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A product of yeast *RAS2* gene is a guanine nucleotide binding protein

(oncogenes/evolution)

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ABSTRACT **Yeast *Saccharomyces cerevisiae* contains two genes, *RAS1* and *RAS2*, which show remarkable homology to mammalian *ras* genes. To characterize these gene products, we have expressed the *RAS2* gene in yeast using an inducible *GAL10* promoter. After labeling with [^{35}S]methionine and immunoprecipitating with a monoclonal antibody Y13-259, which reacts with p21 encoded by mammalian *ras* genes, a major band having an apparent molecular weight of 41,000 is detected. This band has also been identified in cell-free translation products of polyadenylylated RNA extracted from yeast cells grown in the presence of galactose. Crude extracts of cells expressing the *RAS2* gene exhibit guanine nucleotide binding activity. This is detected by incubation with [^{3}H]GDP followed by immunoprecipitation with the antibody Y13-259. The binding of labeled GDP is inhibited by a 20-fold excess of GDP, GTP, and, to a lesser extent, by UTP, a characteristic similar to that possessed by the mammalian ras proteins. However, the activity of the yeast protein differs from that of the mammalian proteins in its strong dependence on temperature. The guanine nucleotide binding activity provides an assay to purify the yeast protein.**

Transfection of NIH 3T3 cells with DNAs from a variety of human tumors has resulted in the detection of transforming genes (1), most of which have been shown to be related to the oncogenes carried by Harvey and Kirsten viruses (2–5). These *ras* genes encode a M_r 21,000 protein (p21), which is localized at the inner surface of plasma membrane (6–8). The p21 contains a lipid moiety detected by palmitic acid labeling (9). An established biochemical activity of the p21 is its ability to bind guanine nucleotides (10, 11). In the case of viral p21, it also exhibits autophosphorylation activity using GTP as a phosphate donor (11).

Oncogenic variants of p21 are found as naturally occurring mutants or as a result of *in vitro* mutagenesis (reviewed in ref. 12). These studies indicate that there are five sites (the 12th, 13th, 59th, 61st, and 63rd amino acid residues) that could be altered to convert a normal p21 to a form active in the transformation of NIH 3T3 cells. At least for the change at the 12th amino acid it is known that the change does not significantly affect its guanine nucleotide binding activity (13, 14). Recently, Gibbs *et al.* (15) reported that the autophosphorylation of viral p21 is modulated by amino acid residue 12. However, a detailed biochemical study is needed to examine the difference between the wild-type protein and the oncogenic variants. To aid such a study, expression of the p21 in *Escherichia coli* has been accomplished recently (16, 17).

The yeast *Saccharomyces cerevisiae* contains two genes, *RAS1* and *RAS2*, which are highly homologous to mammalian *ras* genes (18, 19). Powers *et al.* (19) have shown that yeast cells overproducing the *RAS2* gene product contain proteins having molecular weights of 42,000 and 30,000 that can be immunoprecipitated by the monoclonal antibody Y13-259. Papageorge *et al.* (20) detected three proteins, each having a molecular weight of about 30,000, from wild-type yeast cells that cross-react with the monoclonal antibody. Neither the *RAS1* nor *RAS2* gene itself is essential for the growth of yeast, but yeast cells that have both *RAS1* and *RAS2* genes destroyed are incapable of vegetative growth (21, 22). The amino acid residues responsible for the transforming activity of the p21 are conserved in the yeast genes. Conversion of glycine to valine at the 19th amino acid of the yeast *RAS2* affects the physiology of yeast, including sporulation efficiency (22). This strongly suggests that the yeast *RAS* products function in a manner similar to that of the mammalian p21. Thus, yeast provides a convenient system for the enzymological study of ras proteins. In addition, powerful genetic approaches are available to confirm biochemical findings.

In this paper, we report characterization of the yeast RAS2 protein overproduced in yeast. The protein exhibits guanine nucleotide binding activity similar to that possessed by the mammalian p21. The activity provides a simple assay for the purification of the yeast protein. This is just the first step in ultimately understanding the biochemical activity of the yeast RAS proteins and the difference between wild-type protein and mutant proteins, which cause alterations in the growth of yeast.

MATERIALS AND METHODS

Chemicals and DNAs. [^{3}H]GDP (7.8 Ci/mmol; 1 Ci = 37 GBq) and [^{35}S]methionine (1106.6 Ci/mmol) were obtained from New England Nuclear. ATP, GTP, GDP, GMP, CTP, and UTP were purchased from Sigma. Plasmid YEp51 was obtained from J. Broach (23), and the *Hin*cII fragment containing the *RAS2* gene has been described (19).

Cell Labeling and Immunoprecipitation. *S. cerevisiae* KPPK-1 [derived from SP1 and SX 50-1C (22)] containing the YEp51-RAS2 were first grown at 31°C in a synthetic medium containing 2% (vol/vol) glycerol, 0.67% nitrogen base, and appropriate amino acids. Cells were then grown to 2.4×10^7 cells per ml in a synthetic medium containing 5% galactose or 2% glucose instead of glycerol. The cells were collected and resuspended in an appropriate synthetic medium lacking leucine and methionine. After growing at 31°C for 30 min, 250 μCi of [^{35}S]methionine was added to the 10-ml culture. The labeled cells were collected by centrifugation, washed once with phosphate buffered saline (P_i/NaCl) and kept frozen. After thawing, the cells were broken by mixing on a Vortex with glass beads in P_i/NaCl/1% Triton X-100/0.5% deoxycholate/1 mM phenylmethylsulfonyl fluoride/aprotinin (0.1 mg/ml) (Sigma). The extract was clarified and immunoprecipitated with a monoclonal antibody Y13-259 (8) and analyzed by $NaDodSO_4$/PAGE as described (5).

RNA Isolation and Cell-Free Translation. A 100-ml culture of cells grown to a density of 6×10^7 cells per ml was har-

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vested, washed with cold water, and resuspended in 1 ml of 20 mM Tris·HCl (pH 7.5)/0.3 M sucrose/10 mM EDTA/heparin (0.5 mg/ml). An equal volume of cold glass beads was added, and the mixture was mixed on a Vortex for 1 min. Ten volumes of 50 mM Tris·HCl (pH 7.5)/100 mM NaCl/5 mM EDTA/1% $NaDodSO_4$/heparin (0.5 mg/ml) was added. After mixing, the solution was extracted once with phenol, twice with phenol/chloroform, and once with ether. Nucleic acids were precipitated from the aqueous phase by adding NaCl to 0.3 M and an equal volume of isopropanol. The precipitate was resuspended in 1 ml of 50 mM Tris·HCl (pH 7.5)/10 mM EDTA/500 mM NaCl/0.1% $NaDodSO_4$, loaded onto a 0.5 ml oligo(dT) cellulose column, washed with 2 ml of 50 mM Tris·HCl (pH 7.5)/10 mM EDTA/500 mM NaCl, and eluted with 2 ml of H_2O. Cell-free translation of the polyadenylylated RNA using a reticulocyte lysate was carried out according to Stillman *et al.* (24).

Preparation of Extracts. KPPK-1 cells carrying YEp51-RAS2 were grown at 31°C in synthetic medium containing 5% galactose to 3×10^7 cells per ml. The cells were collected, washed with P_i/NaCl and frozen at −20°C. After thawing, the cells were resuspended in 2/3 vol of buffer A (10 mM Tris·HCl (pH 7.4)/50 mM NaCl/0.1 mM EDTA/0.1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride), 1% Triton X-100, and 5 mM $MgCl_2$. Glass beads (2/3 vol) were added and the cells were broken by mixing on a Vortex. The extract was clarified by centrifugation at 100,000 × *g* for 60 min. The protein concentration of such an extract was ≈10 mg/ml. The extract was stable when kept at −70°C but lost 70% of the activity when kept on ice for a week.

Nucleotide Binding Assay. Each reaction mixture contained buffer A, 5 mM $MgCl_2$, 1% Triton X-100, 20 μM [^{3}H]GDP (specific activity, 7.8 Ci/mmol), and various amounts of extracts in a volume of 50 μl. After incubation at a temperature indicated in the figure legends, 3 μl of monoclonal antibody Y13-259 was added and the mixture was rotated for 2 hr in a cold room. The sample was diluted 1:5 with buffer A and 5 mM $MgCl_2$, 1% Triton X-100, and 10 μl of rabbit anti-rat IgG (4 mg/ml from Cappel Laboratories, Cochranville, PA) was added. After 1 hr on ice, 40 μl of protein A Sepharose (Pharmacia) was added, and the sample was rotated in a cold room. The immune complexes were precipitated by centrifugation and washed 4 times with 1 ml each of buffer A, 5 mM $MgCl_2$, 0.01% Triton X-100. The precipitates were suspended in 60 μl of Laemmli solution (25), transferred to new tubes, and boiled for 3 min. After centrifugation, the supernatant was recovered and assayed for radioactivity in a Beckman scintillation counter. Under this condition, 5648 cpm represents 1 pmol of [^{3}H]GDP.

RESULTS

Cloning of *RAS2* Gene Under a *GAL10* Promoter. Since the *RAS2* gene is not heavily transcribed in normal cells (19), we decided to overproduce the gene product in yeast. A *GAL10* expression system (23) was chosen for this purpose, because a sharp induction of the expression is obtained. The expression is from a gene on a vector that replicates as an extrachromosomal plasmid. The strategy of cloning the *RAS2* gene into this expression vector is shown in Fig. 1. In addition to a *GAL10* promoter, which is inducible by the addition of galactose, the expression vector YEp51 contains a transcription termination site from the 2-μm circle, located distal to the promoter. Therefore, a 1161-base-pair *Hin*cII fragment containing the *RAS2* gene was inserted between the promoter and the termination site, using *Sal* I and *Bcl* I sites of YEp51. The *RAS2 Hin*cII fragment contains 39 base pairs upstream of the initiating ATG, in which there are no other initiation or termination codons. The orientation of insertion was verified by digesting the construct with *Pst* I. The clone

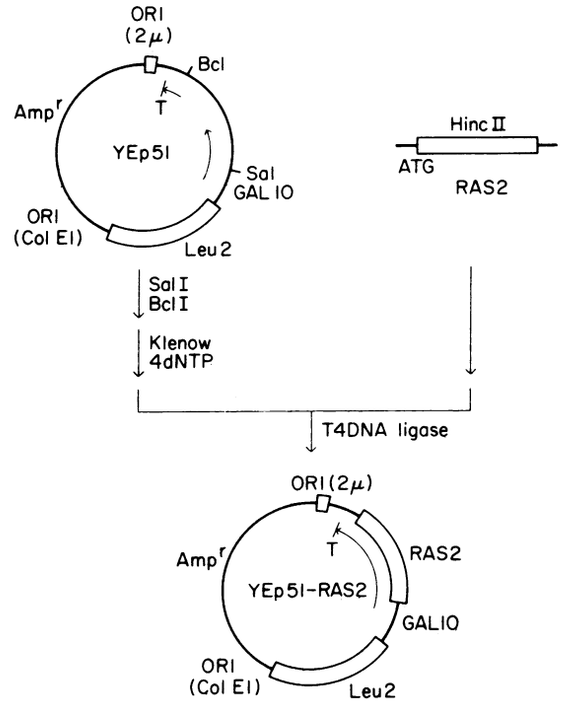

FIG. 1. Cloning of the yeast *RAS2* gene under control of *GAL10* promoter. Yeast expression vector YEp51 contains the ColE1 origin of replication and the gene for β-lactamase to facilitate its propagation in *E. coli*. The plasmid is grown in a *dam*$^-$ strain of *E. coli*. In addition, it contains the *LEU2* gene of yeast, the 2-μm circle origin of replication, and the *REP3* locus, as well as the *GAL10* promoter and transcription termination site (T). The YEp51 DNA was digested with *Sal* I and *Bcl* I, filled in with the Klenow fragment of *E. coli* DNA polymerase I, and ligated to the *Hin*cII fragment containing the yeast *RAS2* gene. A *Pst* I site is present in the *RAS2* gene 195 nucleotides from the ATG.

YEp51-RAS2 was then used to transform a leucine-requiring strain of yeast.

Expression of *RAS2* Gene in Yeast. To examine the expression of the *RAS2* gene, yeast cells carrying YEp51-RAS2 were grown in medium containing either galactose or glucose. The cells were labeled with [^{35}S]methionine, and the labeled proteins were subjected to immunoprecipitation with the monoclonal antibody Y13-259, which reacts with mammalian ras proteins. This antibody cross-reacts with yeast proteins related to the mammalian ras proteins (19, 20). As shown in Fig. 2*A*, immunoprecipitable bands shown by arrows were detected when the cells were grown in the presence of galactose but not detected when the cells were grown in the presence of glucose. A major band was observed at the position corresponding to M_r ≈41,000. Minor bands, one at M_r 38,000 and three others at M_r ≈30,000, were also observed. Only a faint band was detected at the M_r 41,000 protein position when cells containing the vector YEp51 were used (unpublished data).

To investigate which band represents the primary translation product, we carried out the labeling for a brief period. As can be seen in Fig. 2*B*, virtually identical labeling patterns were obtained whether cells were labeled for 1, 2, 10, or 60 min or labeled for 10 min and chased with a 7000-fold excess of unlabeled methionine for 100 min. Therefore, no apparent precursor–product relationship is found between these bands. The chase experiment suggests that the expressed RAS2 protein is stable for at least 100 min.

We have also compared the labeled bands with cell-free translation products. RNA was extracted from yeast cells and polyadenylylated RNA was prepared by oligo(dT) cellu-

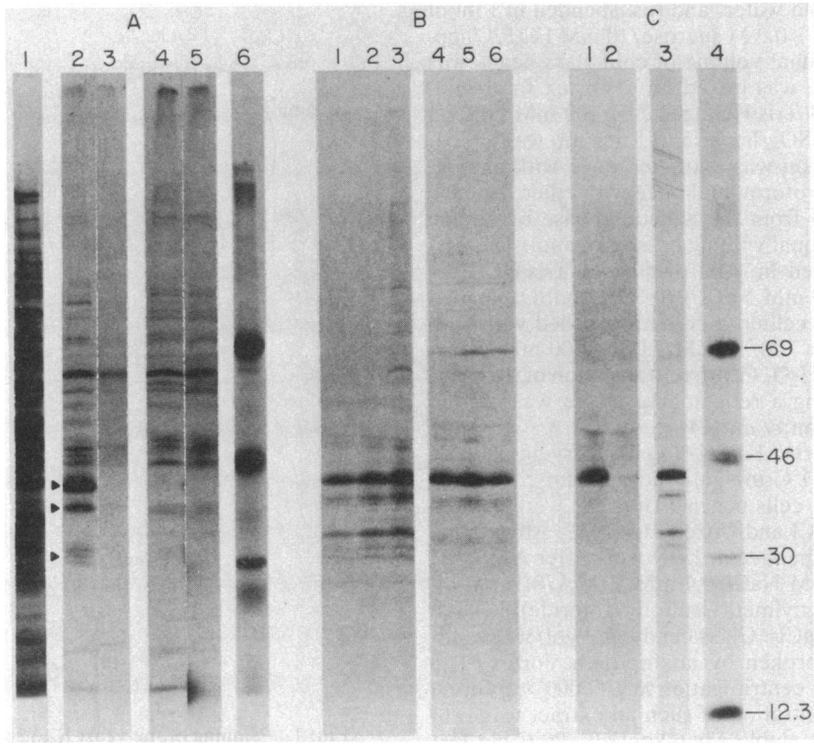

FIG. 2. Identification of yeast *RAS2* gene products. (*A*) Galactose-dependent expression of the RAS2 proteins. Yeast cells containing YEp51-RAS2 were grown at 31°C to 2 × 10^7 cells per ml in galactose-containing medium (lanes 2 and 3) or in glucose-containing medium (lanes 4 and 5). The cells were labeled with [^{35}S]methionine for 3 hr, disrupted by glass beads, and immunoprecipitated with the monoclonal antibody Y13-259 (lanes 2 and 4). The same extracts were used to carry out mock immunoprecipitation without the Y13-259 (lanes 3 and 5). Lane 1 shows total ^{35}S-labeled proteins. Lane 6 shows size markers (M_r × 10^{-3}): albumin, 69; ovalbumin, 46; carbonic anhydrase, 30; and cytochrome C, 12.3. (*B*) Labeling of RAS2 proteins with [^{35}S]methionine. Yeast cells containing YEp51-RAS2 were grown in galactose-containing medium at 31°C to 2 × 10^7 cells per ml and labeled with [^{35}S]methionine for 1 min (lane 1), 2 min (lane 2), and 10 min (lane 3). In another set of experiments, the cells were also labeled for 10 min (lane 4) or 60 min (lane 6) with [^{35}S]methionine or for 10 min with [^{35}S]methionine followed by a chase for 100 min with 160 μM unlabeled methionine (lane 5). The cells were quickly chilled on ice and collected. After breaking the cells, immunoprecipitation was carried out with the Y13-259. (*C*) Cell-free translation of polyadenylylated RNA. Polyadenylylated RNA was isolated from yeast cells carrying YEp51-RAS2 grown in galactose-containing medium. The RNA was translated by reticulocyte lysate and ^{35}S-labeled proteins were immunoprecipitated with (lane 1) or without (lane 2) monoclonal antibody Y13-259. Lane 3 shows immunoprecipitates from [^{35}S]methionine-labeled cells grown in galactose-containing medium run on the same gel. Lane 4 shows size markers identical to those in lane 6 of *A*. Numbers indicate M_r × 10^{-3}.

lose chromatography. RNA blot hybridization of the polyadenylylated RNA separated on formaldehyde gel with nick-translated *RAS2* probe indicated that the *RAS2* transcript is accumulated in the preparation extracted from cells grown in the presence of galactose but not in the preparation from glucose-grown cells (unpublished data). When the polyadenylylated RNA was translated by reticulocyte lysate and the immunoprecipitation of products using the monoclonal Y13-259 was performed, a single band migrating around M_r 41,000 was observed (Fig. 2*C*). Therefore, the major methionine-labeled band having an apparent molecular weight of 41,000 appears to be the primary translation product. As determined by immunoprecipitation, RAS protein represents 0.5% of radioactively labeled proteins during a 60-min pulse (unpublished data). We do not know the degree of amplification of *RAS2* expression we have obtained. However, this level of amplification does not seem to affect the growth of yeast, because the doubling time and shape of the yeast appeared normal.

Guanine Nucleotide Binding Activity of the RAS2 Protein. A high degree of amino acid homology between the yeast RAS proteins and the mammalian ras proteins raises the possibility that the yeast protein contains guanine nucleotide binding activity. To investigate this possibility, extracts were incubated with [^{3}H]GDP, immunoprecipitated with the monoclonal antibody 259, and the radioactivity in the precipitates was determined. As can be seen in Fig. 3, extracts prepared from cells grown in galactose-containing medium exhibited strong GDP binding activity. On the other hand, extracts prepared from cells grown in glucose-containing medium did not show any significant activity. Adding glucose extracts to galactose extracts did not affect the GDP binding activity of the galactose extracts, indicating that there are no inhibitory factors in the glucose extracts. The GDP binding activity of the RAS2 protein is strongly dependent on incubation temperature, as can be seen in Table 1. When the incubation with GDP was carried out at 0°C, no significant binding was detected even after 1 hr of incubation. This is in contrast to the activity of mammalian p21, with which essentially the same level of binding is detected at 0°C as at 37°C (10).

Characterization of the Guanine Nucleotide Binding Activity. To characterize further the guanine nucleotide binding activity of the RAS2 protein, crude extracts prepared from cells grown in the presence of galactose were cleared by high speed centrifugation and then applied onto a column of phosphocellulose. As shown in Fig. 4, the activity bound to the column and was eluted at the salt concentration of 0.17 M. The overall recovery was ≈70%, and the chromatography resulted in 20-fold purification over the high speed supernatant (S100). Examination of proteins in the peak fractions suggested that the M_r 41,000 protein was enriched. Thus, the

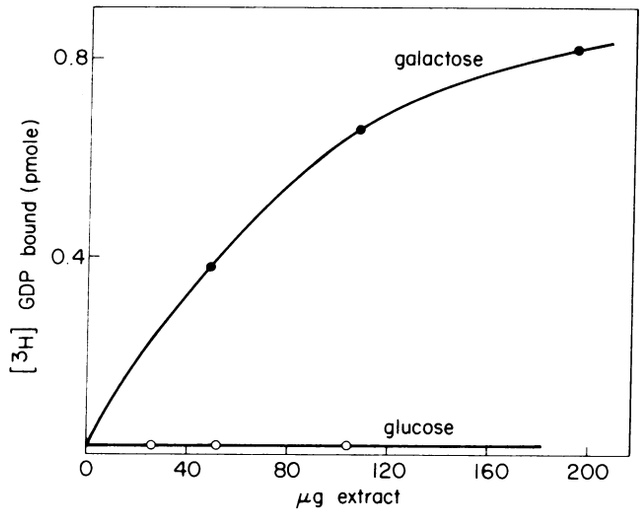

FIG. 3. Galactose-dependent expression of guanine nucleotide binding activity. Appropriate amounts of crude extracts prepared from cells grown in the presence of galactose (●) or glucose (○) were incubated for 1 hr at 30°C with [^{3}H]GDP and immunoprecipitated with the monoclonal antibody Y13-259.

guanine nucleotide binding activity provides an assay to purify the RAS2 protein.

Using the phosphocellulose purified protein, we have also examined the ability of other nucleotides to compete with GDP. As can be seen in Table 2, 20-fold excess of GTP or GDP completely abolished the activity. GMP did not show any competition. Thus, at least two phosphate groups are needed for the binding. The binding appears to be specific for guanine nucleotides, because ATP, CTP, and UTP competed far less efficiently than GTP. In fact, virtually no competition was seen with ATP. It is interesting to note that UTP competed to some extent. Such an observation is also made with the mammalian v-Ha-ras protein (10). Therefore, the GTP binding property of the yeast RAS2 protein is strikingly similar to that of the mammalian ras proteins.

DISCUSSION

Yeast *RAS* genes were originally identified by their sequence homology with mammalian *ras* genes. The observation that changing the 19th amino acid from glycine to valine alters the physiology of yeast (22) points to a close similarity in the way the yeast and mammalian proteins function. Our finding that a product of one of the yeast *RAS* genes exhibits guanine nucleotide binding activity indicates the similarity of biochemical activity of the two proteins. This further substantiates the idea that yeast could serve as a model system to understand the biochemical mechanisms of *ras* function.

Table 1. Effect of incubation temperature on the guanine nucleotide binding activity

Incubation temperature, °C	[^{3}H]GDP bound, pmol
0	0.058
30	0.815
37	1.378
45	1.035

Crude extracts (194 μg of total protein) prepared from cells grown in the presence of galactose were incubated with [^{3}H]GDP for 1 hr at the indicated temperature.

Since the level of RAS2 protein in yeast is low (see Fig. 2*A*), we have overproduced the protein using an inducible *GAL10* promoter. We have identified a major band having an apparent M_r of 41,000 as the primary translation product by performing a brief labeling with [^{35}S]methionine and by translating polyadenylylated RNA by reticulocyte lysate. The apparent molecular weight is slightly higher than that predicted by its amino acid sequence. However, this could be due to an anomalous electrophoretic mobility of the protein. We have also detected minor bands after labeling with methionine: one around M_r 38,000 and three bands around M_r 30,000. The latter could be identical to the three bands detected by Papageorge *et al.* (20). These minor bands were observed even after a 1-min pulse, and we have not been able to establish any precursor–product relationship. We have also examined the possibility of glycosylation by treating the immunoprecipitated proteins with trifluoromethanesulfonic acid. Again, we saw no change in the pattern of immunoprecipitated proteins (unpublished data). Thus, it is more likely that the minor bands represent degradation products of the primary translation product. If so, it is interesting to note that the degradation occurs in a very specific way.

The overproduction of the RAS2 protein enabled us to demonstrate unambiguously that the yeast protein exhibits

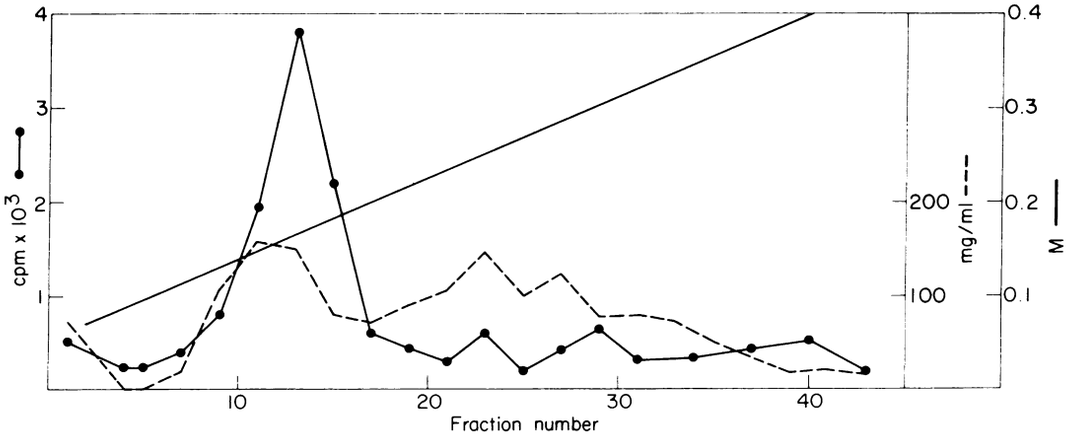

FIG. 4. Phosphocellulose column chromatography of the guanine nucleotide binding activity. Crude extracts (9.5 ml) were prepared from 5 g of cells grown in galactose-containing medium. The extracts were centrifuged for 60 min at 40,000 rpm in a 50 Ti rotor. The recovery of the activity in this step was almost 100%. This high speed supernatant (7 ml) was loaded onto a 10-ml column of phosphocellulose (P11), equilibrated with buffer A and 5 mM $MgCl_2$. After washing with 10 ml of buffer A and 5 mM $MgCl_2$, elution was carried out by the linear gradient of buffer A/5 mM $MgCl_2$ and buffer A/5 mM $MgCl_2$/0.5 M NaCl (50 ml each). Fraction volume was 2 ml. The microliters from each fraction was assayed for guanine nucleotide binding activity by incubating for 1 hr at 37°C with [^{3}H]GDP. ●, [^{3}H]GDP bound; – – – –, protein concentration; ——, salt concentration.

Table 2. Effects of addition of other nucleotides on binding of [^{3}H]GDP

Competitor added	[^{3}H]GDP bound, pmol
None	0.68
GTP	0.01
GDP	0.03
GMP	0.64
ATP	0.57
CTP	0.49
UTP	0.31

Phosphocellulose fraction 13 (20 μl) (Fig. 4) was incubated with [^{3}H]GDP for 1 hr at 37°C. The potential competing nucleotide was added prior to the [^{3}H]GDP and was in 20-fold molar excess. Immunoprecipitation was carried out after the incubation.

guanine nucleotide binding activity. The binding activity is specific to di- and triphosphate forms of guanine nucleotide, and a weak competition of UTP with GDP is seen. This is in remarkable agreement with the mammalian ras protein (10). We have also observed that the guanine nucleotide binding activity of the yeast RAS protein is strongly dependent on incubation temperature. This is in contrast to the mammalian protein, for which the activity is easily detected at 0°C. We do not know the reason for this temperature dependence. It may be that the GTP binding site of the yeast protein is buried within the protein so that local unfolding is needed for the GTP to bind.

The guanine nucleotide binding activity provides a simple assay to purify the yeast RAS2 protein, which enables us to characterize further its biochemical activity. An intriguing question is whether there is any difference in guanine nucleotide binding activity between the wild-type protein and mutant proteins that alter the physiology of yeast. To attack this problem, we have overproduced mutant forms of the RAS2 protein and are studying their comparative biochemical properties. A variety of proteins are known that exhibit high affinity for guanine nucleotides. The "G" proteins of adenylate cyclase bind GTP, which results in the dissociation of interacting proteins. This enables the interacting proteins to stimulate the catalytic subunit of adenylate cyclase (26). A similar mechanism is proposed for the G protein of cyclic GMP-dependent phosphodiesterase (27). Elegant studies have been carried out with other GTP binding proteins, EF-Tu and EF-G. The binding of GTP alters the conformation of these proteins, thus affecting their interaction with ribosomes (reviewed in ref. 28). Whether the yeast RAS2 protein functions in a similar manner and whether the GTP binding affects its protein conformation will become clearer after further purification and characterization of the protein.

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