Differential Activation of Yeast Adenylate Cyclase by Wild-Type and Mutant RAS Proteins

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Summary
In these experiments we demonstrate that purified RAS proteins, whether derived from the yeast RAS1 or RAS2 or the human H-ras genes, activate yeast adenylate cyclase in the presence of guanine nucleotides. These results confirm the prediction of earlier genetic and biochemical data and for the first time provide a complete biochemical assay for RAS protein function. Furthermore, we observe a biochemical difference between the RAS2 and RAS2\textsuperscript{val19} proteins in their ability to activate adenylate cyclase after preincubation with GTP.

Introduction
The members of the ras gene family were first identified in mammals as cellular homologs of the Harvey and Kirsten sarcoma virus oncogenes (Ellis et al., 1981). They encode closely related 21,000 dalton proteins, which are localized in the plasma membrane and bind guanine nucleotides (Shih et al., 1980, 1982; Papageorge et al., 1982). Mutant H-, K-, or N-ras genes, capable of the morphologic and tumorigenic transformation of NIH 3T3 cells, have been isolated from a wide variety of human tumor cell lines (Tabin et al., 1982; Reddy et al., 1982; Tapperowsky et al., 1982, 1983; Shimizu et al., 1983; Yuasa et al., 1983; Capon et al., 1983). These oncogenic ras genes differ from their normal counterparts by single missense mutations that account for their oncogenic potential. The normal ras proteins have a GTPase activity, which is greatly reduced in the oncogenic variants (Sweet et al., 1984; McGrath et al., 1984; Gibbs et al., 1984). Although the function of ras in vertebrates remains a mystery, normal ras genes presumably have essential cellular functions, since they are expressed at virtually all levels of development in mammals (Mueller et al., 1982, 1983) and are highly conserved in evolution (Shilo and Weinberg, 1981; Defeo-Jones et al., 1983; Powers et al., 1984; Neuman-Silberberg et al., 1984). The yeast Saccharomyces cerevisiae contains two genes, RAS1 and RAS2, which encode proteins highly homologous to the mammalian ras proteins (Defeo-Jones et al., 1983; Powers et al., 1984; Dhar et al., 1984).

Recent work indicates that yeast cells are a good model system for studying the biochemical function of the mammalian ras proteins. At least one functional yeast RAS gene is essential for cell viability and proliferation (Kataoka et al., 1984, 1985; Tatchell et al., 1984), and expression of the mammalian H-ras protein suffices for this function (Kataoka et al., 1985). Moreover, yeast cells carrying the RAS2\textsuperscript{val19} gene, a mutant analogous to the H-ras\textsuperscript{val2} mutant of the human bladder carcinoma cell line T24 (Taparowsky et al., 1982), have a defective response to nutritional deprivation (Kataoka et al., 1984; Toda et al., 1985).

Previous genetic studies of yeast cells carrying mutant yeast RAS genes strongly suggest that RAS proteins are involved in the cyclic AMP effector pathway (Toda et al., 1985). First, cells with disrupted RAS genes are phenotypically similar to cells deficient in adenylate cyclase. Second, cells with the RAS2\textsuperscript{val19} gene have a phenotype similar to cells containing the IAC mutation (Uno et al., 1982; Scott Powers, unpublished data), which have high levels of adenylate cyclase activity, and to cells containing the boyl mutation, which fail to make a functional regulatory subunit of the cAMP-dependent protein kinase (Uno et al., 1982). Third, the boyl mutation, which suppresses the lethality resulting from loss of adenylate cyclase (Maumoto et al., 1982), also suppresses the lethality resulting from the disruption of both endogenous yeast RAS genes (Toda et al., 1985). Thus boyl ras1\textsuperscript{val1} ras2\textsuperscript{val2} cells are viable.

Previous biochemical studies of yeast cells carrying mutant RAS proteins strongly suggest that RAS proteins are required for and regulate adenylate cyclase activity (Toda et al., 1985). First, cAMP levels are very low in boyl ras1\textsuperscript{val1} ras2\textsuperscript{val2} yeast as compared with levels in wild-type yeast, whereas they are elevated in RAS2\textsuperscript{val19} yeast. Second, membranes prepared from boyl ras1\textsuperscript{val1} ras2\textsuperscript{val2} have wild-type levels of adenylate cyclase when assayed in the presence of manganese ions but have negligible adenylate cyclase activity when assayed in the presence of magnesium and GTP.

We demonstrate that the addition of purified yeast RAS and human H-ras proteins to membranes prepared from boyl ras1\textsuperscript{val1} ras2\textsuperscript{val2} cells restores the response of adenylate cyclase to magnesium ions and guanine nucleotides. We use this observation as an assay to compare various RAS proteins and their guanine nucleotide requirements.

Results
Copurification of Yeast RAS2 Proteins and an Adenylate-Cyclase-Activating Factor
We tested whether purified yeast RAS2 proteins synthesized in E. coli could activate the adenylate cyclase in membranes prepared from boyl ras1\textsuperscript{val1} ras2\textsuperscript{val2} yeast cells. For this purpose we used f-RAS2, a fusion protein of RAS2 synthesized in E. coli. This protein contains 20 additional amino acids N-terminal to the complete wild-type RAS2 protein (see Experimental Procedures). It was partially purified as described previously and concentrated by ammonium sulfate precipitation (Tamanoi, Samiy, Rao, and...
Walsh, 1985). The resolubilized protein was then fractionated on a Sephacryl S-300 gel filtration column. A peak of \(^{3}H\)-GDP binding activity was detected in fractions that contained a major protein species with a relative mobility of 42,000 daltons (Figure 1). This protein was identified as the f-RAS2 protein by immunoprecipitation with the anti-ras monoclonal antibody Y13-259. Lesser amounts of lower molecular weight bands were also seen in these fractions, which we have shown are degradation fragments of the f-RAS2 protein (data not shown).

We assayed proteins in fractions from the S-300 column for the ability to activate yeast adenylate cyclase in the presence of magnesium ions and the nonhydrolyzable GTP analog, guanosine-5'\(\gamma\)-imino)triphosphate (Gpp(NH)p). For the assay we used crude membranes prepared from bcy1 ras\(^{1}\) ras\(^{2}\) yeast cells. The bcy1 mutation is needed to maintain viability in ras\(^{1}\) ras\(^{2}\) yeast cells, but it does not affect adenylate cyclase (Uno et al., 1983). Crude membranes prepared from these cells have wild-type levels of adenylate cyclase activity when assayed in the presence of magnesium ions, but negligible levels when assayed in the presence of magnesium ions with or without the addition of Gpp(NH)p (Toda et al., 1985). Analysis of fractions from the Sephacryl S-300 column revealed a single peak of GDP binding activity, which co-eluted with a factor that activates yeast adenylate cyclase in bcy1 ras\(^{1}\) ras\(^{2}\) membranes (Figure 1). Similar results were obtained for the fusion protein f-RAS2\(^{val19}\) (data not shown), in which valine replaces glycine at position 19 of RAS2 (Kataoka et al., 1984).

To confirm that the activation of adenylate cyclase which we observed was due to the f-RAS2 and f-RAS2\(^{val19}\) proteins, we separately pooled active fractions of these proteins and assayed fraction supernatants either after immunoprecipitation with the broad spectrum anti-ras monoclonal antibody Y13-259, which does not cross-react with the RAS2 proteins, or with phosphate-buffered saline (None). Ten microliters of the resulting supernatants was incubated with or without Gpp(NH)p at 37°C for 30 min followed by incubation with 30 \(\mu\)g of bcy1 ras\(^{1}\) ras\(^{2}\) membranes at 0°C for 30 min. After the addition of ATP to a final concentration of 0.5 mM the samples were incubated at 27°C for 15 min. The addition of a mixture containing \(\alpha\)\(^{32}\)P-ATP started the 30 min reaction.

### Table 1. Adenylate Cyclase Activation by Proteins Pretreated with Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Mg</th>
<th>Mg + Gpp(NH)p</th>
<th>Mg</th>
<th>Mg + Gpp(NH)p</th>
<th>Buffer G Mg</th>
<th>Buffer G Mg + Gpp(NH)p</th>
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</thead>
<tbody>
<tr>
<td>Y13-259</td>
<td>0.9</td>
<td>1.4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Y13-238</td>
<td>2.4</td>
<td>44.4</td>
<td>4.1</td>
<td>50.8</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>None</td>
<td>3.1</td>
<td>72.0</td>
<td>4.8</td>
<td>68.0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Values are expressed in units of adenylate cyclase activity. The peak fractions containing f-RAS2 or f-RAS2\(^{val19}\) or 20 mM Tris (pH 7.5), 5 mM MgCl\(_2\), 1 mM jMSH (buffer G) were separately immunoprecipitated as described in Experimental Procedures with monoclonal antibody Y13-259 (which reacts with RAS2 proteins), monoclonal antibody Y13-238 (an H-ras-specific antibody which does not cross-react with the RAS2 proteins), or with phosphate-buffered saline (None). Ten microliters of the resulting supernatants was incubated with or without Gpp(NH)p at 37°C for 30 min followed by incubation with 30 \(\mu\)g of bcy1 ras\(^{1}\) ras\(^{2}\) membranes at 0°C for 30 min. After the addition of ATP to a final concentration of 0.5 mM the samples were incubated at 27°C for 15 min. The addition of a mixture containing \(\alpha\)\(^{32}\)P-ATP started the 30 min reaction.

**Figure 1. Co-purification of RAS2 and Adenylate-Cyclase-Stimulating Activity**

Partially purified RAS2 protein was applied to a Sephacryl S-300 column and 1 ml fractions were collected. \(^{3}H\)-GDP binding activity was determined with 5 \(\mu\)l aliquots of fractions. For reconstitution of adenylate cyclase activity, 5 \(\mu\)l of indicated fractions were incubated with 1 \(\mu\)l 1 mM Gpp(NH)p for 30 min at 37°C. The samples were chilled and incubated with 30 \(\mu\)g of bcy1 ras\(^{1}• ras\(^{2}\) membrane proteins at 0°C for 30 min, followed by a 15 min incubation at 27°C in the presence of 0.5 mM ATP. The addition of a mixture containing \(\alpha\)\(^{32}\)P-ATP started the 40 min reaction. The final concentrations of components in the reaction are described in Experimental Procedures. SDS-polyacrylamide gel electrophoresis of Sephacryl S-300 column fractions (inset) shows a 42,000 molecular weight protein, identified as f-RAS2 by immunoprecipitation, eluting at fractions containing maximal GDP binding activity and adenylate-cyclase-stimulating activity.

**Activation of Yeast Adenylate Cyclase with Yeast RAS1 and Mammalian H-Ras Proteins**

Genetic analysis indicates that the intact RAS1 or RAS2 gene is sufficient for yeast viability and proliferation (Kataoka et al., 1984, 1985; Tatchell et al., 1984). To test whether purified RAS1 protein also stimulates yeast adenylate cyclase, we utilized f-RAS1, a RAS1 fusion protein, synthesized in and purified from E. coli (see Experimental Procedures). In the presence of magnesium
Table 2. Maximal Adenylate Cyclase Stimulation with RAS Proteins

<table>
<thead>
<tr>
<th>Protein Added</th>
<th>Mg</th>
<th>Mg + Gpp(NH)p</th>
</tr>
</thead>
<tbody>
<tr>
<td>f-RAS2</td>
<td>3.1</td>
<td>72</td>
</tr>
<tr>
<td>f-RAS2val19</td>
<td>4.8</td>
<td>68</td>
</tr>
<tr>
<td>RAS2</td>
<td>3.4</td>
<td>66</td>
</tr>
<tr>
<td>RAS2val19</td>
<td>4.5</td>
<td>73</td>
</tr>
<tr>
<td>H-ras</td>
<td>0.7</td>
<td>19.3</td>
</tr>
<tr>
<td>H-rasval12</td>
<td>0.4</td>
<td>15.7</td>
</tr>
<tr>
<td>f-RAS1</td>
<td>0.4</td>
<td>21.3</td>
</tr>
<tr>
<td>H-rasval12/RAS2</td>
<td>3.8</td>
<td>76</td>
</tr>
<tr>
<td>None</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Ten units of RAS proteins (in 10 μl of buffer G) were incubated with or without 1 μl of 1 mM Gpp(NH)p at 37°C for 30 min. The samples were then incubated on ice for 30 min with 30 μg of f-RAS1 membrane proteins (in 30 μl buffer D) followed by the addition of 2.5 μl of 20 mM ATP and incubated at 27°C for 15 min. The 30 min reaction was started by the addition of a mixture containing 32P-ATP started the reaction 2.5 μl of 20 mM ATP and incubated at 27°C for 15 min. The 30 min reaction was started by the addition of a mixture containing [32P]ATP (see Experimental Procedures). The values given are expressed in units of adenylate cyclase activity (pmol cAMP/min/mg of membrane protein).

Relative Stimulation of Adenylate Cyclase with Various RAS Proteins

Varying amounts of three purified RAS proteins, f-RAS2, f-RAS2val19, and H-ras, were separately added to a fixed amount of cell membrane prepared from bcy1 ras1 ras2 cells, and the resulting stimulation of adenylate cyclase in the presence of magnesium ion was measured with or without the addition of Gpp(NH)p. For all three proteins, activation of adenylate cyclase was saturable and dependent on the presence of Gpp(NH)p. Moreover, all the RAS proteins induced half-maximal activation in the range of 1–3 units of RAS protein per 30 μg of membrane protein (Figure 3, where 1 unit of RAS protein binds 1 pmol of 3H-GDP under conditions described in Experimental Procedures. These results suggest that there are sites for protein interaction in yeast cell membranes, and both the human H-ras and yeast RAS proteins appear to have a high affinity for these sites.

The maximal level of adenylate cyclase activity induced by the various RAS proteins in the presence of Gpp(NH)p differed considerably (see Table 2). RAS2, RAS2val19, f-RAS2, and f-RAS2val19 showed comparable maximal

Figure 2. Copurification of H-ras and Adenylate-Cyclase-Stimulating Activity

Partially purified H-rasval19 protein was applied to a Sephadex G75 gel filtration column, and the fractions containing H-rasval19 protein were identified by 3H-GDP binding activity (Figure 2), by SDS-polyacrylamide gel electrophoresis (Figure 2, inset), and by immunoprecipitation (data not shown). Aliquots of column fractions were added to membranes prepared from bcy1 ras1 ras2 yeast, and adenylate cyclase activity was assayed. These studies showed a peak of a guanine-nucleotide-dependent adenylate-cyclase-activating factor that coincided with the peak of GDP binding activity (Figure 2). Thus, the human H-ras and yeast RAS proteins are all able to activate yeast adenylate cyclase.

ion and guanine nucleotides, the f-RAS1 protein activates the adenylate cyclase of crude membranes (Table 2) prepared from bcy1 ras1 ras2 yeast cells.

Genetic analysis also has shown that the human H-ras protein expressed in yeast can substitute for the loss of both endogenous RAS genes, demonstrating a functional homology between the human and yeast RAS genes (Kataoka et al., 1985). We tested directly whether the human Ras proteins are functionally homologous to the yeast Ras proteins in their ability to activate yeast adenylate cyclase. Human H-rasval12 protein expressed in E. coli was partially purified by DEAE cellulose chromatography, as previously described (Gross et al., 1985), and concentrated by spin dialysis. The concentrated proteins were fractionated on a Sephadex G75 gel filtration column, and the fractions containing H-rasval19 protein were identified by 3H-GDP binding activity (Figure 2), by SDS-polyacrylamide gel electrophoresis (Figure 2, inset), and by immunoprecipitation (data not shown). Aliquots of column fractions were added to membranes prepared from bcy1 ras1 ras2 yeast, and adenylate cyclase activity was assayed. These studies showed a peak of a guanine-nucleotide-dependent adenylate-cyclase-activating factor that coincided with the peak of GDP binding activity (Figure 2). Thus, the human H-ras and yeast RAS proteins are all able to activate yeast adenylate cyclase.
the H-ras protein (Kataoka et al., 1985). Finally, a chimeric RAS (H-rasval12/RAS2) protein that consists of the first 73 amino acids of H-ras and the remaining 242 amino acids of RAS2 stimulates adenylate cyclase to the same maximal level as the RAS2 protein and its derivatives. This, too, agrees with our previous genetic data, which indicate that yeast cells with disrupted endogenous RAS genes grow vigorously if they express the chimeric RAS protein (Kataoka et al., 1985).

### Requirement of Guanine Nucleotides for Activation of Adenylate Cyclase

We compared the activity of adenylate cyclase in the boy1L ras1 ras2 membranes after the addition f-RAS2val19 protein preincubated with or without guanine nucleotides (Gpp(NH)dp, GTP, or GDP) as described in the legend to Figure 4. The presence of guanine nucleotides is required for efficient stimulation of adenylate cyclase. In the absence of added guanine nucleotide we observe less than 10% of the activity observed in the presence of GDP. This low level of activity may result from residual guanine nucleotides bound to the purified proteins. As seen in Figure 4, maximal rates of cAMP production were observed in membranes to which we added Gpp(NH)dp. When Gpp(NH)dp cannot be hydrolyzed, we conclude that GTP hydrolysis is not required for activation of RAS.

When f-RAS2val19 was preincubated with GTP it stimulated adenylate cyclase to the same extent as when prein-
cubated with Gpp(NH)p. However, when f-RAS2\textsuperscript{val19} protein was preincubated with GDP, it induced a rate of cAMP production approximately one-half of that induced by f-RAS2\textsuperscript{val19} protein preincubated in the presence of guanine triphosphate (Figure 4). Thin layer chromatographic analysis of radiolabeled GDP showed no GTP had been produced during the reaction (data not shown). Also, a GDP analog, guanosine-5'-O-(2-thiodiphosphate), GDP\textsubscript{2S}, which cannot be directly converted to a triphosphate, gave a rate of cAMP production identical to that observed in the presence of GDP (data not shown). We conclude that guanine diphosphates do activate f-RAS2\textsuperscript{val19}, but not to the same extent as guanine triphosphates.

The f-RAS2 protein, like the f-RAS2\textsuperscript{val19} protein, induced a rate of cAMP production after preincubation with Gpp(NH)p that was twice that observed with GDP (Figure 4). However, we observed one significant difference between the RAS2 protein and the RAS2\textsuperscript{val19} protein. While f-RAS2\textsuperscript{val19} preincubated with GTP stimulated adenylate cyclase to the same extent as when preincubated with Gpp(NH)p, f-RAS2 protein preincubated with GTP stimulated adenylate cyclase to the same extent as when preincubated with GDP. Similar results were observed with the intact RAS2 protein, which does not contain the additional 20 amino acids found in the f-RAS2 protein (data not shown). This difference is likely due to the inability of the RAS2\textsuperscript{val19} protein to hydrolyze GTP under conditions in which the RAS2 protein is an effective GTPase (Tamanoi et al., 1985).

Discussion

All RAS proteins we have studied dramatically activate yeast adenylate cyclase in the presence of magnesium ions and guanine nucleotides. Maximal activation is achieved at low protein concentrations. However, not all RAS proteins stimulate adenylate cyclase to the same extent. The most stimulatory are the proteins derived from yeast RAS2. In the presence of the nonhydrolyzable GTP analog, Gpp(NH)p, we see the same level of activation with the RAS2 and RAS2\textsuperscript{val19} proteins as with the N-terminal fusion proteins f-RAS2\textsuperscript{f} and f-RAS2\textsuperscript{2}. From these studies it appears that the addition of amino acids at the N terminus of the f-RAS2 protein does not seriously perturb their function as reflected in this assay system. The RAS1- and H-10-derived proteins are less stimulatory: they maximally activate adenylate cyclase to one-fourth the level seen with RAS2 proteins. We cannot rule out the possibility that the additional seven amino acids in the f-RAS1 protein affect its ability to activate adenylate cyclase. Finally, we observe that the chimeric protein, H-10\textsubscript{10}/RAS2, containing the first 73 N-terminal positions of the H-10 and the last 242 C-terminal positions of RAS2, behaves like the intact RAS2 proteins. These results are largely consistent with previous genetic data. First, RAS1\textsuperscript{f} RAS2 cells have a premature sporulation phenotype not seen in RAS1\textsuperscript{f} RAS2 cells (Toda et al., 1985), and they have considerably lower intracellular cAMP levels than the latter (Toda et al., 1985). Second, RAS1\textsuperscript{f} RAS2 cells grow poorly if they express the intact H-ras protein, but grow like wild-type cells if they express the chimeric H-ras/RAS2 protein (Kataoka et al., 1985).

All RAS proteins we have studied require guanine nucleotides in order to stimulate adenylate cyclase efficiently. Because the nonhydrolyzable GTP homolog, Gpp(NH)p, can fulfill this requirement, we may conclude that RAS proteins are not kinases which utilize GTP as a phosphate donor. GDP serves well in the assay system. This is not due to regeneration of GTP from GDP First, this regeneration does not occur under our assay condition as determined by analysis of radiolabeled GDP using thin layer chromatography (data not shown). Second, the GDP analog, GDP\textsubscript{2S}, serves as well as GDP in this assay system (data not shown), and it is not possible to regenerate a triphosphate directly from GDP\textsubscript{2S}. Nevertheless, RAS proteins complexed with guanine diphosphates activate yeast adenylate cyclase only half as well as proteins complexed with Gpp(NH)p. These results strongly suggest that guanine nucleotides are regulators of RAS function and that there is a component in yeast membranes that can distinguish RAS proteins complexed to guanine triphosphates from RAS proteins complexed to guanine diphosphates.

We observed one significant difference between the RAS2 and RAS2\textsuperscript{val19} proteins. RAS2\textsuperscript{val19} protein preincubated with GTP stimulated adenylate cyclase twice as much as RAS2 protein preincubated with GTP. This difference can be explained by the GTPase activity of RAS2 protein, an activity greatly reduced in the mutant RAS2\textsuperscript{val19} protein (Tamanoi et al., 1985), and by the observation that the RAS2 proteins are less stimulatory when complexed to a guanine diphosphate than when complexed to a guanine triphosphate. We do not know if this is a sufficient explanation for all the phenotypic differences we observe between wild-type cells and cells carrying the RAS2\textsuperscript{val19} allele. Although we observe only a 2-fold difference under in vitro conditions between the activities of RAS proteins bound to guanine triphosphates and those bound to guanine diphosphates, this difference may be greater in vivo, as suggested by a 4-fold elevation of cAMP levels in RAS2\textsuperscript{val19} cells with respect to wild-type cells (Toda et al., 1985).

Our studies have demonstrated that the human H-ras protein can activate yeast adenylate cyclase. At least two mammalian guanine nucleotide binding proteins, Gs and Ga, have been identified that can modulate adenylate cyclase (Gilman, 1984). Are the mammalian ras proteins related to these or other G proteins? The N-terminal regions of ras and two G proteins, Ga and transducin, show some sequence homology (Hurley et al., 1984). The G proteins are oligomers comprising an a subunit that binds guanine nucleotides and a \(\beta\gamma\) complex that presumably inhibits a subunit function. Among the various G proteins, the \(\beta\gamma\) complexes are functionally interchangeable (Gilman, 1984). However, in preliminary experiments, we have failed to observe any functional interaction between the yeast or mammalian ras proteins and purified \(\beta\gamma\) complexes (J. N. and D. B., unpublished results). Thus the connection between ras and G proteins is unclear. Furthermore, RAS bound to GDP under our as-
say condition is a potent activator of yeast adenylate cyclase, while the effect of GDP bound to Gα on mammalian adenylate cyclase remains unclear (Eckstein et al., 1979; Iyengar et al., 1980).

Preliminary experiments have also failed to demonstrate any functional interaction between mammalian or yeast ras proteins and mammalian adenylate cyclase (J. N. and Carmen Birchmeier, unpublished results). Although our preliminary results strongly suggest that the immediate effector interactions of RAS proteins have been conserved in evolution, we have not determined whether the RAS proteins interact directly with yeast adenylate cyclase. In fact, several pathways can be envisioned by which RAS proteins stimulate yeast adenylate cyclase, and such models make different predictions about which protein interactions might be conserved in evolution. Since we now have an in vitro assay for RAS protein function, we can in principle isolate the protein targets with which RAS proteins directly interact. These target proteins, we argue, will have domains that are conserved in evolution.

Experimental Procedures

RAS Protein Expression Systems

The construction of the expression systems for H-RAS2 and H-RAS2\textsuperscript{Val12} are described elsewhere in detail (Tamanoi et al., 1985). The H-RAS2 and RAS2\textsuperscript{Val12} proteins were produced in E. coli by inserting their genes into the same ZPL heat-inducible promoter vector system RAS2 (Toda et al., 1985). The\textsuperscript{RAS1} and\textsuperscript{RAS2} fragment was inserted into a pUC8 vector (Messing and Vieira, 1982) to produce the H-RAS1 protein and contains these seven additional amino acids N-terminal to the first methionine of the intact RAS1 protein: Met Thr Met Ile Thr Asn Ser. The yeast RAS1 fragment was inserted into a pUC8 vector (Messing and Vieira, 1982) to produce the H-RAS1 protein and contains these seven additional amino acids N-terminal to the first methionine of the intact RAS2 protein: Met Thr Met Ile Thr Asn Ser. The construction of the H-RAS and H-ras\textsuperscript{Val12} expression systems has been described (Gross et al., 1985). The H-ras\textsuperscript{Val12}/H-RAS2 chimeric gene was described previously (Toda et al., 1985). The H-ras\textsuperscript{Val12}/H-RAS2 chimeric protein and the intact RAS2 and RAS2\textsuperscript{Val12} proteins were produced in E. coli by inserting their genes into the same pJ6 heat-inducible promoter vector system used to express the intact H-ras proteins.

Purification of RAS Proteins

The yeast H-RAS2 proteins synthesized in E. coli were purified by diethylaminoethyl (DEAE) Sephacel (Pharmacia) chromatography and gel filtration on a Sephacryl S-300 (Pharmacia) column (column volume 60 ml) as described (Tamanoi et al., 1985). The H-ras protein was purified as described (Gross et al., 1985). The intact RAS2 proteins and the chimeric H-ras\textsuperscript{Val12}/RAS2 protein were purified from E. coli containing a temperature-inducible expression system (Gross et al., 1985). Twelve grams of E. coli cells (wet weight) was suspended in 50 ml of 20 mM Tris-HCl (pH 7.5), 5 mM MgCl\textsubscript{2}, 2 mM 2-mercaptoethanol (2-ME), 20 mM KCl, 10 \mu M GDP (Buffer O) and passed through a French press at 20,000 psi. The purification of the chimeric RAS protein utilized Buffer O plus 1% Triton X-100 for the extraction from E. coli. The disrupted cell suspension was centrifuged at 105,000 \times g, and the supernatant was applied to a DEAE Sephacel column preequilibrated with Buffer O. Proteins were eluted with a linear gradient from 20 mM to 500 mM KCl. Fractions containing RAS2 proteins were identified by immunoprecipitation of bound \textsuperscript{3H}-GDP. The peak fractions contain RAS2 proteins were pooled, and the RAS proteins were found to precipitate between 45% and 55% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} saturation. The resolubilized RAS2 proteins were dialyzed against 20 mM Tris-HCl (pH 7.5), 2 mM 2-ME, 20 mM KCl, 1 \mu M NHS (buffer G) prior to use. One unit of RAS protein is defined as that which will bind 1 pmol of GDP, as determined by nitrocellulose filter binding, after 30 min of incubation at 37\degree C in buffer G in the presence of 3 \mu M \textsuperscript{3}H-GDP. Both of the H-ras proteins (H-ras and H-ras\textsuperscript{Val12}) as well as the yeast RAS2 proteins (H-RAS2, H-RAS2\textsuperscript{Val12}, RAS2, RAS2\textsuperscript{Val12}) have GDP binding characteristics consistent with single site binding. Furthermore, as determined by a nitrocellulose filter binding assay, 1 pmol of RAS protein binds 0.6-0.8 pmol of \textsuperscript{3}H-GDP.

While searching for conditions to store the RAS proteins (H-ras, H-RAS2, and RAS2) we found that storage of the RAS proteins in buffer G at -20\degree C completely inactivated the adenylate cyclase stimulatory activity of the protein, although little or no loss of \textsuperscript{3}H-GDP binding activity was observed (data not shown). Consequently all of the experiments described here were carried out on proteins stored on ice in buffer G for less than 48 hr after the final purification step. Subsequent to these experiments we found the RAS protein stored at -20\degree C in the presence of 50% glycerol retained more than 85% activity after 3 weeks.

Preparation of Yeast Membranes

Yeast cells were grown in YPD media as previously described (Toda et al., 1985). Membrane extracts were prepared as described (Toda et al., 1985) with the following modifications. Instead of disruption of cells by preparing spheroplasts and spheroplast homogenization, the yeast cell pellets were suspended in 2 vol of 50 mM 2(N-morpholino)ethanesulfonic acid (MES), pH 6.2, 0.1 mM MgCl\textsubscript{2}, 0.1 mM EGTA, 1 \mu M \textsuperscript{3}H-GDP, 2 mM phenylmethyl sulfonyl fluoride, and 1 \mu g/ml soybean trypsin inhibitor (buffer C), and passed through a French press at 20,000 psi. The crude membrane preparations were stored in aliquots at -70\degree C in buffer C containing 10% glycerol (buffer D). Protein content was determined by the method of Lowry et al., 1951.

Adenylate Cyclase Assays

Adenylate cyclase activity was assayed as described previously (Casperson et al., 1983), and \textsuperscript{32}P-cAMP production was determined as described (Solomon et al., 1973). All adenylate cyclase assays contained 100 \mu l final volume with final concentrations of the following components: 20 units per ml creatine phosphokinase, 20 mM phosphocreatine, 2.5 mM MgCl\textsubscript{2}, 10 mM theophylline, 1 mM \textsuperscript{3}H-cAMP (20,000 cpm per reaction); 0.1 mg per ml bovine serum albumin and 1 \mu M \textsuperscript{3}H-Mesh. The additions of RAS proteins and of guanine nucleotides were carried out as described in the figure legends. The reactions were stopped by the addition of 0.9 ml of a solution containing 10% SDS, 5 mM ATP, 0.18 mM cAMP.

Immunoprecipitation of an Adenylate-Cyclase-Activating Factor with Monoclonal Antibody

One hundred microliters of H-RAS2 and H-RAS2\textsuperscript{Val12} (2 units of 1RAS2 protein per \mu l) in buffer G lacking GDP and KC\textsubscript{1} were incubated separately with 15 \mu l of monoclonal antibody Y13-259 (10 mg/ml), Y13-238 (10 mg/ml), or phosphate-buffered saline at 4\degree C on an orbital shaker for 2 hr. Monoclonal antibodies were a gift from M. Furth (Furth et al., 1982). Fifty microliters of a 50% suspension of protein A-Sepharose (Boehringer Mannheim), presaturated with rabbit anti-rat IgG, was then added, and the mixture was allowed to incubate for 30 min at 4\degree C. The samples were centrifuged for 1 min at 12,000 \times g, the supernatants were mixed with yeast membranes as described in the legend to Table 2, and adenylate cyclase activity was assayed as described above.

Other Procedures

Guanine nucleotides were separated by thin layer chromatography as previously described (Tamanoi et al., 1985). Sodium dodecylsulfate-polyacrylamide gel electrophoresis was carried out using the method of Laemmli (1970). Centricon microconcentrators (Amicon) were used to concentrate dilute protein solutions by centrifugation at 8,000 rpm in an SS-34 rotor (Sorval).

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