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## Purification of a RAS-Responsive Adenylyl Cyclase Complex from *Saccharomyces cerevisiae* by Use of an Epitope Addition Method

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**We developed a method for immunoaffinity purification of *Saccharomyces cerevisiae* adenylyl cyclase based on creating a fusion with a small peptide epitope. Using oligonucleotide technology to encode the peptide epitope we constructed a plasmid that expressed the fusion protein from the *S. cerevisiae* alcohol dehydrogenase promoter ADH1. A monoclonal antibody previously raised against the peptide was used to purify adenylyl cyclase by affinity chromatography. The purified enzyme appeared to be a multisubunit complex consisting of the 200-kilodalton adenylyl cyclase fusion protein and an unidentified 70-kilodalton protein. The purified protein could be activated by RAS proteins. Activation had an absolute requirement for a guanine nucleoside triphosphate.**

We have been studying the two RAS genes, *RAS1* and *RAS2*, of *Saccharomyces cerevisiae* as models for the mammalian *ras* oncogenes. The yeast RAS proteins are structurally, functionally, and biochemically similar to their mammalian counterparts and at least one of their effector systems is known (3, 7, 8, 18, 24, 34, 36, 38). The yeast RAS genes were originally isolated by using mammalian *ras* genes to screen libraries from *S. cerevisiae* (7, 24). They encode proteins that are highly homologous to the mammalian *ras* proteins, particularly in their amino-terminal half. Both yeast and mammalian RAS proteins undergo similar processing events and localize to membrane fractions (6, 11, 25, 30, 42). All RAS proteins bind guanine nucleotides and possess an intrinsic GTPase activity. The GTPase activity is reduced in a number of oncogenic forms of mammalian *ras* proteins containing point mutations (12, 21, 29, 33, 34). Analogous mutations in the yeast RAS proteins also result in substantially reduced GTPase activity (36). The enzymatic properties of RAS proteins have led to their inclusion in the class of signal-transducing proteins that bind guanine nucleotides and activate a variety of effector systems. It is generally assumed that these proteins are turned off by GTP hydrolysis (reviewed in reference 13).

We have previously demonstrated that RAS proteins are potent activators of yeast adenylyl cyclase (3, 38). In our previous studies, we carried out in vitro measurements of adenylyl cyclase activity in crude yeast membrane extracts supplemented with purified RAS proteins bound to various guanine nucleotides. Under these conditions, RAS2 protein activated adenylyl cyclase when bound to GTP and GTP analogs, but not when bound to the stable GDP analog GDP- $\beta$ S [guanosine-5'-O-(2-thiodiphosphate)]. RAS2 protein bound to GDP itself gave intermediate levels of activation, presumably because GTP was being generated during the assay from GDP by factors present in the crude membrane extract (3, 9, 10). These studies, while consistent with the

G-protein model described above, have not rigorously excluded the possibility that GDP-bound RAS2 protein is capable of activating adenylyl cyclase. In addition, because crude yeast membrane extracts were used it was not certain that RAS2 protein interacts directly with adenylyl cyclase or through intermediate proteins. To understand the interaction between RAS and adenylyl cyclase in greater detail, we undertook to reconstitute the reaction with purified components. Adenylyl cyclase has proven difficult to purify by conventional methods, so we used the epitope addition method described below.

The epitope addition method of protein purification takes advantage of the powerful technique of monoclonal antibody affinity chromatography. The first step in our method was to construct a vector that encodes an epitope fusion protein. This fusion protein consists of a peptide epitope linked to the N-terminus of adenylyl cyclase. Next, a monoclonal antibody directed against the peptide epitope was used to purify the fusion protein. The choice for the epitope was based on the work of Green et al. (14) who demonstrated that small peptides corresponding to sequences from the hemagglutinin of the influenza virus (HA1) could be used to raise antibodies that reacted with the HA1 protein itself. Niman et al. (23) subsequently developed several monoclonal antibodies to one of the peptide sequences, HA1 (75-110), and Wilson et al. (43) demonstrated that a short 9-amino-acid sequence, YPYDVPDYA, contained the complete antigenic determinant of the immunizing 36-mer peptide. This peptide can be used to release the fusion protein from the monoclonal antibody.

### MATERIALS AND METHODS

**Vectors and strains.** pT7/TPK1, the TPK1 expression vector, was a gift of M. Zoller (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). This vector contains the *SalI-HindIII* fragment of the cyclic AMP-dependent protein kinase TPK1 (37) cloned into the T7 promoter-based vector pT7 (16, 32) which had been digested with *SalI* and *HindIII*. pPK1 then was constructed by inserting the synthetic oligonucleotide pair

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5' T ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TTG GGT GGT CC  
3' AC ATG GGT ATG CTA CAA GGT CTA ATG CGA TCG AAC CCA CCA GGA T

into pT7/TPK1 at the *NdeI* site which marks the start codon for *TPK1*. These oligonucleotides encode the HA1 epitope. pPK5 was next constructed by first digesting pPK1 partially with *NdeI* and completely with *EcoRV* and isolating the fragment containing the HA1 *TPK1* fusion protein sequences. This fragment was ligated into the *HindIII* site of pAD1, a yeast expression plasmid containing the alcohol dehydrogenase I promoter (1). Both insert and vector were filled in with Klenow fragment before ligation. As a consequence, a *BamHI-SalI* fragment of pPK5 contains the ADHI promoter and the epitope coding sequences. In parallel to these constructions, the oligonucleotide pair

5' TCG AGG ATC CGC GTC GAC GC 3'  
3' CC TAG GCG CAG CTG CGAGCT 5'

was inserted into the *SalI* site of YRp7-*CYR1* (10), a plasmid containing both the *CYR1* gene and the *TRP1* gene, to create pBM11. This addition created a *BamHI* site and a properly phased *SalI* site while destroying the endogenous *SalI* sites. pBM11 was digested with *SalI* and *BamHI* and ligated to the *BamHI-SalI* fragment of pPK5, described above. The resulting plasmid, pEF-*CYR1*, was capable of suppressing the loss of adenylyl cyclase and also the loss of both *RAS1* and *RAS2*. The auxotrophic marker *TRP1* was therefore not required to maintain the plasmid in certain mutant strains of *S. cerevisiae*. For the studies described here, we used two strains interchangeably: JF27A [(*MATa his3 leu2 ura3 trp1 ade8 cyr1::URA3(pEF-CYR1)*)] and JF36A [(*MATa his3 leu2 ura3 trp1 ade8 can1 ras1::HIS3 ras2::URA3(pEF-CYR1)*)].

**Immunological methods.** Monoclonal antibody 12CA5, a subclone of H26D08 (mouse immunoglobulin G2b), was raised against influenza hemagglutinin peptide HA1 (75-110) as described previously (23). Cells expressing this antipeptide antibody were injected into mice to induce tumors, and the antibody was purified from ascites fluid. Western blots (immunoblots) were visualized with reagents from Bio-Rad Laboratories (Richmond, Calif.). In Fig. 2, goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase was used to identify the immunoreactive bands. In Fig. 3, colloidal gold conjugated to goat anti-mouse immunoglobulin G was used (Bio-Rad). The peptide YPYDVPDYA was synthesized on an Applied Biosystems synthesizer by Dan Marshak (Cold Spring Harbor, N.Y.).

**Preparation of *RAS2* proteins and adenylyl cyclase assays.** Adenylyl cyclase activity was measured and *RAS2* proteins were prepared from *Escherichia coli* expression systems as described previously (3, 27). Protein from *RAS2*<sup>Val-19</sup>, a mutant of *RAS2*, was used in some experiments to test activation of adenylyl cyclase. Protein determinations were by the method of Bradford (2) except when detergents were present. In these cases, the amido black method was used (28). Each experiment described in this report was repeated at least twice. Similar results were obtained with two preparations of antibody and peptide and more than five preparations of yeast extracts.

**Adenylyl cyclase solubilization.** Solubilization of adenylyl cyclase from yeast membrane extracts was done by a modification of the method of Varimo and Londesborough (41). First, yeast membranes were prepared as described previously (3, 38) from 1 to 5 liters of cells grown to a density

of  $2 \times 10^7$  cells per ml in YPD medium (1% yeast extract, 2% glucose, 2% peptone [Difco Laboratories, Detroit, Mich.]). Membranes were brought to a volume of 20 ml/liter of cells with buffer C (200 mM MES [2-(*N*-morpholino)ethanesulfonic acid, pH 6.2], 0.1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) containing 1% Lubrol PX and 0.5 M NaCl. Then the membranes were centrifuged for 60 min at 35,000 rpm in a Beckman type 50 Ti rotor. The supernatant, which contained about 50% of the adenylyl cyclase activity, was stored at -80°C until needed.

**Adenylyl cyclase purification.** An extract from 5 liters of cells was prepared as described above to obtain fraction 1 (crude extract, 100 ml). This was passed through a 1-ml Sepharose CL-2B (Pharmacia, Uppsala, Sweden) column to remove proteins that nonspecifically bound Sepharose and was then loaded twice onto a monoclonal antibody 12CA5 column that had been prepared by cross-linking 2 mg of monoclonal antibody 12CA5 to 2 ml of CNBr-activated Sepharose 4B (Pharmacia) under conditions recommended by the manufacturer. The resin was prepared in a column that was surrounded by a water jacket and had been equilibrated with buffer C containing 1% Lubrol PX and 0.5 M NaCl at 4°C. After loading, the column was washed with 100 ml of buffer C containing 0.1% Lubrol PX and 0.5 M NaCl. To elute the bound protein, the column resin was mixed with 1 mg of the peptide YPYDVPDYA dissolved in 2.5 ml of the column buffer and prewarmed to 30°C. The jacket on the column was then flooded with water at 30°C and incubated for 15 min at 30°C. Care was taken during this step to mix the resin and the elution buffer to release air bubbles caused by the rapid warming of the resin. Fractions were collected, and the column was washed with 3 ml of column buffer at 30°C. The elution step was carried out twice and the peak fractions were pooled to obtain fraction 2 (immunoaffinity purified, 3.2 ml). More than 70% of the activity recovered was found in the first elution. Except for the relatively brief 30°C incubation described above, all steps were done at 0 to 4°C. After use, the column was regenerated by washing with 100 mM glycine (pH 2.9) followed by buffer C containing 1% Lubrol PX and 0.5 M NaCl. We have carried out this procedure more than five times with the same column.

A portion of fraction 2 (1.0 ml) was concentrated by centrifugation through a Centricon 10 microconcentrator (Amicon Corp., Danvers, Mass.) to a volume of 0.2 ml and then layered onto a 5-ml 10 to 35% glycerol gradient (buffer C containing 0.1% Lubrol PX and 0.5 M NaCl). The gradient was centrifuged for 13 h at 35,000 rpm in a Beckman SW50.1 rotor. After centrifugation, the tube was punctured and fractionated. Fractions were analyzed by silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (20-μl samples) and also assayed for adenylyl cyclase activity (5-μl samples). Recovery of activity was about 50% for the concentration step and >50% for the gradient itself. A separate marker gradient was run in parallel and analyzed by SDS-polyacrylamide gel electrophoresis. Marker proteins were catalase, 11.3S; alcohol dehydrogenase, 7.6S; bovine serum albumin, 4.4S; ovalbumin, 3.5S; cytochrome *c*, 1.7S.

## RESULTS

**Construction of an epitope fusion protein.** The plasmid pEF-CYR1 which expresses the adenylyl cyclase fusion protein is shown in Fig. 1, along with the relevant sequences of the fusion protein and the sequence of the peptide. The peptide epitope is fused to the amino terminus of adenylyl cyclase. The amino acid sequence GGP was used to connect the peptide to the rest of the protein. This sequence was chosen as a linker because we thought it would cause the antigen to protrude from the protein. We did not test whether this linker is required for antigenic recognition. A total of 20 amino acids were added to adenylyl cyclase. To test the complementing activity of this expression system, we carried out plasmid exchange experiments. The adenylyl cyclase construction described above could suppress the loss of adenylyl cyclase and also the loss of *RAS1* and *RAS2* (data not shown). This is consistent with previous observations that wild-type adenylyl cyclase expressed on a similar plasmid could also suppress these otherwise lethal mutations (10, 17, 19, 35).

We first tested for the presence of the peptide epitope by subjecting extracts from the appropriate strains to Western blot analysis. When blots were probed with the anti-peptide monoclonal antibody 12CA5, a band of 200 kilodaltons (kDa) was observed in cells containing pEF-CYR1 (Fig. 2). This is close to the predicted molecular size of 220 kDa (17). A band of this molecular size was not seen in extracts from cells that overexpressed adenylyl cyclase without the epitope fused to it. It should be noted that Western blots of some yeast extracts yielded additional bands reacting with the monoclonal antibody (Fig. 2, lane 1). These bands were not present in all extracts and were even seen in extracts from cells that did

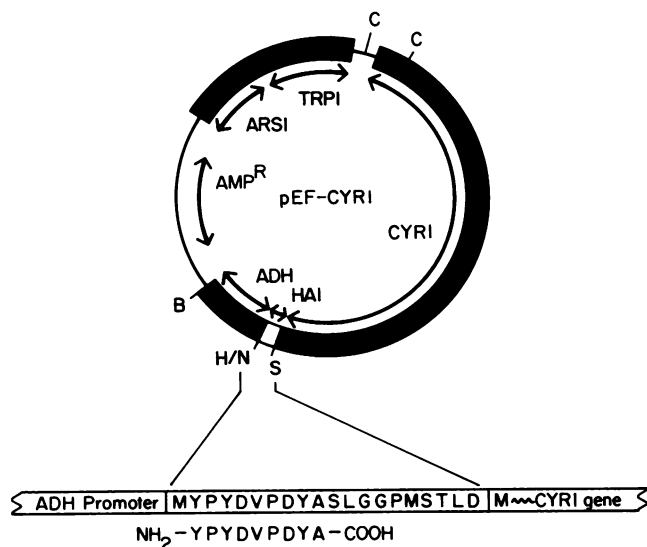


FIG. 1. Construction of the epitope fusion protein expression vector used in this study. See Materials and Methods for details. The final plasmid was constructed to express the peptide epitope fused to yeast adenylyl cyclase (*CYR1*), using the yeast alcohol dehydrogenase I (*ADH*) promoter. The antibody-binding sequence YPYDVPDYASL is from the influenza hemagglutinin *HAI*, but the antibody also recognizes the shorter sequence YPYDVPDYA. *S. cerevisiae* sequences are shown by heavy lines, and *E. coli* sequences are shown by light lines. The oligonucleotide encoding the epitope is shown by an open box. Abbreviations for restriction sites are as follows: B, *Bam*HI; H/N, *Hind*III-*Nde*I junction; S, *Sal*I; C, *Clal*. *AMP<sup>R</sup>*, Ampicillin resistance.

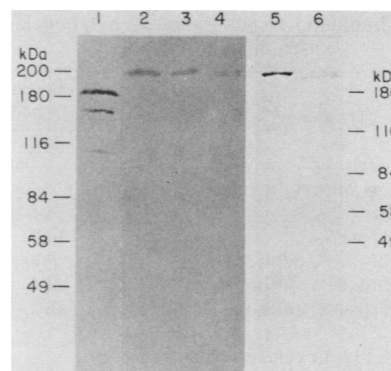


FIG. 2. Western blot analysis of antigen fusion proteins. In lanes 1 through 4 and 6, membrane extracts from *S. cerevisiae* harboring the indicated plasmids expressing the yeast adenylyl cyclase gene, *CYR1*, were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose paper. In lane 5, membranes from *S. cerevisiae* cells harboring pEF-CYR1 were solubilized with 1% Lubrol PX and 0.5 M NaCl as described in Materials and Methods, immunoprecipitated with monoclonal antibody 12CA5, subjected to electrophoresis on an SDS-polyacrylamide gel, and transferred to nitrocellulose paper. Lane 1, pADH-CYR1, (which encodes wild-type adenylyl cyclase), 300  $\mu$ g of membrane extract; lane 2, pEF-CYR1, 400  $\mu$ g of membrane extract; lane 3, pEF-CYR1, 200  $\mu$ g of membrane extract; lane 4, pEF-CYR1, 100  $\mu$ g of membrane extract; lane 5, pEF-CYR1, 500  $\mu$ g of extract immunoprecipitated; lane 6, pEF-CYR1, 200  $\mu$ g of membrane extract. The nitrocellulose was probed with monoclonal antibody 12CA5 and then visualized with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. Prestained molecular size markers were myosin, 200 kDa;  $\alpha$ 2-macroglobulin, 180 kDa;  $\beta$ -galactosidase, 116 kDa; fructose-6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarate, 48.5 kDa.

not contain pEF-CYR1. Thus, they appear to be caused by cross-reactivity of the antibody with other yeast proteins.

**Immunoprecipitation and purification of adenylyl cyclase.** We continued our studies with the epitope-fused adenylyl cyclase to see whether it could be purified by monoclonal antibody affinity chromatography. First, it was necessary to determine whether the adenylyl cyclase could interact with the monoclonal antibody in a soluble system. To do this, we solubilized the normally membrane-associated adenylyl cyclase with Lubrol PX and NaCl, immunoprecipitated with antibody, and then subjected the samples to Western blot analysis as described in Materials and Methods. In Fig. 2, lanes 5 and 6, the solubilized and immunoprecipitated fraction is compared with a membrane fraction. Both fractions yielded the expected 200-kDa band, indicating that soluble adenylyl cyclase could react with the antibody. Enzymatic assays of adenylyl cyclase immunoprecipitation experiments are shown in Table 1. About 50% of the epitope-fused adenylyl cyclase activity was depleted from a solubilized extract by the antibody 12CA5. No activity was depleted when immunoprecipitations were done in the presence of a large excess of the peptide YPYDVPDYA, which is known to bind to the antibody, or from extracts that overexpress wild-type adenylyl cyclase. The activity not depleted from the extract probably represents proteolytic fragments of adenylyl cyclase that have lost the epitope but are still catalytically active. To determine whether the immunoprecipitated adenylyl cyclase was still active, we carried out enzymatic assays on the agarose beads used to deplete the extracts (Table 1). More than 50% of the activity depleted by the antibody could be found in the immobilized fraction.

TABLE 1. Immunoprecipitation of adenylyl cyclase activity with anti-peptide antibody<sup>a</sup>

Treatment	Mn activity (pmol of cyclic AMP/30 min)	
	Soluble	Immobilized
1. Epitope fusion adenylyl cyclase		
No incubation	904	
Protein A-agarose	960	26
Protein A-agarose + peptide	988	26
Protein A-agarose + antibody	504	338
Protein A-agarose + antibody + peptide	1,092	39
2. Wild-type adenylyl cyclase		
Protein A-agarose + antibody	1,340	6.7
Protein A-agarose + antibody + peptide	1,199	9.4

<sup>a</sup> Membranes from yeast strains expressing epitope-fused adenylyl cyclase (experiment 1) or wild-type adenylyl cyclase (experiment 2) were treated with buffer C containing 1% Lubrol PX and 0.5 M NaCl to solubilize adenylyl cyclase. Where indicated, 200  $\mu$ l of extract was then incubated with 30  $\mu$ g of monoclonal antibody 12CA5 or 50  $\mu$ g of peptide YDYDVPDYA for 5 h at 4°C followed by a second incubation with 50  $\mu$ l of 50% protein A-agarose for 3 h at 4°C. The pellets were washed twice with solubilization buffer and brought to a final volume of 130  $\mu$ l. Adenylyl cyclase assays were done with 50  $\mu$ l of supernatant or 10  $\mu$ l of pellet. The data are expressed as if the entire sample has been assayed.

Encouraged by the observations that the solubilized adenylyl cyclase reacted with the monoclonal antibody and was still active when immobilized by the antibody, we sought to develop an immunoaffinity purification step. We began by testing on an analytical scale a number of commonly used methods for immunoaffinity chromatography elutions. However, urea concentrations of more than 2.0 M or glycine buffers lower than pH 3.5 inactivated the adenylyl cyclase activity in these experiments. The peptide YPYDVPDYA could elute the antibody-bound adenylyl cyclase activity, but only when incubated at the relatively high temperature of 30°C (data not shown). To carry out the purification, we cross-linked the antibody to a Sepharose matrix and then subjected solubilized yeast extracts to a column chromatography procedure. Elution from the column was done by competing away the immobilized enzyme with a large excess of the peptide YPYDVPDYA incubated at 30°C for 15 min. Table 2 shows the results of a purification procedure. Overall purification was about 700-fold. Recovery was about 20% of the starting activity or about 40% of the bound activity. Most of the activity lost was probably due to heat inactivation by the 30°C temperature used for elution.

We further characterized the immunopurified fraction by subjecting it to SDS-polyacrylamide gel electrophoresis. When visualized with silver stain, major bands of 200, 150, and 70 kDa were observed along with several minor bands (Fig. 3A). This pattern of bands was the same in preparations from *ras1 ras2 CYR1*(pEF-CYR1) cells (JF36A) as well as preparations from *RAS1 RAS2 cyr1*(pEF-CYR1) cells (JF27A). Neither of these strains yielded bands in the size range of *RAS1* or *RAS2* (data not shown). To determine which of these bands corresponded to the adenylyl cyclase fusion protein, we electrophoresed samples through an SDS-polyacrylamide gel and then transferred the proteins to nitrocellulose paper. We stained a portion of the blot for total protein using colloidal gold (Fig. 3B) and subjected another portion of the blot to Western blot analysis using the monoclonal antibody 12CA5 (Fig. 3B). In the portion stained for total protein, we detected a pattern of bands nearly

TABLE 2. Purification of adenylyl cyclase<sup>a</sup>

Fraction	Total U (pmol/min)	Protein (mg)	Sp act (pmol/min per mg)	Fold purification	% Recovery
1. Crude extract	6,120	90	68	1.0	100
2. Immunoaffinity purified	998	<0.021	>47,500	>700	16.3

<sup>a</sup> Extracts from a yeast strain harboring pEF-CYR1 were chromatographed on a monoclonal antibody column as described in Materials and Methods. The recovery of activity actually bound to the column was 45%. Assays were done with Mn<sup>2+</sup> as the divalent cation. The protein concentration of fraction 2 was too low to measure.

identical to that in the silver-stained gel, while in the portion probed with monoclonal antibody 12CA5, we detected only a single band of about 200 kDa. Thus, the 200-kDa band seen after visualizing total protein is probably the adenylyl cyclase fusion protein. The exact assignment of the 200-kDa band was not possible because in the total protein blot a doublet was observed in this region, while in the Western blot only a single band was observed. To determine whether the 200-kDa protein binds to either of the other two major proteins in our preparation, we subjected a portion of the preparation to glycerol gradient sedimentation, fractionated the gradient, and then assayed the various fractions in the gradient (Fig. 4). The adenylyl cyclase activity sedimented with an S value of about 15S which is close to the value of 12.5S previously reported for solubilized adenylyl cyclase (15). A silver-stained SDS-polyacrylamide gel indicated that the 200- and 70-kDa bands, but not the 150-kDa band, cosedimented with the peak fractions of activity. We are presently investigating what relationship, if any, the 70-kDa protein has to adenylyl cyclase. Heideman et al (15) have determined the molecular size of solubilized adenylyl cy-

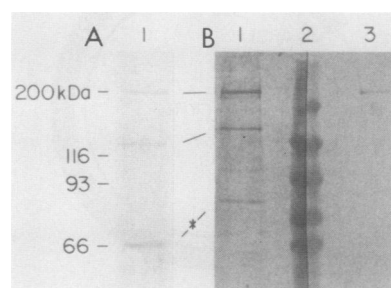


FIG. 3. SDS-polyacrylamide gel and Western blot analysis of purified adenylyl cyclase. (A) A 20- $\mu$ l sample of purified protein (fraction 2) visualized by a silver staining method (Pierce Chemical Co., Rockford, Ill.) after SDS-polyacrylamide gel electrophoresis. Marker proteins were myosin, 200 kDa;  $\beta$ -galactosidase, 116 kDa; phosphorylase *b*, 93 kDa; bovine serum albumin; 66 kDa. (B) Purified adenylyl cyclase (20  $\mu$ l) was electrophoresed through an SDS-polyacrylamide gel and was then transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper was cut through the marker lanes for later alignment. Samples were visualized in lane 1 and the left half of lane 2 by using a colloidal gold protein stain. Samples were visualized in the right half of lane 2 and lane 3 by using the monoclonal antibody 12CA5 followed by colloidal gold-conjugated goat anti-mouse antibody. The marker proteins in lane 2 (Sigma prestained molecular size markers) from top to bottom are 180, 116, 84, 58, and 49 kDa. These are the same as those used in Fig. 2. The three major bands are identified by the lines between the two gels. The asterisk (\*) indicates the 70-kDa band discussed in text.

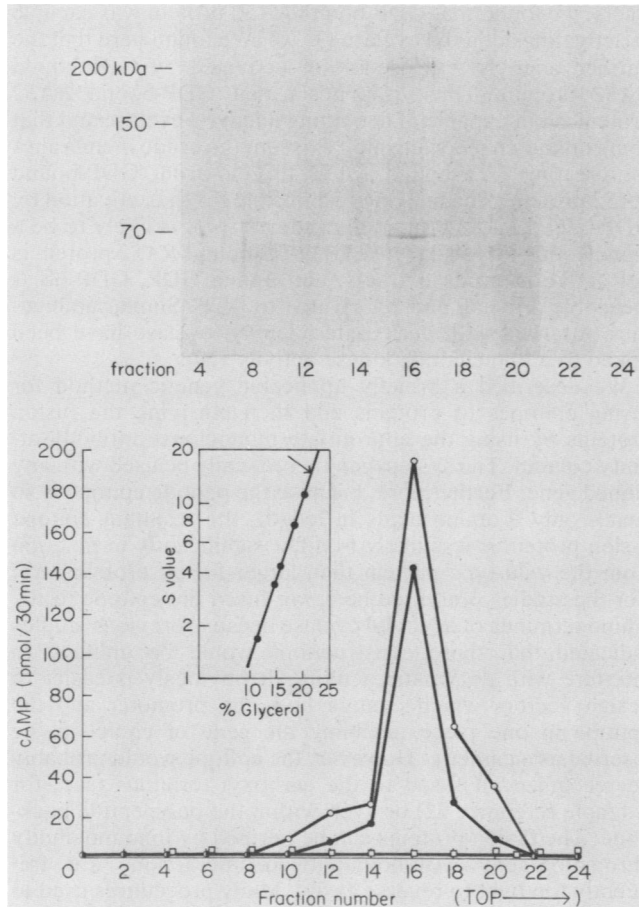


FIG. 4. Glycerol gradient sedimentation of immunopurified adenylyl cyclase. Gradients were run as described in Materials and Methods. The gradient fractions were analyzed on silver-stained SDS-polyacrylamide gels (above) and also by adenylyl cyclase assays (below). Inset, S value determination by comparison with standards. Arrow indicates peak of adenylyl cyclase activity. Symbols:  $\circ$ ,  $Mn^{2+}$ ;  $\square$ ,  $Mg^{2+}$ ;  $\bullet$ ,  $Mg^{2+}$  plus 12  $\mu g$  of  $RAS2^{Val-19}$  protein. cAMP, Cyclic AMP.

clase to be 594 kDa, and Varino and Londesborough have determined the molecular size of adenylyl cyclase to be 450 kDa (41). Thus, adenylyl cyclase appears to consist of a multisubunit complex containing the 200-kDa *CYR1* gene product and an unidentified 70-kDa polypeptide.

**Activation of adenylyl cyclase by *RAS2* protein and guanine nucleotides.** Purified yeast *RAS2* and *RAS2<sup>Val-19</sup>* proteins are known to activate wild-type adenylyl cyclase in crude membrane fractions. Optimum activation requires *RAS2* or *RAS2<sup>Val-19</sup>* protein bound to GTP and takes place when  $Mg^{2+}$  is used as the divalent cation (3, 9, 10). When  $Mn^{2+}$  is used, as in the experiments described above, *RAS2* protein is not needed for activation (3, 5). To test the *RAS2* protein responsiveness of adenylyl cyclase, we used a mutant form of *RAS2* protein, *RAS2<sup>Val-19</sup>* protein, purified from an *E. coli* expression system. This protein is deficient in its GTPase activity. We found it fully capable of stimulating adenylyl cyclase without the addition of exogenous GTP, presumably because it is predominantly in its GTP-bound state. *RAS2<sup>Val-19</sup>* protein activation of various epitope-fused adenylyl cyclase fractions is shown in Table 3. The crude

membrane preparation, solubilized membrane preparation, immobilized fraction, and purified protein were all greatly stimulated by *RAS2<sup>Val-19</sup>* protein when  $Mg^{2+}$  was present as the divalent cation. The peaks of  $Mn^{2+}$ -dependent activity and *RAS2<sup>Val-19</sup>*-responsive activity also cosedimented through a glycerol gradient (Fig. 4). When the ratio of  $Mn/Mg^{2+}$  *RAS2<sup>Val-19</sup>* protein activity was compared, we found that about half of the *RAS2* protein activation was lost during the solubilization procedure (Table 3). This is probably caused by the addition of NaCl, which is required, along with Lubrol PX, to solubilize adenylyl cyclase. Other experiments have shown that NaCl, but not Lubrol PX, inhibits adenylyl cyclase in the *RAS2* protein activation assay, but not in the  $Mn^{2+}$  assay (data not shown). Thus, the purified adenylyl cyclase complex is fully activated by *RAS2<sup>Val-19</sup>* protein.

We also tested the ability of wild-type *RAS2* protein to activate the purified adenylyl cyclase. We previously found that GDP-bound wild-type *RAS2* protein was capable of partially activating adenylyl cyclase in crude membrane preparations. However, *RAS2* protein bound to the stable analog of GDP, GDP- $\beta$ S, was inactive (3, 10). To determine whether the partial activation of GDP-bound *RAS2* protein was still observed with highly purified adenylyl cyclase, we tested various guanine nucleotides in *RAS2* protein activation assays (Fig. 5). *RAS2* protein activated adenylyl cyclase when bound to GTP, but not when bound to GDP or GDP- $\beta$ S. *RAS2* protein incubated without any exogenously added nucleotide was not capable of activating adenylyl cyclase, suggesting that it is not bound to GTP when we purify it from *E. coli* expression systems. Taken together, these results indicate that *RAS2* protein activates adenylyl cyclase when bound to GTP, but not GDP, and that in crude membranes some unknown mechanisms converts GDP-bound *RAS2* protein to GTP-bound *RAS2* protein.

## DISCUSSION

We have been studying the *RAS* protein interactions with the adenylyl cyclase of *S. cerevisiae* as a model for mammalian *ras* effector interactions. Uno et al. (40) have reported the expression of a fragment of adenylyl cyclase in *E. coli* which they claim is stimulated by *RAS* proteins, sug-

TABLE 3. Activation of various fractions of epitope-fused adenylyl cyclase by *RAS2<sup>Val-19</sup>* protein<sup>a</sup>

Fraction	Activity (pmol/30 min) with:			
	$Mn^{2+}$	$Mg^{2+}$	$Mg^{2+}$ + <i>RAS2</i>	$(Mg^{2+}$ + <i>RAS2</i> )/ $Mn$
Expt 1				
Membranes	66	4.0	47	0.71
Soluble extract	38	<1.0	12.9	0.33
Agarose beads	29	<1.0	8.9	0.31
Expt 2				
Soluble extract	44	<1.0	17.8	0.40
Immunopurified	112	<1.0	28.8	0.31

<sup>a</sup> The indicated fractions were assayed in the presence of  $Mn^{2+}$ ,  $Mg^{2+}$ , or  $Mg^{2+}$  and 12  $\mu g$  of *RAS2<sup>Val-19</sup>* protein bound to GTP. Experiment 1 compares a membrane extract, a soluble fraction, and a solubilized membrane extract that was immobilized to agarose beads with monoclonal antibody 12CA5. All three samples were from the same crude cell lysate. Experiment 2 compares a solubilized membrane fraction and a purified adenylyl cyclase fraction prepared from the same original crude cell lysate. The amount of each extract tested was chosen to give roughly comparable levels of activity in the presence of  $Mn^{2+}$ .

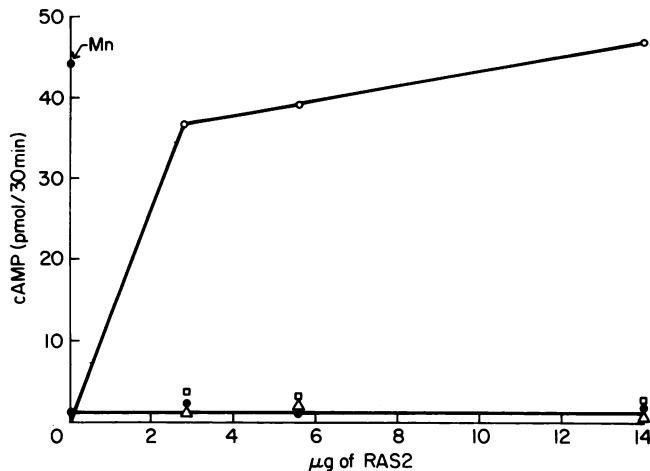


FIG. 5. Activation of purified adenylyl cyclase by wild-type *RAS2* protein bound to various guanine nucleotides. The indicated amounts of wild-type *RAS2* protein were incubated with 5  $\mu$ l of purified adenylyl cyclase (fraction 2).  $Mg^{2+}$  was the divalent cation except where indicated by  $Mn^{2+}$ . Before incubation with adenylyl cyclase and the reaction mix, *RAS2* proteins were preincubated for 30 min at 20°C in a volume of 50  $\mu$ l with no nucleotide ( $\square$ ), 50  $\mu$ M GTP ( $\circ$ ), 50  $\mu$ M GDP ( $\bullet$ ), or 50  $\mu$ M GDP- $\beta$ S ( $\Delta$ ). cAMP, Cyclic AMP.

gesting a direct interaction between *RAS* protein and adenylyl cyclase. We have been unable to duplicate these results and are not able to express a *RAS*-responsive adenylyl cyclase in *E. coli* (17). Indeed, the fragment of adenylyl cyclase which they express in *E. coli* does not appear to be *RAS* responsive when we express it in yeasts (unpublished data). We have utilized *S. cerevisiae* expression systems to study this problem. In this report, we describe the purification of adenylyl cyclase from *S. cerevisiae* using an immunoaffinity procedure. After a single column passage, there is enrichment of at least 700-fold. A second procedure, glycerol gradient sedimentation, increases the purification severalfold and suggests that the purified enzyme is a multisubunit complex containing perhaps two copies of the 200-kDa *CYR1* gene product and some number of an unidentified 70-kDa protein. The purified enzyme was fully activated by *RAS2* protein. Although the purified adenylyl cyclase was still activated by *RAS2* protein, it is still not certain whether *RAS2* protein interacts directly with adenylyl cyclase, through the 70-kDa protein, or by acting on the complex itself. The mechanism of transduction activation of cyclic GMP phosphodiesterase sets a precedent for complex interactions. Transducin is thought to act by releasing inhibition of activity by the  $\gamma$  subunit of the phosphodiesterase complex (31). We are presently trying to determine whether the 70-kDa protein is required for *RAS2* protein stimulation of adenylyl cyclase, has other functions related to adenylyl cyclase, or is merely an artifact of purification.

It is widely believed that *RAS* proteins are members of signal transduction pathways and are capable of activating their effectors when bound to GTP but not when bound to GDP. The guanine nucleotide content is believed to be regulated by *RAS* itself through its GTPase activity or possibly by other proteins (4, 26, 39). The *in vitro* adenylyl cyclase assay enabled us to test the guanine nucleotide response of *RAS* protein directly. Previously, when we measured adenylyl cyclase activity in crude membrane ex-

tracts, we found that GDP-bound *RAS2* protein was capable of activating adenylyl cyclase (3, 10). We found here that the purified adenylyl cyclase is not activated by GDP-bound *RAS2* protein. Thus, it appears that GDP-bound *RAS2* protein is not capable of activating adenylyl cyclase and that some unknown mechanism(s) exists in the crude membranes for creating GTP-bound *RAS2* protein from GDP-bound *RAS2* protein. One enzyme responsible for the activation by GDP-bound *RAS2* protein in crude extracts is likely to be a dinucleotide kinase because GDP- $\beta$ S-bound *RAS2* protein is not active in crude extracts, and unlike GDP, GDP- $\beta$ S is incapable of being phosphorylated to GTP. Similar ambiguities observed with vertebrate adenylyl cyclase have been linked to a dinucleotide kinase activity (20).

We described a broadly applicable genetic method for fusing epitopes to proteins and then purifying the fusion proteins by using the appropriate monoclonal antibody affinity column. The system can theoretically be used with any cloned gene. Furthermore, because the peptide epitope is so small, only 9 amino acids in length, the resultant epitope fusion protein is less likely to differ significantly in function from the wild-type protein than larger fusion proteins are. For the studies presented here, we fused the epitope to the amino terminus of adenylyl cyclase because previous studies indicated that these constructions would be unlikely to interfere with enzymatic activity. It obviously is easier to design vectors which contain both the promoter and the epitope in one piece, enabling the gene of choice to be inserted as a cassette. However, the epitope would probably be recognized if fused to the carboxyl terminus (see, for example reference 22) or even within the polypeptide backbone. The fusion proteins can be purified by immunoaffinity chromatography. Antibody-antigen interactions are frequently too tight to reverse easily. Many procedures used to elute material from antibody affinity columns exploit extreme pHs or chaotropic agents and subsequently inactivate the enzyme being purified. We found that adenylyl cyclase was inactivated by a number of these procedures. The method finally chosen, incubation with the synthetic peptide epitope, was relatively gentle and should be useful with other proteins as well. Should it be necessary to separate the synthetic peptide from the purified protein, one could use a number of procedures designed to exploit their size differences.

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