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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*^{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; Taparowsky 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 (Muller 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*^{val19} gene, a mutant analogous to the H-*ras*^{val12} 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*^{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 *bcy1* mutation, which fail to make a functional regulatory subunit of the cAMP-dependent protein kinase (Uno et al., 1982). Third, the *bcy1* mutation, which suppresses the lethality resulting from loss of adenylate cyclase (Matsumoto et al., 1982), also suppresses the lethality resulting from the disruption of both endogenous yeast *RAS* genes (Toda et al., 1985). Thus *bcy1 ras1*$^-$ *ras2*$^-$ cells are viable.

Previous biochemical studies of yeast cells carrying mutant *RAS* genes strongly suggest that *RAS* proteins are required for and regulate adenylate cyclase activity (Toda et al., 1985). First, cAMP levels are very low in *bcy1 ras1*$^-$ *ras2*$^-$ yeast as compared with levels in wild-type yeast, whereas they are elevated in *RAS2*^{val19} yeast. Second, membranes prepared from *bcy1 ras1*$^-$ *ras2*$^-$ 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 *bcy1 ras1*$^-$ *ras2*$^-$ 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 *bcy1 ras1*$^-$ *ras2*$^-$ 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

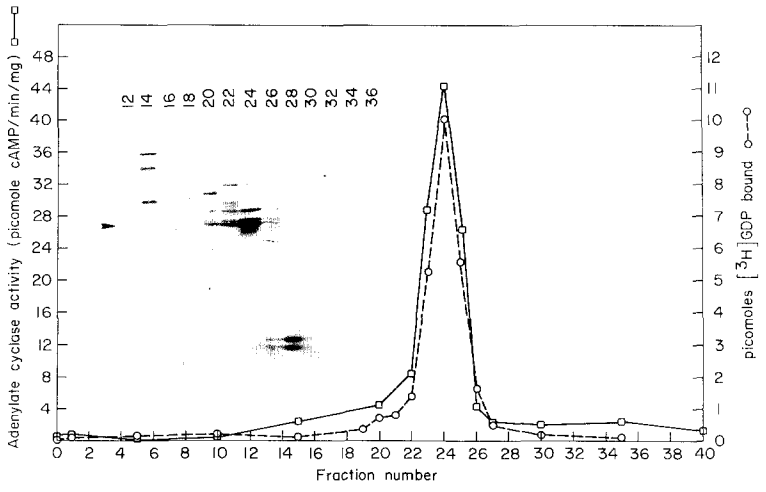

Figure 1. Copurification of *RAS2* and Adenylate-Cyclase-Stimulating Activity

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 μl aliquots of fractions. For reconstitution of adenylate cyclase activity, 5 μl of indicated fractions were incubated with 1 μl 1 mM Gpp(NH)p for 30 min at 37°C. The samples were chilled and incubated with 30 μg of *bcy1 ras1*$^-$ *ras2*$^-$ 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 α-^{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.

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

	f-*RAS2*^{val19}		f-*RAS2*		Buffer *G*	
Antibody	Mg	Mg + Gpp(NH)p	Mg	Mg + Gpp(NH)p	Mg	Mg + Gpp(NH)p
Y13–259	0.9	1.4	0.4	0.5	0.2	0.4
Y13–238	2.4	44.4	4.1	50.8	0.4	0.4
None	3.1	72.0	4.8	68.0	0.2	0.2

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 βMSH (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 μg of *bcy1 ras1*$^-$ *ras2*$^-$ 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 α-^{32}P-ATP started the 30 min reaction.

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′(β,γ-imino)triphosphate (Gpp(NH)p). For the assay we used crude membranes prepared from *bcy1 ras1*$^-$ *ras2*$^-$ yeast cells. The *bcy1* mutation is needed to maintain viability in *ras1*$^-$ *ras2*$^-$ 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 manganese 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 coeluted with a factor that activates yeast adenylate cyclase in *bcy1 ras2*$^-$ *ras2*$^-$ 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 cross-reacts with yeast *RAS2* protein (Powers et al., 1984), or after immunoprecipitation with the H-*ras*-specific antibody Y13-238, which does not cross-react with the yeast *RAS2* protein (data not shown). Immunoprecipitation with Y13-259, but not with Y13-238, eliminates the activating factor from pooled fractions (Table 1).

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

Protein Added	Mg	Mg + Gpp(NH)p
f-*RAS2*	3.1	72
f-*RAS2*^{val19}	4.8	68
RAS2	3.4	66
RAS2^{val19}	4.5	73
H-*ras*	0.7	19.3
H-*ras*^{val12}	0.4	15.7
f-*RAS1*	0.4	21.3
H-*ras*^{val12}/*RAS2*	3.8	76
None	0.2	0.1

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 *bcy1 ras1*$^-$ *ras2*$^-$ membrane proteins (in 30 μl buffer D) followed by the addition 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 [^{32}P]ATP (see Experimental Procedures). The values given are expressed in units of adenylate cyclase activity (pmol cAMP/min/mg of membrane protein). See Experimental Procedures for a description of purified *RAS* proteins.

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* proteins (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-*ras*^{val12} 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-*ras*^{val12} protein were identified by ^{3}H-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.

Relative Stimulation of Adenylate Cyclase with Various *RAS* Proteins

Varying amounts of three purified *RAS* proteins, f-*RAS2*, f-*RAS2*^{val19}, 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 ^{3}H-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*, *RAS2*^{val19}, f-*RAS2*, and f-*RAS2*^{val19} showed comparable maximal

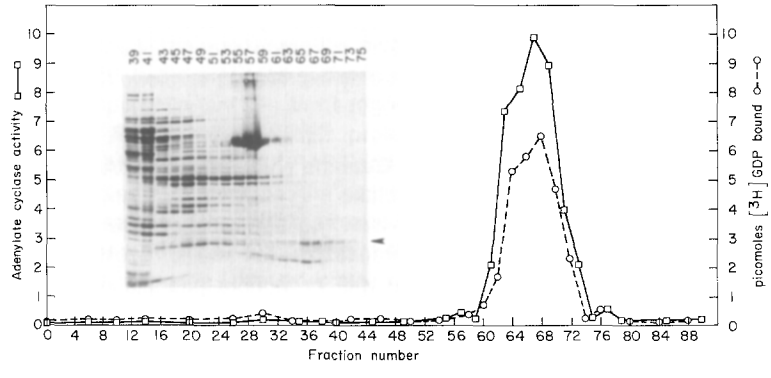

Figure 2. Copurification of H-*ras* and Adenylate-Cyclase-Stimulating Activity

Partially purified H-*ras*^{val12} protein was applied to a Sephadex G75 gel filtration column (95 cm × 2.5 cm) and 4.5 ml fractions collected. ^{3}H-GDP binding activity was determined on 5 μl aliquots of column fractions using a nitrocellulose filter binding assay. Column fractions were also assayed for the ability to restore adenylate cyclase activity to *bcy1 ras1*$^-$ *ras2*$^-$ membranes. Five microliters of column fractions were incubated with 1 μl of 1 mM Gpp(NH)p at 37°C for 30 min. After the samples were chilled on ice, 30 μl of *bcy1 ras1*$^-$ *ras2*$^-$ membranes (30 μg protein) were added, and the incubation continued for 30 min at 0°C. Then ATP was added to a final concentration of 0.5 mM and the samples were incubated at 27°C for 15 min. The addition of a mixture containing α-^{32}P-ATP started the 30 min reaction. SDS-polyacrylamide gel electrophoresis of the Sephadex G75 column fractions shows a 21,000 molecular weight protein identified as H-*ras*^{val19} by immunoprecipitable GDP binding activity which elutes at fractions containing GDP binding activity and a factor which stimulates adenylate cyclase. Adenylate cyclase activity is expressed in units, where 1 unit is equal to the production of 1 pmol of cAMP/min/mg of membrane protein.

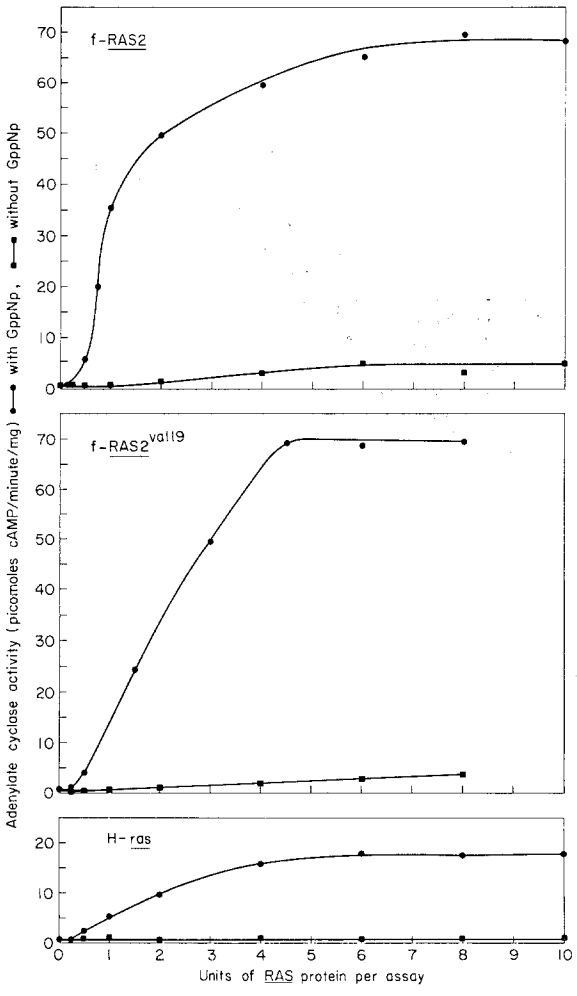

Figure 3. Stimulation of Adenylate Cyclase with Increasing Concentrations of *RAS* Proteins

Increasing amounts of *RAS* proteins, (A) f-*RAS2*, (B) f-*RAS2*^{val19} or (C) human H-*ras* were used to induce adenylate cyclase activity in *bcy1 ras1*$^-$ *ras2*$^-$ membranes. The *RAS* proteins (0.125 to 10 units) in 10 μl of buffer G were incubated with (●—●) or without (■—■) Gpp(NH)p at 37°C for 30 min. After the samples were chilled, 30 μg of *bcy1 ras1*$^-$ *ras2*$^-$ membranes in 30 μl buffer D were added and the mixture incubated on ice for 30 min. ATP (2.5 μl, 20 mM) was added, and the samples were incubated at 27°C for 30 min. The addition of a mixture containing α-^{32}P-ATP started the reaction. The reactions were carried out at 32°C for 30 min. One unit of *RAS* is defined as that which will bind 1 pmol of ^{3}H-GDP in 30 min at 37°C (see Experimental Procedures), and 1 unit of adenylate cyclase activity is defined as the production of 1 pmol of cAMP/min/mg of membrane protein.

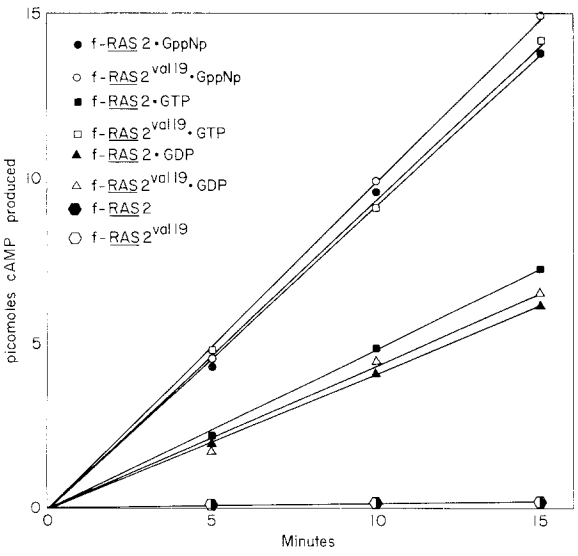

Figure 4. Guanine Nucleotide Requirements for *RAS* Function

Two units of f-*RAS2* and f-*RAS2*^{val19} were incubated with 5 pmol of Gpp(NH)p, GTP, GDP, or without nucleotide in 10 μl of buffer G for 30 min at 37°C (conditions that hydrolyzed essentially all GTP bound to f-*RAS2* and approximately 10% of that bound to *RAS2*^{val19}). After addition of 30 μg of *bcy1 ras1*$^-$ *ras2*$^-$ membrane protein in 30 μl buffer D, the samples were set on ice for 30 min followed by the addition of 2.5 μl of 20 mM ATP and incubation at 27°C for 15 min. The reactions were initiated by the addition of a mixture containing α-^{32}P-ATP. The reactions were stopped as described under Experimental Procedures, and the amount of cAMP produced was determined at 5, 10, and 15 min. Each data point is the average of duplicate samples that did not differ by more than 7%.

stimulation of adenylate cyclase activity, approximately 70 pmol cAMP/min/mg membrane protein. On the other hand, the maximal stimulation observed with f-*RAS1* was approximately one-quarter of this value. Thus, f-*RAS1* appears to be a less potent activator of adenylate cyclase than f-*RAS2*, an observation that is consistent with our previous genetic and biochemical data (see below). The maximal activity observed using the H-*ras* and H-*ras*^{val12} proteins was also comparatively low, approximately 17 pmol cAMP/min/mg membrane protein. This agrees with our previous findings that yeast cells with disrupted endogenous *RAS* genes grow only poorly when expressing the H-*ras* protein (Kataoka et al., 1985). Finally, a chimeric *RAS* (H-*ras*^{val12}/*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 *bcy1 ras1*$^-$ *ras2*$^-$ membranes after the addition f-*RAS2*^{val19} protein preincubated with or without guanine nucleotides (Gpp(NH)p, 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 f-*RAS2*^{val19} protein preincubated with Gpp(NH)p. Since Gpp(NH)p cannot be hydrolyzed, we conclude that GTP hydrolysis is not required for activation of *RAS*.

When f-*RAS2*^{val19} was preincubated with GTP it stimulated adenylate cyclase to the same extent as when prein-

cubated with Gpp(NH)p. However, when f-*RAS2*^{val19} protein was preincubated with GDP, it induced a rate of cAMP production approximately one-half of that induced by f-*RAS2*^{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′-0-(2-thiodiphosphate), GDPβS, 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*^{val19}, but not to the same extent as guanine triphosphates.

The f-*RAS2* protein, like the f-*RAS2*^{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*^{val19} protein. While f-*RAS2*^{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*^{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*^{val19} proteins as with the N-terminal fusion proteins f-*RAS2* and f-*RAS2*^{val19}. From these studies it appears that the addition of amino acids at the N terminus of the f-*RAS2* proteins does not seriously perturb their function as reflected in this assay system. The *RAS1*- and H-*ras*-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-*ras*^{val12}/*RAS2*, containing the first 73 N-terminal positions of the H-*ras* 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*$^{+}$ *ras2*$^{-}$ cells have a premature sporulation phenotype not seen in *ras1*$^{-}$ *RAS2*$^{+}$ cells (Toda et al., 1985); and they have considerably lower intracellular cAMP levels than the latter (Toda et al., 1985). Second, *ras1*$^{-}$ *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, GDPβS, serves as well as GDP in this assay system (data not shown), and it is not possible to regenerate a triphosphate directly from GDPβS. 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*^{val19} proteins. *RAS2*^{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*^{val19} protein (Tamanoi et al., 1985), and by the observation that the *RAS* 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*^{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*^{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, G_s and G_i, 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, G_o and transducin, show some sequence homology (Hurley et al., 1984). The G proteins are oligomers comprising an α subunit that binds guanine nucleotides and a β-γ complex that presumably inhibits α subunit function. Among the various G proteins, the β-γ 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 β-γ 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 G_s 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 f-*RAS2* and f-*RAS2*^{val19} are described elsewhere in detail (Tamanoi et al., 1985). The f-*RAS2* proteins contain these 20 additional amino acids N-terminal to the first methionine of the intact *RAS2* protein: Met Thr Met Ile Thr Asn Ser Asn Arg Phe Arg Ile Glu Arg Arg Tyr Thr Glu Lys Lys. The yeast *RAS1* fragment was inserted into a pUC8 vector (Messing and Vieira, 1982) to produce the f-*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 construction of the H-*ras* and H-*ras*^{val12} expression systems has been described (Gross et al., 1985). The H-*ras*^{val12}/*RAS2* chimeric gene was described previously (Toda et al., 1985). The H-*ras*^{val12}/*RAS2* chimeric protein and the intact *RAS2* and *RAS2*^{val19} proteins were produced in E. coli by inserting their genes into the same λP_L heat-inducible promotor vector system used to express the intact H-*ras* proteins.

Purification of *RAS* Proteins

The yeast f-*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 were purified as described (Gross et al., 1985). The intact *RAS2* proteins and the chimeric H-*ras*^{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 $MgCl_2$, 2 mM 2-mercaptoethanol (βMSH); 20 mM KCl, 10 μM 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 × 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 ^{3}H-GDP. The peak fractions contain *RAS2* proteins were pooled, and the *RAS* proteins were found to precipitate between 45% and 55% $(NH_4)_2SO_4$ saturation. The resolubilized *RAS2* proteins were dialyzed against 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM βMSH (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°C in buffer G in the presence of 3 μM ^{3}H-GDP. Both of the H-ras proteins (H-*ras* and H-*ras*^{val12}) as well as the yeast *RAS2* proteins (f-*RAS2*, f-*RAS2*^{val19}, *RAS2*, *RAS2*^{val19}) 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 [^{3}H]-GDP.

While searching for conditions to store the *RAS* proteins (H-*ras*, f-*RAS2*, and *RAS2*) we found that storage of the *RAS* proteins in buffer G at −20°C completely inactivated the adenylate cyclase stimulatory activity of the protein, although little or no loss of ^{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°C in the presence of 50% glycerol retained more than 80% 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 dounce 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 $MgCl_2$, 0.1 mM EGTA, 1 mM βMSH, 2 mM phenylmethyl sulfonyl fluoride, and 1 μg/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°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 ^{32}P-cAMP produced was determined as described (Solomon et al., 1973). All adenylate cyclase assays contained 100 μl final volume with final concentrations of the following components: 20 units per ml creatine phosphokinase, 20 mM phosphocreatine, 20 mM MES (pH 6.2), 2.5 mM $MgCl_2$, 10 mM theophylline, 1 mM ^{3}H-cAMP (20,000 cpm per reaction); 0.1 mg per ml bovine serum albumin and 1 mM βMSH. 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 0.25% SDS, 5 mM ATP, 0.18 mM cAMP.

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

One hundred microliters of f-*RAS2* and f-*RAS2*^{val19} (2 units of f-*RAS2* protein per μl) in buffer O lacking GDP and KCl were incubated separately with 15 μl of monoclonal antibody Y13-259 (10 mg/ml), Y13-238 (10 mg/ml), or phosphate-buffered saline at 4°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°C. The samples were centrifuged for 1 min at 12,000 × 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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References

Capon, D., Seeburg, P., McGrath, J., Hayflick, J., Edman, U., Levinson, A., and Goeddel, D. (1983). Activation of Ki-*ras*2 gene in human colon and lung carcinoma by different point mutations. Nature *304*, 507–513.

Casperson, G., Walker, N., Brasier, A., and Bourne, H. (1983). A guanine nucleotide-sensitive adenylate cyclase in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. *258*, 7911–7914.

Defeo-Jones, D., Scolnick, E., Koller, R., and Dhar, R. (1983). *ras*-Related gene sequences identified and isolated from *Saccharomyces cerevisiae*. Nature *306*, 707–709.

Dhar, R., Nieto, A., Koller, R., Defeo-Jones, D., and Scolnick, E. (1984). Nucleotide sequence of two ras^H related-genes isolated from the yeast *Saccharomyces cerevisiae*. Nucl. Acids Res. *12*, 3611–3618.

Eckstein, F., Cassel, D., Levkovitz, H., Lowe, M., and Selinger, Z. (1979). Guanosine 5′-O-(2-thiodiphosphate): an inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. J. Biol. Chem. *254*, 9829–9834.

Ellis, R., DeFeo, D., Shih, T., Gonda, M., Young, H., Tsuchida, N., Lowy, D., and Scolnick, E. (1981). The p21 *src* genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature *292*, 506–511.

Furth, M., Davis, L., Fleurdelys, D., and Scolnick, E. (1982). Monoclonal antibodies to the p21 products of the transforming gene of Harvey sarcoma virus and of the cellular *ras* family. J. Virol. *43*, 294–304.

Gibbs, J., Sigal, I., Poe, M., and Scolnick, E. (1984). Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA *81*, 5704–5708.

Gilman, A. G. (1984). G proteins and dual control of adenylate cyclase. Cell *36*, 577–579.

Gross, M., Sweet, R., Sathe, G., Yokoyama, S., Fasano, O., Goldfarb, M., Wigler, M., and Rosenberg, M. (1985). Purification and characterization of human H-*RAS* proteins expressed in E. coli. Mol. Cell. Biol., in press.

Hurley, J., Simon, M., Teplow, D., Robishaw, J., and Gilman, A. (1984). Homologies between signal transducing G proteins and *ras* gene products. Science *226*, 860–862.

Iyengar, R., Abramowitz, J., Bordelon-Riser, M., Blume, A., and Birnbaumer, L. (1980). Regulation of hormone–receptor coupling to adenylyl cyclase: effects of GTP and GDP. J. Biol. Chem. *255*, 10312–10321.

Kataoka, T., Powers, S., McGill, C., Fasano, O., Strathern, J., Broach, J., and Wigler, M. (1984). Genetic analysis of yeast *RAS*1 and *RAS*2 genes. Cell *37*, 437–445.

Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J., and Wigler, M. (1985). Functional homology of mammalian and yeast *RAS* genes. Cell *40*, 19–26.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–686.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951). Protein measurement with a phenol reagent. J. Biol. Chem. *193*, 265–275.

Matsumoto, K., Uno, I., Oshima, Y., and Ishikawa, T. (1982). Isolation and characterization of yeast mutants deficient in adenylate cyclase and cyclic AMP dependent protein kinase. Proc. Natl. Acad. Sci. USA *79*, 2355–2359.

McGrath, J., Capon, D., Goeddel, D., and Levinson, A. (1984). Comparative biochemical properties of normal and activated human *ras* p21 protein. Nature *310*, 644–655.

Messing, J., and Vieira, J. (1982). A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene *19*, 269–276.

Muller, R., Slamon, D., Tremblay, J., Kline, M., and Verma, I. (1982). Differential expression of cellular oncogenes during pre- and postnatal development of the mouse. Nature *299*, 640–644.

Muller, R., Slamon, D., Adamson, E., Tremblay, J., Muller, D., Cline, M., and Verma, I. (1983). Transcription of c-onc genes c-ras^{Ki} and c-fms during mouse development. Mol. Cell. Biol. *3*, 1062–1069.

Neuman-Silberberg, F., Schejter, E., Hoffmann, F., and Shilo, B.-Z. (1984). The drosophila *ras* oncogenes: structure and nucleotide sequence. Cell *37*, 1027–1033.

Papageorge, A., Lowy, D., and Scolnick, E. (1982). Comparative biochemical properties of p21 *ras* molecules coded for viral and cellular *ras* genes. J. Virol. *44*, 509–519.

Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. (1984). Genes in S. cerevisiae encoding proteins with domains homologous to the mammalian *ras* proteins. Cell *36*, 607–612.

Reddy, E., Reynolds, R., Santos, E., and Barbacid, M. (1982). A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature *300*, 149–152.

Shih, T., Papageorge, A., Stokes, P., Weeks, M., and Scolnick, E. (1980). Guanine nucleotide-binding and autophosphorylating activities associated with the $p21^{src}$ protein of Harvey murine sarcoma virus. Nature *287*, 686–691.

Shih, T., Stokes, P., Smythes, G., Dhar, R., and Oroszian, S. (1982). Characterization of the phosphorylation sites and the surrounding amino acid sequences of the p21 transforming proteins coded for by the Harvey and Kirsten strains of murine sarcoma viruses. J. Biol. Chem. *257*, 11767–11773.

Shilo, B., and Weinberg, R. (1981). DNA sequences homologous to vertebrate oncogenes are conserved in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA *78*, 6789–6792.

Shimizu, K., Birnbaum, D., Ruley, M., Fasano, O., Suard, Y., Edlund, L., Taparowsky, E., Goldfarb, M., and Wigler, M. (1983). The structure of the K-*ras* gene of the human lung carcinoma cell line Calu-1. Nature *304*, 497–500.

Solomon, Y., Landos, C., and Rodbell, M. (1973). A highly sensitive adenylate cyclase assay. Anal. Biochem. *58*, 541–548.

Sweet, R., Yokoyama, S., Kamata, T., Feramisco, J., Rosenberg, M., and Gross, M. (1984). The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature *311*, 273–275.

Tabin, C., Bradley, S., Bargmann, C., Weinberg, R., Papageorge, A., Scolnick, E., Dhar, R., Lowy, D., and Chang, E. (1982). Mechanism of activation of a human oncogene. Nature *300*, 143–148.

Tamanoi, F., Samiy, N., Rao, M., and Walsh, M. (1985). Enzymatic properties of yeast *RAS2* protein. In Cancer Cells III: Growth Factors and Transformation, J. Feramisco, B. Ozanne, and L. Stiles, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 251–256.

Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M., and Wigler, M. (1982). Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature *300*, 762–765.

Taparowsky, E., Shimizu, K., Goldfarb, M., and Wigler, M. (1983). Structure and activation of the human N-*ras* gene. Cell *34*, 581–586.

Tatchell, K., Chaleff, D., Defeo-Jones, D., and Scolnick, E. (1984). Requirement of either of a pair of *ras*-related genes of Saccharomyces cerevisiae for spore viability. Nature *309*, 523–527.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, *RAS* proteins are controlling elements of the cyclic AMP pathway. Cell *40*, 27–36.

Uno, I., Matsumoto, K., and Ishikawa, T. (1982). Characterization of cyclic AMP-requiring yeast mutants altered in the regulatory subunit of protein kinase. J. Biol. Chem. *257*, 14110–14115.

Uno, I., Matsumoto, K., Aduchi, K., and Ishikawa, T. (1983). Genetic and biochemical evidence that trehalase is a substrate of cAMP-dependent protein kinase in yeast. J. Biol. Chem. *258*, 10867–10872.

Yuasa, Y., Srivastava, S., Dunn, C., Rhim, J., Reddy, E., and Aaronson, S. (1983). Acquisition of transforming properties by alternative point mutations within C-*bas/has* human proto-oncogene. Nature *303*, 775–779.