Guanine nucleotide activation of, and competition between, RAS proteins from Saccharomyces cerevisiae.

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Guanine Nucleotide Activation of, and Competition between, RAS Proteins from *Saccharomyces cerevisiae*

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In the yeast *Saccharomyces cerevisiae*, yeast RAS proteins are potent activators of adenylate cyclase. In the present work we measured the activity of adenylate cyclase in membranes from *Saccharomyces cerevisiae* which overexpress this enzyme. The response of the enzyme to added RAS2 proteins bound with various guanine nucleotides and their analogs suggests that RAS2 proteins are active in their GTP-bound form and are virtually inactive in their GDP-bound form. Also, active RAS2 protein is not inhibited by inactive RAS2, suggesting that the inactive form does not compete with the active form in binding to its effector.

Although the normal *ras* genes are not oncogenic, they too can cause the malignant transformation of cells when expressed at abnormally high levels (7). In mammals, RAS proteins are 21,000-dalton molecules that are localized to the cytoplasmic side of the cell membrane (44). They bind GTP and GDP (30) and have an intrinsic GTPase activity (14, 21, 35). Moreover, some oncogenic forms of Ras protein are deficient in GTPase activity (14, 21, 35). Based on these properties, and reasoning by analogy to other known guanine nucleotide-binding proteins, the G proteins (reviewed in reference 15), most investigators think that Ras proteins act as transducers to convey extracellular signals to an intracellular effector pathway. According to this model, Ras protein bound to GTP activates its effector, and then shuts itself off through its intrinsic GTPase activity. Mutant Ras proteins which are defective in GTPase would thus cause abnormally prolonged stimulation of the effector system, explaining their oncogenicity.

The RAS1 and RAS2 proteins of the yeast *Saccharomyces cerevisiae* provide an ideal system for testing aspects of this model. They are structurally, biochemically, and functionally similar to the mammalian Ras proteins, and at least one of their effector systems is known (3, 9, 10, 18, 24, 37, 41, 42). The yeast RAS genes were originally isolated by using mammalian *ras* genes as probes to screen libraries of *S. cerevisiae* genomic DNA. They encode proteins which are highly homologous to the mammalian Ras proteins, particularly at their amino termini (9, 24). They undergo processing events very similar to those of their mammalian counterparts and localize to membrane fractions (8, 13, 25, 31, 44). Like the mammalian proteins, they bind guanine nucleotides; they have an intrinsic GTPase activity; and this activity is reduced in the mutant RAS2Val19Gln protein which has an amino acid substitution analogous to one of the oncogenic forms of the mammalian Ras proteins (37, 41; unpublished observations). RAS1 and RAS2 proteins are activators of yeast adenylate cyclase, and this appears to be their major physiological effector (3, 42).

We have previously shown that yeast and mammalian Ras proteins purified from *Escherichia coli* vector expression systems are potent activators of the adenylate cyclase present in crude fractions of yeast membranes (3). Surprisingly, we observed that RAS2 protein bound to GDP appeared to activate adenylate cyclase although activation was only one half to one third that of RAS2 protein bound to a nonhydrolyzable GTP analog. We have since cloned the yeast gene encoding adenylate cyclase (17), and that has enabled us to create yeast strains which greatly overexpress that enzyme. As a result, we have been able to develop a more sensitive biochemical assay for Ras proteins. Using this system we have reexamined the guanine nucleotide dependence of RAS2 protein and conclude that RAS2 may be active only in its GTP-bound form. Moreover, we have found that RAS2 bound with a GDP analog does not compete with RAS2 bound with a GTP analog for activating its effector. This finding has relevance for understanding how overexpression of a normal Ras protein can be oncogenic.

**MATERIALS AND METHODS**

**Construction of plasmid pADH-CYRI.** The entire coding sequence of the *CYRI* gene was isolated from pCYRI-2(SalI) (17) by complete digestion with SalI and partial digestion with *ClaI*. The 8.1-kilobase fragment was cloned into YRp7, which was digested with *SalI* and *ClaI* to produce YRp7-CYRI as shown in Fig. 1. YRp7 contains the *TRPI* marker and *ARS1* for replication as a multicopy plasmid in *S. cerevisiae* (34). Then YRp7-CYRI was cleaved with *SalI* and ligated with a 400-base-pair *Sphl*-HindIII fragment containing the yeast alcohol dehydrogenase promoter (1) after their cohesive ends were filled in or removed by treatment with Klenow fragment of *E. coli* DNA polymerase I. The *Sphl*-HindIII fragment had been isolated from pLD94 obtained from L. Davidow and M. Gollaher of Pfizer Central Research (Groton, Conn.). The direction of the *ADHI* promoter inserted was confirmed by the reconstruction of a *SalI* cleavage site at the junction of the filled-in *Sall* and *Sphl* sites.

**Growth of S. cerevisiae and construction of strain TK-B111.** *S. cerevisiae* was grown in YPD (1% yeast extract, 2% peptone, and 2% glucose) or, to maintain selective pressure for plasmids, in synthetic medium (0.67 g of yeast nitrogen base per liter, 2% glucose, and appropriate auxotrophic.
supplements). General genetic manipulations of *S. cerevisiae* were performed as described by Mortimer and Hawthorne (23). Yeast transformations were performed as described (16). The strain TK-B111 (MATa ras1::HIS3 ras2::URA3 trp1 leu2 ade8 can1 pADH-CYRI) was constructed by transforming the plasmid pADH-CYRI into strain SPK1 (MATa ras1::HIS3 ras2::URA3 trp1 leu2 ade8 can1 pTLC-RAS2), followed by screening for segregants that lost the RAS2-bearing plasmid but retained pADH-CYRI. The plasmid pTLC-RAS2 contains the yeast RAS2 gene cloned into a plasmid that confers sensitivity to canavanine. Details of this construction will be described elsewhere.

**Preparation of yeast extracts and purification of RAS2.** The preparation of yeast membrane extracts has been previously described (3, 42). RAS2 and RAS2Val-19 were purified from *E. coli* isolates containing expression systems as previously described (3). These *E. coli* strains express complete RAS2 proteins using the lambda pL promoter.

**Assays.** Adenylate cyclase assays were performed as described (3, 28, 42) except that an ATP-generating system was omitted. The legend to Fig. 3 describes a typical assay in more detail. All assays were carried out with 10 μg of yeast extracts. Divalent cations, RAS2, and guanine nucleotides were varied as indicated in the figure legends. Incubations were for 30 min at 30°C. The data are expressed as units of adenylate cyclase, where 1 unit catalyzed the formation of 1.0 pmol of cyclic AMP per min per mg of yeast membrane protein. Similar results were obtained with at least two preparations each of RAS2, RAS2Val-19, and yeast membrane extracts. The experiments reported here were carried out with the same preparations of reagents and repeated at least twice. Mn²⁺ activity and maximum RAS activation varied by less than 50% between experiments, and duplicate data points within an experiment varied by less than 20%. Guanine nucleotide binding was measured using a nitrocellulose filter binding assay. Protein assays were performed by the method of Bradford (2) with reagents from BioRad Laboratories (Richmond, Calif.).

**RESULTS**

**Assay for RAS using membranes from cells which overexpress adenylate cyclase.** Our present assays for RAS protein stimulation of adenylate cyclase differ from our previous assays in several significant respects. First, we use RAS2 protein extracted from an *E. coli* expression system intended to express the intact RAS2 protein. Previous studies utilized mainly an N-terminal fusion protein called f-RAS2 (3). Second, our source of yeast adenylate cyclase derives from membranes of yeast cells which greatly overexpress a cloned adenylate cyclase gene. As a consequence, the current assay has greater sensitivity. Thus, third, we have been able to omit an ATP-regenerating system. Fourth, we have performed guanine nucleotide exchange experiments with RAS2 in the presence of EDTA, since we have observed that nucleotide exchange is quite slow in the presence of free Mg²⁺ ions, and we monitored results with radiolabeled nucleotides.

The RAS2 proteins used in these studies were purified from an *E. coli* expression system which was previously described. Sodium dodecyl sulfate-polyacrylamide gels of the purified RAS2 and RAS2Val-19 proteins shown in Fig. 2 indicate that about 90% of the protein in this preparation is in a band of 37 kilodaltons (kDa), somewhat smaller than the 42-kDa protein predicted from the nucleotide sequence of RAS2. Indeed, Western blot analysis of the purified protein with an anti-RAS2 antiserum revealed two bands, one at 42 kDa and another at 37 kDa (data not shown), suggesting that
the 37-kDa form of the RAS2 is a degradation product. Amino-terminal sequence analysis indicated that the RAS2 protein began with the second amino acid predicted from the DNA sequence (24). Thus the 37-kDa product may lack up to 50 amino acids from the carboxyl terminus, including the cysteine residue(s) likely to be essential for posttranslational modifications and membrane targeting of Ras proteins in vivo (45). In Coomassie blue-stained gels, the percentage of the 42-kDa band to the total RAS2 protein varied from less than the 10% shown in Fig. 2 to more than 50% in other preparations. However, all preparations were equally potent in binding guanine nucleotides and in activating adenylate cyclase. Apparently, modifications dependent upon an intact C terminus are not required for RAS2 function in vitro, as discussed by Powers et al. (25). Under saturating guanine nucleotide exchange conditions, 1 pmol of RAS2 or RAS2Val19 bound 0.5 to 1.0 pmol of GDP (data not shown).

Our current assay system uses membranes from ras1-ras2 cells which express greatly elevated levels of adenylate cyclase. To create such cells, we expressed the cloned yeast adenylate cyclase gene. CYRI (17), using the powerful yeast alcohol dehydrogenase promoter (1) and a high-copy-number plasmid, pADH-CYRI (Fig. 1). This plasmid is capable of suppressing the lethality which otherwise results from loss of both RAS genes (data not shown) (19, 40). Membranes from the resulting ras1-ras2 pADH-CYRI-bearing strain contain approximately 20-fold higher levels of adenylate cyclase activity than wild-type strains when assayed with Mn2+ as the divalent cation. The Mn2+-dependent adenylate cyclase activity is not dependent on RAS proteins (3, 6, 42).

The adenylate cyclase expressed in abundance in the ras1-ras2 pADH-CYRI strain is fully RAS responsive. In the absence of added RAS2 protein, we observed low Mg2+-dependent adenylate cyclase activity in membranes prepared from these strains. However (Fig. 3), activity could be stimulated 20- to 50-fold by purified RAS2 protein in the presence of both Mg2+ and GTP. As in previous experiments (3), stimulation was saturable, and at the maximal level of stimulation, the Mg2+-RAS2-GTP-stimulated activity was about equal to the Mn2+-stimulated activity. The maximum level of stimulation by RAS2 protein was 20- to 50-fold higher than the maximum previously observed in ras1-ras2 membranes. These experiments suggest that if RAS proteins act on adenylate cyclase through an intermediate protein, this intermediate protein is not present in limiting amounts in the pADH-CYRI-bearing strain.

In most of the assays presented below, we monitored the extent of guanine nucleotide binding to RAS2 protein. To do this, we first bound a small amount of a radioactive "tracer" nucleotide to RAS2 protein. We then incubated the protein with the unlabeled nucleotide we wished to bind. The extent to which the tracer was displaced was determined by a nitrocellulose filter binding assay. As our work progressed it became clear that RAS2 protein purified from E. coli must come already bound with a guanine nucleotide, and that guanine nucleotide binding is actually an exchange reaction was 25 mM during guanine nucleotide exchange, and the concentration of MgCl2 used to chelate the additional EDTA was 40 mM. Also, the concentration of GDP-βS was increased to 1 mM. These changes were required to maximize exchange of the [α-32P]GTP. The 100% point in this experiment corresponds to 51,200 cpm. Amounts of original nucleotide retained: with GTP added, 13%; with GDP-βS, 5.5%.

FIG. 3. Response of yeast adenylate cyclase to RAS2 proteins and guanine nucleotides (with accelerated exchange). (A) Effect of wild-type protein. Guanine nucleotide exchange reactions were monitored as follows. A 140-μg sample of RAS2 was mixed with 10 μCi of [3H]GDP, the "tracer" nucleotide, at 7 Ci/mmol in a total volume of 100 μl of buffer G (20 mM Tris hydrochloride buffer, pH 7.5, 20 mM NaCl, 5 mM β-mercaptoethanol, 3 mM MgCl2) supplemented with 7.5 mM EDTA and then incubated for 30 min at 37°C. A sample of 1.0 μl was removed, and the [3H]GDP bound to RAS2 was measured by binding to nitrocellulose filters. This value (18,300 cpm) was used as the 100% point. Next, 15 μl of the above reaction mixture was incubated with 15,000 pmol of the indicated "exchange" nucleotide in a volume of 30 μl for 60 min at 37°C and then transferred to 0°C. A portion (2 μl) of this mixture was removed, and the [3H]GDP remaining bound to RAS2 was determined by filter binding. This determined the extent of nucleotide exchange or replacement (amounts of original nucleotide retained: with GTP added, 0.8%; with GDP, 7.7%; with GDP-βS, 5.5%). The remainder of the mixture was added to adenylate cyclase assays as follows. Samples ranging from 0.5 to 10.0 μl were mixed with equal volumes of 15 mM MgCl2 to give an excess of MgCl2 over EDTA. Next, the samples were diluted to 20 μl of buffer G containing the indicated guanine nucleotide, such that the final concentration of the nucleotide was 250 μM. Ten-microgram samples of membranes from ras1-ras2 pADH-CYRI yeast strains (see Materials and Methods) were added in a volume of 30 μl, and the samples were incubated for 15 min at 0°C. Next, 50 μl of assay mix was added to the following final concentrations: 20 mM MES (2-(N-morpholino)ethanesulfonic acid; pH 6.2), 1 mM β-mercaptoethanol, 2.5 mM MgCl2 (or, where indicated, 2.5 mM MnCl2), 0.5 mM cyclic AMP, 1 mM ATP, and 20 μCi of [α-32P]ATP (810 Ci/mmol) to a specific activity of 100 to 300 cpm/pmol. Reactions were stopped, and cyclic AMP was measured.

(B) Effects of RAS2Val19 protein. This experiment was performed as described for panel A with the following modifications: RAS2Val19 was used instead of RAS2, 10 μCi of [α-32P]GTP was used instead of [3H]GDP as the "tracer" nucleotide, the concentration of EDTA
TABLE 1. Guanine nucleotide exchange in presence of MgCl₂

<table>
<thead>
<tr>
<th>Exchange nucleotide</th>
<th>Labeled tracer nucleotide</th>
<th>% Label retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>[³H]GDP</td>
<td>100</td>
</tr>
<tr>
<td>GDP</td>
<td>[³H]GDP</td>
<td>70</td>
</tr>
<tr>
<td>GTP</td>
<td>[³H]GDP</td>
<td>81</td>
</tr>
<tr>
<td>Gpp(NH)₃p</td>
<td>[³H]GDP</td>
<td>81</td>
</tr>
<tr>
<td>GDP·BS</td>
<td>[³H]GDP</td>
<td>81</td>
</tr>
</tbody>
</table>

None [³P]GTP 100 100
GDP [³P]GTP 73 83
GTP [³P]GTP 70 83
Gpp(NH)₃p [³P]GTP 76 89
GDP·BS [³P]GTP 72 88

* RAS proteins were preincubated for 60 min with 1 μM either [α-³²P]GTP (60,000 cpm/pmol) or [³H]GDP (3,000 cpm/pmol) in the presence of buffer G (2 mM Tris hydrochloride buffer, pH 7.5, 20 mM NaCl, 5 mM β-mercaptoethanol, 3 mM MgCl₂). RAS2 (1.5 μg) or RAS2Val-19 (5.2 μg) was subsequently incubated with 50 μM of the indicated exchange nucleotides. Incubations (50 μl) were for 30 min at 37°C in the presence of buffer G. The samples were then filtered through nitrocellulose filters. The value of 100% label retained represents the following absolute values: 24.3 pmol of GDP·BS-bound GDP, 22.9 pmol of GDP·BS-bound GDP, 14.5 pmol of GDP·BS-bound GDP, and 14.8 pmol of GDP·BS-bound GDP.

which occurs slowly in the presence of free magnesium ions (Table 1). In the presence of EDTA, an Mg⁺ ion chelator, nucleotide exchange was greatly accelerated (see legend to Fig. 3). The slow exchange in the presence of free Mg⁺ was exploited in one experiment (see Fig. 4).

Guanine nucleotide dependence of RAS2 proteins. We measured the ability of wild-type RAS2 and RAS2Val-19 proteins to stimulate the Mg²⁺-dependent adenylate cyclase activity when bound to various guanine nucleotides. Adenylate cyclase was greatly stimulated by the addition of RAS2 or RAS2Val-19 proteins bound to GTP (Fig. 3). Saturation of the Mg²⁺-dependent adenylate cyclase activity was achieved at about 5 μg of either RAS2-GTP or RAS2Val-19-GTP. As we have observed previously (3), RAS2 protein bound to GDP was able to activate adenylate cyclase, although only one fourth as well as protein bound to GTP (Fig. 3A). One possible explanation for this is that RAS2 bound to GDP is indeed a weak stimulator of adenylate cyclase. Another explanation is that, due to an ubiquitous dinucleotide kinase in cell membranes (20), the activity seen with RAS2 bound to GDP results from the conversion of free GDP to GTP followed by nucleotide exchange. Support for the latter explanation comes from experiments with the nucleotide GDP·BS [guanosine-5'-O-(2-thiodiphosphate)], which cannot be directly converted to GTP. Very little stimulation of adenylate cyclase was observed after the addition of up to 7 μg of RAS2 or 16 μg of RAS2Val-19 protein bound to GDP·BS (Fig. 3). The inactive RAS2 proteins bound to GDP·BS could activate adenylate cyclase after the readdition of GTP, indicating that the failure of RAS2-GDP·BS complexes to stimulate adenylate cyclase was not due to denaturation of the RAS2 proteins (data not shown). In experiments in which RAS2 was compared directly with RAS2Val-19, both activated adenylate cyclase with identical dose-response curves when in their GTP-bound state (data not shown). These experiments suggest that RAS2 and RAS2Val-19 proteins in their GDP-bound form are potent activators of yeast adenylate cyclase, whereas these proteins in their GTP-bound form may be virtually inactive. The difference in potency of the two forms may be as great as 20-fold. Recently, De Vendittis et al. reported similar results using wild-type yeast RAS2 (11).

RAS2 bound with GDP·BS does not inhibit stimulation of adenylate cyclase by RAS2 bound with Gpp(NH)₃p. The observations that nucleotide exchange occurs slowly in the presence of Mg²⁺ (Table 1) allowed us to test the competition between active and inactive forms of RAS2 (Fig. 4). To do this we bound wild-type RAS2 with Gpp(NH)₃p [guanosine-5'-β,γ-imino]triphosphate], a nonhydrolyzable GTP analog. Under conditions in which essentially complete nucleotide exchange could be demonstrated, Gpp(NH)₃p-bound RAS2 activated adenylate cyclase as well as GDP·BS-bound RAS2 (not shown). We bound another sample of RAS2 to GDP·BS. Both nucleotides were bound to RAS2 in the presence of EDTA to accelerate exchange, and then the proteins were dialyzed to remove unbound nucleotide and EDTA. Adenylate cyclase assays were then performed without exogenously added nucleotide. As expected, Gpp(NH)₃p-bound RAS2 activated adenylate cyclase, and GDP·BS-bound RAS2 did not activate adenylate cyclase. In competition assays the total amount of RAS2 was maintained at a saturating level, while the ratios of active to inactive RAS2 were varied over a 10-fold range. Essentially no inhibition was seen which could be attributed to competition between the active and inactive forms of RAS2, although noncompetitive inhibition was observed in some, but not all experiments. Thus we conclude that the inactive form of RAS2 does not compete with the active form of RAS2 for the stimulation of adenylate cyclase. Essentially identical results were obtained using GDP·BS-bound RAS2 and GTP-bound RAS2Val-19 (data not shown).

![FIG. 4. Competition between Gpp(NH)₃p-bound RAS2 and GDP·BS-bound RAS2. Wild-type RAS2 protein was treated with either GDP·BS or Gpp(NH)₃p in the presence of 25 mM EDTA in buffer G and then dialyzed against buffer G containing 50% glycerol to remove unbound nucleotides. [³H]GDP tracer binding indicated that 95% exchange had occurred for GDP·BS and 70% exchange had occurred for Gpp(NH)₃p. When re-exchanged with GTP, each protein could activate adenylate cyclase as well as untreated protein (not shown). (●) Gpp(NH)₃p-treated RAS2 alone; (○) GDP·BS-treated RAS2; (△) Gpp(NH)₃p-treated RAS2 plus GDP·BS-treated RAS2 (total RAS2 protein constant at 24 μg). The inset shows that RAS2 bound with GDP·BS is inactive.](http://mcb.asm.org/Downloaded from http://mcb.asm.org)
FIG. 5. Model for RAS2 activation of adenylate cyclase. See text for details.

DISCUSSION

We have modified the system for the in vitro biochemical assay of RAS protein function by using membranes from an *S. cerevisiae* strain overexpressing the adenylate cyclase gene. Interestingly, when overexpressed in *S. cerevisiae*, adenylate cyclase was fully responsive to RAS2. Thus, if there is an intermediate between RAS2 and adenylate cyclase, it is not present in limiting amounts. Another group has concluded that *S. cerevisiae* RAS proteins interact directly with adenylate cyclase, based on their report that the yeast adenylate cyclase gene, when expressed in *E. coli* is still activated by RAS (43). We have been unable to confirm these results (17). Moreover, the adenylate cyclase plasmid they used lacked regions of the gene which we know confer RAS regulation in *S. cerevisiae* (unpublished data). Further studies are required to determine whether RAS acts directly or indirectly on adenylate cyclase.

Our guanine nucleotide activation experiments (Fig. 3 and 4) suggest that both RAS2 and RAS2Val19 activate yeast adenylate cyclase when in their GTP-bound state. As we previously reported, GDP-bound RAS is capable of weakly stimulating adenylate cyclase (3). However, based on results with the GDP analog GDP-βS (Fig. 3 and 4), we believe that this weak stimulation results from rebinding of GTP generated from GDP. RAS2 bound to GDP-βS is at most 10% as active as RAS2 bound to GTP. Although we cannot completely exclude that RAS2 bound to the GDP analog assumes a nonphysiological conformation, RAS2 in this form can be reactivated by the addition of GTP.

Our competition data suggest that the active GTP-bound form of RAS2 binds its effector, while the inactive, GDP-bound form does not bind or does so less effectively. A simple consequence of the competition experiment is that the cellular signal due to RAS2 will be a function of the presence of the active form only, and not a function of the ratio of active to inactive forms. This is consistent with the genetic data in *S. cerevisiae* that the RAS2Val19 phenotype is dominant even in the presence of wild-type RAS2 (19). Taken together, our studies of RAS2 protein-adenylate cyclase interactions support the model, shown in Fig. 5, in which RAS2 shuttles between a conformationally active GTP-bound state and an inactive GDP-bound state. The amount of time spent in the active state may be determined by the intrinsic GTP hydrolysis rate as well as by other proteins which may interact with RAS2. Only the active conformation binds its effector.

The mammalian H-ras protein is capable of functioning in the *S. cerevisiae* system both in vivo and in vitro (3, 10, 18), so it is tempting to extend our conclusions to the mammalian cell. Some oncogenic forms of mammalian ras genes encode proteins that, like the yeast RAS2 Val19 proteins, have reduced GTPase activity (14, 21, 35). It has been proposed that transformation by these proteins results from a defective turn-off mechanism. Our experiments support this model, since we suggest that the GDP-bound form of RAS2 is virtually inactive. Moreover, mammalian cells can be transformed by overexpression of wild-type ras genes (7). Our competition data suggest that transformation in this case might result from an increase in the total amount of GTP-bound Ras, even though the ratio of the GTP-bound Ras to the GDP-bound form might not change.

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