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

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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<sup>val19</sup>, in the yeast *Saccharomyces cerevisiae*. All these mutants encode unprocessed H-ras proteins that remain in the cytoplasm. One of the mutants, H-ras<sup>arg186</sup>, was examined in detail. H-ras<sup>arg186</sup> protein is a competitive inhibitor of RAS2<sup>val19</sup> protein. It does not interfere with processing and membrane localization of RAS2<sup>val19</sup>, nor does it appear to compete with RAS protein for its proposed regulator, the CDC25 protein. By several criteria the RAS2<sup>val19</sup> adenylate cyclase interaction is unaffected by H-ras<sup>arg186</sup>. We infer from our results that H-ras<sup>arg186</sup> protein interferes with an alternative function of RAS2<sup>val19</sup>.

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

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Introduction

The RAS genes are highly conserved in evolution (see Barbacid, 1987, for review). They encode guanine nucleotide binding proteins with weak GDPase 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 (Barricci, 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 proteins are primarily required for the activation of adenylate cyclase (Brock 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functions besides the activation of adenylate cyclase (Toda et al., 1987; Wigler et al., 1988; T. Toda and S. Powers, unpublished observations).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<sup>val12</sup>, the RAS<sup>val19</sup> gene has a dominant phenotype (Kataoka et al., 1985; Toda et al., 1985). Cells carrying the RAS<sup>val19</sup> 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<sup>val19</sup>. The RAS<sup>val19</sup> 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; Matsuzaki 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 RAS<sup>val19</sup> 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<sup>val19</sup>, however, do not require the CDC25 gene product, presumably because RAS2<sup>val19</sup> 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 RAS<sup>val19</sup> 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<sup>val19</sup> 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*)...
plasmids that can suppress the heat shock sensitivity induced by \( RAS^2^{val19} \). Pools of the mutagenized library were transformed into the strain TK161-R2V, which contains the activated \( RAS^2^{val19} \) 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 these 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 \( RAS^2^{val19} \). This plasmid carried a mutant \( H\text{-}ras \), called \( RVS \) (\( RAS\text{-valine-suppressor} \)), that was capable of conferring heat shock resistance to TK161-R2V (Figure 1). Other \( RAS^2^{val19} \)-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 (Tapaowlowsky et al., 1983). The Cys-A-A-X sequence is required for the fatty 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, \( H\text{-}ras^{arg186} \) 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 \( RAS^2^{val19} \)

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

**Fig. 1.** Isolation of an \( H\text{-}ras \) mutant that suppresses the heat shock sensitive phenotype of \( RAS^2^{val19} \). Heat shock sensitive \( RAS^2^{val19} \) cells (strain TK161-R2V) were transformed with the following plasmids: (1) pAL1, containing the \( LEU2 \) gene; (2) pADH-\( H\text{-}ras \), a plasmid derived from pAL1 carrying the \( H\text{-}ras \) cDNA under the control of the \( ADH1 \) promoter; and (3) pRVS, a mutated pADH-\( H\text{-}ras \) plasmid encoding \( H\text{-}ras^{arg186} \). 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 \( H\text{-}ras^{arg186} \) and \( RAS^2^{val19} \). Cytoplasmic and membrane extracts were prepared from \( RAS^2^{val19} \) cells (strain TK161-R2V) carrying the plasmids (b) pAL1, (c) pADH-\( H\text{-}ras \) and (d) pRVS (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 \( RAS^2 \) and anti \( H\text{-}ras \) antisera. \( RAS^2^{val19} \) and \( H\text{-}ras \) proteins were identified by probing blot with each of the individual antisera (data not shown). Anti \( RAS^2 \) 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 premaintained mol. wt markers (from the top: 180, 116, 84, 58, 45, 5, 36.5 and 26.6 kD) is depicted on the left-hand side of the figure.
Fig. 3. Sensitivity to heat shock of \( \text{RAS}^2 \text{val}^{19} \) cells containing \( \text{H-ras}^{\text{arg}^{186}} \) and overexpressing \( \text{CDC}25, \text{RAS2}, \text{RAS2}^{\text{val}^{19}} \) or adenylate cyclase genes. (a) \( \text{RAS2}^{\text{val}^{19}} \) cells (strain TK161-R2V) containing pURVS, a plasmid carrying the \( \text{URA}3 \) gene and \( \text{H-ras}^{\text{arg}^{186}} \) under the control of the \( \text{ADH}1 \) promoter, were transformed with the following additional plasmids: (1) pAL1, containing the \( \text{LEU2} \) marker; (2) pALCDC25, containing the \( \text{LEU2} \) gene and the \( \text{CDC}25 \) gene; (3) pARS2-1, carrying the \( \text{LEU2} \) gene and wild-type \( \text{RAS2} \) gene; and (4) pA219, carrying the \( \text{LEU2} \) gene and the activated \( \text{RAS2}^{\text{val}^{19}} \) allele. Four independent transformants were patched onto SC-ura-leu plates and, following 4 days at 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) pURVS (see legend to Figure 1 for plasmid description) were transformed with either (a) pTRP, carrying the \( \text{TRP1} \) gene, or (b) pADH-CYR, carrying \( \text{TRP1} \) and the adenylate cyclase gene under the control of the \( \text{ADH1} \) promoter. Two independent transformants were patched onto SC-leu-trp plates and, following 3 days at 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 \( \text{RAS2}^{\text{val}^{19}} \) cells carrying various \( \text{H-ras} \) alleles

<table>
<thead>
<tr>
<th>Extra-chromosomal plasmid</th>
<th>Experiment</th>
<th>Assay conditions</th>
<th>( \text{Mn}^{2+} )</th>
<th>( \text{Mg}^{2+} )</th>
<th>( \text{Mg}^{2+} + \text{Gpp(NH)}_p )</th>
<th>( \text{Mn}^{2+}/\text{Mg}^{2+} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAL1</td>
<td>1</td>
<td>58</td>
<td>21.8</td>
<td>23.1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>pADH-H-ras</td>
<td>2</td>
<td>81</td>
<td>35.9</td>
<td>38.4</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>pHVS</td>
<td>1</td>
<td>60</td>
<td>25</td>
<td>27.9</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>pURVS</td>
<td>2</td>
<td>66</td>
<td>28.3</td>
<td>29.7</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>pAL1</td>
<td>3</td>
<td>39</td>
<td>14.2</td>
<td>16.9</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>pADH-H-ras</td>
<td>3</td>
<td>70</td>
<td>69.0</td>
<td>58.5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>pHVS</td>
<td>3</td>
<td>75</td>
<td>34.0</td>
<td>37.4</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>

*The indicated plasmids were introduced into TK161-R2V, a strain containing the activated \( \text{RAS2}^{\text{val}^{19}} \) allele. pAL1 carries the \( \text{LEU2} \) gene as a selective marker; pADH-H-ras and pHVS contain in addition to the \( \text{LEU2} \) gene the \( \text{H-ras} \) and \( \text{H-ras}^{\text{arg}^{186}} \) coding regions respectively transcribed from the \( \text{ADH1} \) promoter.

Each experiment was performed on an independent preparation of membranes, assayed in duplicate.

Membranes from the indicated strains were prepared and adenylate cyclase was assayed either in the presence of 2.5 mM \( \text{Mn}^{2+} \), 2.5 mM \( \text{Mg}^{2+} \) or 2.5 mM \( \text{Mg}^{2+} \) in the presence of 50 mM Gpp(NH)p (see Materials and methods). Gpp(NH)p [guanosine 5'-O-(beta, gamma-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.

Discussion

We have demonstrated the existence of mutant forms of the human \( \text{H-ras} \) gene that interfere with the expression of the phenotype of \( \text{RAS2}^{\text{val}^{19}} \) when co-expressed in the yeast, \( \text{S.cerevisiae} \). The interfering forms of \( \text{H-ras} \), such as \( \text{H-ras}^{\text{arg}^{186}} \), are mutated in sequences encoding the Cys-A-A-X consensus sequence that is required for proper \( \text{RAS} \) protein processing (Willsens et al., 1984a; Deschones and Broach, 1987). Since these interfering \( \text{H-ras} \) mutants do not block the phenotype of \( \text{RAS2}^{\text{val}^{19}} \) when \( \text{RAS2}^{\text{val}^{19}} \) is overexpressed, we can conclude that the interfering forms of \( \text{H-ras} \) compete for interaction with a yeast protein (or proteins) that normally interacts with the \( \text{RAS2}^{\text{val}^{19}} \) protein. In principle, the \( \text{H-ras} \) mutants could interfere with \( \text{RAS2}^{\text{val}^{19}} \) function in any of three major ways, as discussed below.

Table III. Intracellular cyclic AMP levels in \( \text{RAS2}^{\text{val}^{19}} \) cells carrying various \( \text{H-ras} \) alleles

<table>
<thead>
<tr>
<th>Extra-chromosomal plasmid</th>
<th>Cyclic AMP level (pmol/mg protein) in experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>pAL1</td>
<td>3.2</td>
</tr>
<tr>
<td>pADH-H-ras</td>
<td>3.2</td>
</tr>
<tr>
<td>pHVS</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*See legend to Table II for plasmid descriptions. Plasmids were introduced into TK161-R2V, a \( \text{RAS2}^{\text{val}^{19}} \) strain, as described in Table II.

*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 \( \text{H-ras} \) proteins could block the processing of yeast \( \text{RAS} \) proteins. The mutant \( \text{H-ras}^{\text{arg}^{186}} \) protein indeed remains in the cytosolic fraction of the cell, and is not processed. However, we have demonstrated directly that cells which express \( \text{H-ras}^{\text{arg}^{186}} \) appear to have normal
Table 1. The effect of expression of various H-ras mutants upon the heat shock sensitivity of TK161-R2V

<table>
<thead>
<tr>
<th>Mutant</th>
<th>C'-Terminal sequence</th>
<th>Heat shock phenotype in TK161-R2V</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-ras186</td>
<td>Lys-Cys-Val-Leu-Ser186</td>
<td>S</td>
</tr>
<tr>
<td>H-ras186</td>
<td>Lys-Arg-Val-Leu-Ser</td>
<td>R</td>
</tr>
<tr>
<td>H-ras186</td>
<td>Lys-Ser-Val-Leu-Ser</td>
<td>R</td>
</tr>
<tr>
<td>H-ras186</td>
<td>Lys</td>
<td>R</td>
</tr>
<tr>
<td>H-ras186</td>
<td>Lys-Cys</td>
<td>R</td>
</tr>
</tbody>
</table>

\(^a\)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\(^{arg186}\), H-ras\(^{arg187}\), and H-ras\(^{arg187}\), 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\(^{arg186}\) 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 only 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\textsuperscript{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\textsuperscript{val19}. Thus, interference appears to result from the cytoplasmic location of H-ras, but not from interference with the processing of RAS2\textsuperscript{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\textsuperscript{val19} protein still interacts normally with adenylyl cyclase in the presence of H-ras\textsuperscript{arg166}. Second, the only upstream element currently known to affect RAS2 is encoded by CDC25. However, the mutationally activated RAS2\textsuperscript{val19} protein does not require the CDC25 product to exert its phenotype and H-ras\textsuperscript{arg166} protein can block the RAS2\textsuperscript{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\textsuperscript{val19} phenotype that results from expression of H-ras\textsuperscript{arg166}.

There remains the possibility that H-ras\textsuperscript{arg166} could block RAS2\textsuperscript{val19} function by competing for one of its effectors. The one known effector for RAS is adenylyl cyclase (Toda et al., 1985). However, three lines of evidence suggest that this effector function is not impaired by H-ras\textsuperscript{arg166}. First, the adenylyl cyclase activity in membranes from cells containing RAS2\textsuperscript{val19} and expressing H-ras\textsuperscript{arg166} is indistinguishable from the activity in membranes from cells containing RAS2\textsuperscript{val19} only. Second, expression of H-ras\textsuperscript{arg166} does not appear to affect cyclic AMP levels in cells containing RAS2\textsuperscript{val19}. Third, overexpression of adenylyl cyclase does not reverse the blockade of the RAS2\textsuperscript{val19} phenotype caused by overexpression of H-ras\textsuperscript{arg166}. Nevertheless, recent experiments (data not presented) strongly suggest that H-ras\textsuperscript{arg166} does compete with RAS2\textsuperscript{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; Cale et al., 1988; Trahey et al., 1988; Vogel et al., 1988). However, GAP does not affect the GTP hydrolysis of some mutant RAS proteins such as H-ras\textsuperscript{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\textsuperscript{ser12} protein in RAS2\textsuperscript{val9} cells and restores the heat shock sensitive phenotype. To determine whether GAP protein reverses the effects of H-ras\textsuperscript{ser12} protein by competing for binding to a yeast protein or by accelerating GTP hydrolysis, we used the mutant H-ras\textsuperscript{val12ser16} protein. This mutant RAS2 protein also inhibits the RAS2\textsuperscript{val19}-induced heat shock sensitivity. However, GAP protein cannot block the effects of H-ras\textsuperscript{val12ser16} protein on the RAS2\textsuperscript{val19} phenotype. These results suggest that H-ras\textsuperscript{ser12} protein must be in its active GTP-bound state to exert its inhibitory effects on the RAS2\textsuperscript{val19} protein.

We are left with a rather unexpected conclusion: H-ras\textsuperscript{arg166} interferes with an effector function of RAS2\textsuperscript{val19} that is required for the manifestation of its heat shock sensitive phenotype, but this function does not appear to be the activation of adenylyl cyclase. We have recently presented evidence based upon genetic analysis that yeast RAS proteins have at least one other essential function besides stimulating adenylyl cyclase (Toda et al., 1987; Wigler et al., 1988; T. Toda and S. Powers, unpublished observations). We can thus propose that H-ras\textsuperscript{arg166} impairs this second function which, together with the activation of adenylyl cyclase, may be required for RAS2\textsuperscript{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, media and genetic manipulation

The strain TK161-R2V (MATa leu2 ura2 his3 trp1 ade8 can1 RAS2\textsuperscript{val19}) was used in most experiments. ST100 (MATa leu2 ura3 his3 trp1 ade8 can1 ras1::TRP1 ras2::ADE8 pSCH9) was generated by S. Powers (unpublished). pSCH9 is a plasmid capable of suppressing loss of RAS function (Toda et al., 1988). Yeast were grown inYPD (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). pRSV is a mutated pADH-H-ras encoding H-ras\textsuperscript{arg166}. pURVS is similar to pRSV but contains the URA3 gene instead of the LEU2 gene. Sequencing and site-directed mutagenesis were performed in plasmid vectors (Vieira and Messing, 1987) carrying the H-ras or the RAS2\textsuperscript{val19} coding sequences. pAL1 is a 2 μ-based LEU2 plasmid that contains the ADH1 promoter. pALCDC25 is a 2 μ-based plasmid that carries the LEU2 gene and the CDC25 gene expressed from the ADH1 promoter. pRAS2-1 and pRAS2\textsuperscript{val19} were described by Broek et al. (1987). pTPR contains the 1.4 kb EcoRI TRP1-ARG1 fragment. The structure of pADH-CYR is described by Field et al. (1988) and pSCH9 by Toda et al. (1988).

Mutagenesis

The H-ras gene was mutated in the Mud4 conditional mutant strain of E. coli, L630 (Silliby et al., 1984). For this purpose, pADH-H-ras was transformed into L630 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'-GAACCCTGCAAGGATGCTCCATGAC-3' and (ii) 5'-GACCTGGCAAGTGCTTCGCTCCCTCGG-3' to convert codon 186 to a serine and to a termination codon respectively; and (iii) 5'-GACCTGCAAGGATGCTCCATGAC-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 x g for 10 min, and the cellular extract was further centrifuged at 45 000 x r.p.m. in a T50 rotor (Beckman) for 30 min. The supernatant or soluble fraction was discarded. 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 microliters of soluble and insoluble protein extracts were prepared for SDS-PAGE by boiling in sample buffer containing SDS and β-mercaptoethanol (Munirat et al., 1982). Samples were transferred to
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. Nucleioids were extracted by a modified method of Oleniuk-Zeece and Freese (1984). Cells were harvested by centrifugation and incubated in 1 M formic acid saturated with 1-hexanol. The supernatant was lyophilized. The cyclic AMP content of the resuspended pellet was determined by a radioimmunoassay (Harper and Brooker, 1975).

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