) carrying the H-*ras* or the H-*ras*^{arg186} coding sequences. pAL1 is a 2 μ-based *LEU2*$^{+}$ plasmid that contains the *ADH1* promoter. pAL*CDC25* is a 2 μ-based plasmid that carries the *LEU2*$^{+}$ gene and the *CDC25* gene expressed from the *ADH1* promoter. p*RAS2*-1 and p*RAS2*^{val19} were described by Broek *et al.* (1987). p*TRP* contains the 1.4 kb *Eco*RI *TRP1–ARS1* fragment. The structure of pADH-CYR is described by Field *et al.* (1988) and p*SCH9* by Toda *et al.* (1988).

Mutagenesis

The H-*ras* gene was mutated in the MutD4 conditional mutator strain of *E.coli*, LE30 (Silhavy *et al.*, 1984). For this purpose, pADH-H-*ras* was transformed into LE30 cells and grown in LB medium for 36 h as described (Fowler *et al.*, 1974). Mutagenized plasmid pools were rescued from *E.coli* and used to transform TK161-R2V cells.

Oligonucleotide-directed mutagenesis was performed by a modification of the procedure of Zoller and Smith (1984) that utilized uracil-containing template DNA (Kunkel, 1985). The following oligonucleotides were used: (i) 5′-GAGCTGCAAGTCTGTGCT-3′ and (ii) 5′-GAGCTGCAAGTGAGTGCTC-3′, to convert codon 186 to a serine and to a termination codon respectively; and (iii) 5′-GAGCTGCAAGTGTTGACTCTCCTG-3′ to convert codon 187 to a termination codon.

DNA sequencing

DNA sequencing was performed according to Sanger *et al.* (1977) using phagemid vectors (Vieira and Messing, 1987).

Cell fractionation and Western blots

Exponentially growing yeast cells were harvested and washed in ice-cold extraction buffer (50 mM potassium phosphate, pH 7.4, 150 mM NaCl, 1 mM β-mercaptoethanol, 1 mM PMSF, 0.1 mM EGTA, 1 μg/ml leupeptin, 0.7 μg/ml pepstatin). An equal volume of glass beads and 200 μl extraction buffer were added to the cell pellet. Cells were disrupted by vortexing four times in 1 min bursts followed by chilling on ice. Cellular debris was removed by centrifugation at 1000 *g* for 10 min, and the cellular extract was further centrifuged at 45 000 r.p.m. in a Ti50 rotor (Beckman) for 30 min. The supernatant or soluble fraction was decanted. The pellet was washed with extraction buffer and centrifuged once more. The washed pellet was resuspended in 200 μl extraction buffer. This constituted the insoluble fraction.

Sixty micrograms of soluble and insoluble protein extracts were prepared for SDS–PAGE by boiling in sample buffer containing SDS and β-mercaptoethanol (Maniatis *et al.*, 1982). Samples were transferred to

nitrocellulose paper (Towbin *et al.*, 1979) and incubated in blocking buffer containing 3% bovine serum albumin and 1% gelatin. The blots were then incubated with anti H-*ras* and anti *RAS2* antisera and then incubated with 10 μCi of [^{125}I]Protein A (ICN). Anti H-*ras* antiserum, raised in rabbits injected with a purified H-*ras* protein, was a gift from D.Bar-Sagi. Anti H-*ras* antiserum reacted only with proteins of mol. wt ~21 kd, and only in yeast strains carrying H-*ras* expression plasmids. Anti *RAS2* antiserum was raised in rabbits injected with *RAS2* protein purified from *E.coli*. The purified *RAS2* protein contains a C'-terminal truncation and co-purifies with traces of other proteins of similar mol. wt (Field *et al.*, 1987). The antiserum raised against this protein preparation binds specifically to yeast *RAS* proteins and to a soluble and abundant yeast protein, which is found even in *ras1*$^-$*ras2*$^-$ cells.

Adenylate cyclase assays

Yeast membrane fractions were prepared as described (Broek *et al.*, 1985). Adenylate cyclase activity was assayed according to Solomon *et al.* (1973) as modified by Broek *et al.* (1985).

Cyclic AMP measurements

Yeast strains were grown in selective media to a cell density of ~0.5 × 10^7 cells/ml. Nucleotides were extracted by a modification of published methods (Olempska-Beer and Freese, 1984). Cells were harvested by centrifugation and incubated in 1 M formic acid saturated with 1-butanol. The supernatant was lyophilized. The cyclic AMP content of the resuspended pellet was determined by a radioimmunoassay (Harper and Brooker, 1975).

Acknowledgements

We thank Scott Powers, Ken Ferguson, Dafna Bar-Sagi, Drs J.Harper and G.Brooker for their generous assistance, and Frank McCormick for cDNA clones of human GAP. We also thank Patricia Bird for her help in preparation of this manuscript. This work was supported by grants from the National Institutes of Health, The Pfizer Biomedical Research Award and the American Cancer Society. T.M. is supported by the Damon Runyon Walter Winchell Cancer Fund Fellowship, DRG-923, J.F. is supported by the Anna Fuller Fund and the National Institutes of Health. M.W. is an American Cancer Society Professor.

References

Barbacid,M. (1987) *Annu. Rev. Biochem.*, **56**, 779–827.
Broek,D., Samiy,N., Fasano,O., Fujiyama,A., Tamanoi,F., Northup,J. and Wigler,M. (1985) *Cell*, **41**, 763–769.
Broek,D., Toda,T., Michaeli,T., Levin,L., Birchmeier,C., Zoller,M., Powers,S. and Wigler,M. (1987) *Cell*, **48**, 789–799.
Cales,C., Hancock,J.F., Marshall,C.J. and Hall,A. (1988) *Nature*, **332**, 548–551.
Camonis,J.H., Kalekine,M., Bernard,G., Garreau,H., Boy-Marcotte,E. and Jacquet,M. (1986) *EMBO J.*, **5**, 375–380.
Cannon,J. and Tatchell,K. (1987) *Mol. Cell. Biol.*, 7, 2653–2663.
DeFeo-Jones,D., Scolnick,E.M., Koller,R. and Dhar,R. (1983) *Nature*, **306**, 707–709.
DeFeo-Jones,D., Tatchell,K., Robinson,L.C., Sigal,I., Vass,W., Lowy,D.R. and Scolnick,E.M. (1985) *Science*, **228**, 179–184.
Deschenes,R.J. and Broach,J.R. (1987) *Mol. Cell. Biol.*, 7, 2344–2351.
Feig,L.A. and Cooper,G.M. (1988) *Mol. Cell. Biol.*, **8**, 3235–3243.
Field,J., Broek,D., Kataoka,T. and Wigler,M. (1987) *Mol. Cell. Biol.*, **7**, 2128–2133.
Field,J., Nikawa,J., Broek,D., MacDonald,B., Rodgers,L., Wilson,I.A., Lerner,R.A. and Wigler,M. (1988) *Mol. Cell. Biol.*, **8**, 2159–2165.
Fowler,R.G., Degnon,G.E. and Cox,E.C. (1974) *Mol. Gen. Genet.*, **133**, 179–185.
Harper,J.F. and Brooker,G.J. (1975) *J. Cyclic Nucleotide Res.*, **1**, 207–218.
Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163–168.
Kataoka,T., Powers,S., McGill,C., Fasano,O., Strathern,J., Broach,J. and Wigler,M. (1984) *Cell*, **37**, 437–445.
Kataoka,T., Powers,S., Cameron,S., Fasano,O., Goldfarb,M., Broach,H. and Wigler,M. (1985) *Cell*, **40**, 19–26.
Kunkel,T. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 488–492.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marshall,M.S., Gibbs,J.S., Scolnick,E.M. and Sigal,I.S. (1987) *Mol. Cell. Biol.*, 7, 2309–2315.
Matsumoto,K., Uno,I. and Ishikawa,T. (1985) *Yeast*, **1**, 15–24.
Nikawa,J., Cameron,S., Toda,T., Ferguson,K.M. and Wigler,M. (1987) *Genes Dev.*, **1**, 931–937.
Olempska-Beer,Z. and Freese,E.B. (1984) *Anal. Biochem.*, **140**, 236–245.
Powers,S., Kataoka,T., Fasano,O., Goldfarb,M., Strathern,J., Broach,J. and Wigler,M. (1984) *Cell*, **36**, 607–612.
Powers,S., Michaelis,S., Broek,D., Anna-A,S.S., Field,J., Herskowitz,I. and Wigler,M. (1986) *Cell*, **47**, 413–422.
Powers,S., O'Neill,K. and Wigler,M. (1989) *Mol. Cell. Biol.*, **9**, in press.
Robinson,L.C., Gibbs,J.B., Marshall,M.S., Sigal,I.S. and Tatchell,K. (1987) *Science*, **235**, 1218–1221.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Sass,P., Field,J., Nikawa,J., Toda,T. and Wigler,M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9303–9307.
Sefton,B.M., Trowbridge,I.S., Cooper,J.A. and Scolnick,E.M. (1982) *Cell*, **31**, 465–474.
Sigal,I.S., Gibbs,J.B., D'Alonzo,J.S., Temeles,G.L., Wolanski,B.S., Socher,S.H. and Scolnick,E.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 952–956.
Silhavy,T.J., Berman,M.L. and Enquist,L.W. (1984) *Experiments with Gene Fusion*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 75–78.
Solomon,Y., Landos,C. and Rodbell,M. (1973) *Anal. Biochem.*, **58**, 541–548.
Taparowsky,E., Shimizu,K., Goldfarb,M. and Wigler,M. (1983) *Cell*, **34**, 581–586.
Toda,T., Brock,D., Field,J., Michaeli,T., Cameron,S., Nikawa,J., Sass,P., Birchmeier,C., Powers,S. and Wigler,M. (1987) In Aaronson,S.A. *et al.* (eds), *Oncogene and Cancer*. Japan Scientific Society Press, Tokyo/VNU Science Press, Utrecht, pp. 253–260.
Toda,T., Cameron,S., Sass,P. and Wigler,M. (1988) *Genes Dev.*, **2**, 517–527.
Toda,T., Uno,I., Ishikawa,T., Powers,S., Kataoka,T., Broek,D., Cameron, S., Broach,J, Matsumoto,K. and Wigler,M. (1985) *Cell*, **40**, 27–36.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Trahey,M. and McCormick,F. (1987) *Science*, **238**, 542–545.
Trahey,M., Wong,G., Halenbeck,R., Rubinfeld,B., Martin,G.A., Ladner, M., Long,M.C., Crosier,W.J., Watt,K., Koths,K. and McCormick,F. (1988) *Science*, **242**, 1697–1700.
Viera,J. and Messing,J. (1987) *Methods Enzymol.*, **153**, 3–11.
Vogel,U.S., Dixon,R.A.F., Schaber,M.D., Diehl,R.E., Marshall,M.S., Scolnick,E.M., Sigal,I.S. and Gibbs,J.B. (1988) *Nature*, **335**, 90–93.
Wigler,M., Field,J., Powers,S., Broek,D., Toda,T., Cameron,S., Nikawa,J., Michaeli,T., Colicelli,J. and Ferguson,K. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 649–655.
Willumsen,B.M., Christensen,A., Hubbert,N.L., Papageorge,A.G. and Lowy,D.R. (1984a) *Nature*, **310**, 583–586.
Willumsen,B.M., Norris,K., Papageorge,A.G., Hubbert,N.L. and Lowy,D.R. (1984b) *EMBO J.*, **3**, 2581–2585.
Zoller,M. and Smith,M. (1984) *DNA*, **3**, 479–488.

Received on February 6, 1989; revised on June 13, 1989