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Analysis of the Function of the 70-Kilodalton Cyclase-Associated Protein (CAP) by Using Mutants of Yeast Adenylyl Cyclase Defective in CAP Binding

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In *Saccharomyces cerevisiae*, adenylyl cyclase forms a complex with the 70-kDa cyclase-associated protein (CAP). By in vitro mutagenesis, we assigned a CAP-binding site of adenylyl cyclase to a small segment near its C terminus and created mutants which lost the ability to bind CAP. CAP binding was assessed first by observing the ability of the overproduced C-terminal 150 residues of adenylyl cyclase to sequester CAP, thereby suppressing the heat shock sensitivity of yeast cells bearing the activated *RAS2* gene (*RAS2*$^{Val-19}$), and then by immunoprecipitability of adenylyl cyclase activity with anti-CAP antibody and by direct measurement of the amount of CAP bound. Yeast cells whose chromosomal adenylyl cyclase genes were replaced by the CAP-nonbinding mutants possessed adenylyl cyclase activity fully responsive to RAS2 protein in vitro. However, they did not exhibit sensitivity to heat shock in the *RAS2*$^{Val-19}$ background. When glucose-induced accumulation of cyclic AMP (cAMP) was measured in these mutants carrying *RAS2*$^{Val-19}$, a rapid transient rise indistinguishable from that of wild-type cells was observed and a high peak level and following persistent elevation of the cAMP concentration characteristic of *RAS2*$^{Val-19}$ were abolished. In contrast, in the wild-type *RAS2* background, similar cyclase gene replacement did not affect the glucose-induced cAMP response. These results suggest that the association with CAP, although not involved in the in vivo response to the wild-type RAS2 protein, is somehow required for the exaggerated response of adenylyl cyclase to activated RAS2.

The yeast *Saccharomyces cerevisiae* has two *RAS* genes, *RAS1* and *RAS2*, whose protein products are structurally, functionally, and biochemically similar to mammalian Ras oncoproteins (6, 10, 11, 25, 38, 46, 47). The yeast RAS proteins are essential controlling elements of adenylyl cyclase (6, 49), which catalyzes the production of cyclic AMP (cAMP), a second messenger critical for yeast cell growth (31). The RAS-adenylyl cyclase pathway has been implicated in transduction of a signal triggered by glucose to an intracellular environment where a protein phosphorylation cascade is induced by cAMP (33, 35, 39). A product of the *CDC25* gene appears to convey the glucose signal to RAS proteins by promoting the GDP-GTP exchange on RAS (7, 8, 23, 35). Yeast cells that possess activated *RAS2* gene *RAS2*$^{Val-19}$ have an elevated level of intracellular cAMP and display abnormal phenotypes, including sensitivity to heat shock, sensitivity to nutritional starvation, and failure to sporulate (26, 49).

Yeast adenylyl cyclase, a product of the *CYR1* gene (31), consists of 2,026 amino acid residues that comprise at least four domains: the N-terminal, the middle repetitive, the catalytic, and the C-terminal domains (24, 53). The middle repetitive domain is composed of a repetition of 23-amino-acid leucine-rich motifs which have homology to the leucine-rich repeat family proteins of yeasts, mammals, and *Drosophila melanogaster* (see references 15 and 52 and references therein). Studies using site-specific mutagenesis showed that any residue at particular positions of the consensus amino acids in the leucine-rich repeats was virtually unmutable without destruction of the interaction with RAS proteins (9, 44, 52). In addition, we recently identified a segment of 14 amino acids that appeared to be involved in activation of adenylyl cyclase by RAS proteins (45).

Adenylyl cyclase forms an 890-kDa complex with RAS protein-dependent activity that is separable from another complex of 670 kDa with Mn^{2+}-dependent activity only (52). The RAS-responsive complex contains the 70-kDa adenylyl cyclase-associated protein CAP (14), or the *SRV2* product (12), as its inherent component (52). CAP is the only protein associated with adenylyl cyclase that has been characterized by molecular cloning (12–14). Although original studies indicated a critical role of CAP in the RAS-adenylyl cyclase interaction (12, 14), we have shown that CAP is not essential for in vitro activation of adenylyl cyclase by RAS proteins (52). However, the result could not explain why disruption of the *CAP* gene in yeast cells bearing *RAS2*$^{Val-19}$ resulted in disappearance of heat shock sensitivity, which was consistently shown by other groups (12, 14, 17) and confirmed by us. Studies of CAP have shown that the protein appears to have another function that is required for normal cell morphology and responsiveness to nutrient deprivation and excess (14, 17). This function appeared to be separable from that in the RAS-adenylyl cyclase pathway on the basis of deletion mutational analysis of CAP (17).

Here, we localized a binding site of CAP on the adenylyl cyclase molecule on the basis of mutational analysis of the gene that encodes it and thereby were able to produce mutant adenylyl cyclases defective in CAP binding. The mutant proteins were used to analyze the function of CAP in the RAS-adenylyl cyclase pathway.

MATERIALS AND METHODS

Cell strains, growth media, and transformation. The *S. cerevisiae* strains used are listed in Table 1. Yeast cells were

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TABLE 1. Yeast strains used in this study

Strain[a]	Genotype
SP1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1*
T50-3A	*MATα his3 leu2 trp1 ura3 cyr1-2*
TK35-1	*MATα his3 leu2 trp1 ura3 cyr1-2 ras2::LEU2*
TK161-R2V	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1* $RAS2^{Val-19}$
TK89-1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1* $RAS2^{Val-19}$ *CYR1(∇1976)*
TK89-2	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1* $RAS2^{Val-19}$ *CYR1(∇2003)*
TK89-3	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1* $RAS2^{Val-19}$ *CYR1(∇2006)*
TK89-4	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1* $RAS2^{Val-19}$ *CYR1(∇2011)*
TK90-1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1 CYR1(∇2011)*

[a] Strains SP1, T50-3A, TK35-1, and TK161-R2V were described previously (24, 26, 44, 49). TK89-1, TK89-2, TK89-3, TK89-4, and TK90-1 were constructed as described in Materials and Methods.

grown in YPD (2% Bacto Peptone, 1% Bacto Yeast Extract, 2% glucose) or yeast synthetic medium (0.67% yeast nitrogen base, 2% glucose) with appropriate auxotrophic supplements. Plates used for 5-fluoroorotic acid selection were prepared as previously described (5). Genetic manipulation of yeast cells was performed as previously described (40). Transformation into yeast cells was done with lithium acetate (22).

Plasmid construction and in vitro mutagenesis. Plasmid YEP24-ADC1-CYR1, bearing the *URA3* selectable marker, which overexpressed the full-length wild-type *CYR1* gene under control of the yeast alcohol dehydrogenase I (*ADC1*) promoter (1), was described previously (44). For overexpression of the C-terminal segment of adenylyl cyclase, the plasmid was cleaved with *Kpn*I and *Stu*I to delete the internal 1,728 amino acid residues (amino acids 41 to 1768) of *CYR1*, and the resulting fragment was resealed with an 8-bp *Mlu*I linker (GACGCGTC) in between after flushing of the *Kpn*I cohesive terminus with the large fragment of *Escherichia coli* DNA polymerase I, producing YEP24-ADC1-CYR1(Δ41-1768). A 2-kb *Nco*I-*Sma*I fragment of YEP24-ADC1-CYR1 encompassing amino acids 1609 to 2026 and the 3′-flanking sequence of *CYR1* was flush ended similarly and cloned into *Sma*I-cleaved pUC19. The resulting plasmid, pUC-CYR1NS, was subjected to 2-amino-acid insertional mutagenesis by using 6-bp synthetic oligonucleotides (3) (TAB linkers; Pharmacia, Uppsala, Sweden) after partial digestion with suitable restriction endonucleases (see Results for details). The locations of the insertions were determined by mapping *Apa*I cleavage sites introduced by the linkers CGGGCC and GGGCCC and by nucleotide sequencing (4). The *Nco*I-*Nhe*I fragments of the plasmids bearing the mutations were subsequently transferred to YEP24-ADC1-CYR1 for expression in *S. cerevisiae*. Mutant forms of YEP24-ADC1-CYR1 which possessed small deletions originating from a *Cla*I cleavage site located at amino acid 1959 of *CYR1* have been described elsewhere (44, 53). Forms of YEP24-ADC1-CYR1 bearing the various C-terminal mutations were used to construct YEP24-ADC1-CYR1(Δ41-1768) plasmids bearing the corresponding mutations by introducing internal deletions as described above. For expression of the C-terminal segments of various lengths in *S. cerevisiae*, YEP24-ADC1-CYR1 plasmids with the 6-bp linker insertion mutations were cleaved by *Kpn*I (at amino acid 41) and *Apa*I (at the insertion sites) and resealed with an *Mlu*I linker in between as described for construction of YEP24-ADC1-CYR1(Δ41-1768). All of the resulting mutant *CYR1* genes were designated *CYR1*(∇*x*) or *CYR1*(Δ*y-z*), where amino acid positions *x*, *y*, and *z* are the location of the 2-amino-acid insertion and the beginning and end of the deletion, respectively. pAD-$RAS2^{Val-19}$, an overexpression vector of $RAS2^{Val-19}$ bearing the *LEU2* selectable marker, was prepared by inserting a 1.2-kb *Hin*cII fragment of the $RAS2^{Val-19}$ gene (26) into pAD4 (14). Similarly, the full-length *S. cerevisiae CDC25* gene (7, 8) was cloned into pAD4, producing pAD-CDC25, for overexpression under control of the *ADC1* promoter. pADH-CAP (14, 17) was used for overexpression of the *CAP* gene.

Replacement of the chromosomal *CYR1* gene. pUC19-URA3-CYR1(∇x) plasmids were constructed by inserting a 1.2-kb *Hin*dIII fragment of the *URA3* gene into a *Hin*dIII cleavage site of pUC19 and then by inserting the *Eco*RI (at amino acid 1822)-*Nhe*I fragments of the *CYR1*(∇*x*) genes between the *Eco*RI and *Xba*I sites of pUC19. The plasmids were used for replacement of the C-terminal region of chromosomal *CYR1* with the corresponding mutant genes by a standard gene transplacement method (5, 41). Briefly, pUC19-URA3-CYR1(∇x) plasmids were linearized by cleavage with *Cla*I at amino acid 1959 and transformed into *S. cerevisiae* TK161-R2V (49) possessing the $RAS2^{Val-19}$ and wild-type *CYR1* genes and into SP1 (26) carrying the wild-type *RAS2* and *CYR1* genes. The resulting Ura^+ transformants were examined for insertional integration of the plasmids at the *Cla*I site by Southern blot analysis of their genomic DNAs (data not shown). Those containing one copy insertion were cultured in YPD and then spread onto 5-fluoroorotic acid plates for selection of Ura^- colonies formed by eviction of the plasmids. Successful replacements of the C-terminal region of the chromosomal gene with the mutants in the Ura^- colonies were verified by Southern blot analysis of their genomic DNAs by using *Apa*I cleavage sites at the linker insertion sites to distinguish between the wild-type and mutant *CYR1* genes (data not shown).

Examination of heat shock sensitivity. Survival of yeast cells after heat shock was examined essentially as described previously (15, 49). Patches of various yeast colonies were made on an agar plate with appropriate media, grown for 2 days at 30°C, and replica plated onto one plate preincubated at 55°C and another preincubated at 30°C. The 55°C plate was further incubated at 55°C for 10 min, subsequently transferred to a 30°C incubator, and cultured for 2 days to observe cells that survived the heat shock. The 30°C replica plate was incubated at the same temperature for 2 days as a control.

Immunoprecipitation and measurement of CAP binding. Affinity-purified rabbit polyclonal antibodies against the whole CAP (anti-CAP), against a segment (amino acids 335 to 596) of adenylyl cyclase (anti-CYR1HP), and against a 15-amino-acid synthetic peptide corresponding to the C terminus of adenylyl cyclase (anti-CYR1CT) were described

previously (45, 52, 53). Polyclonal antiserum against *Schistosoma japonicum* glutathione *S*-transferase (GST), anti-GST, was prepared by immunizing rabbits with GST purified by glutathione-Sepharose affinity chromatography from extracts of *E. coli* bearing plasmid pGEX-2T (42) (Pharmacia). A mouse monoclonal antibody of the immunoglobulin G1 subclass, anti-CYR1NS1, was prepared against a middle segment (amino acids 1315 to 1667) of adenylyl cyclase by a conventional procedure (44a). Yeast cells were disrupted by shaking with glass beads, and crude membrane fractions were prepared as described previously (44, 53). Adenylyl cyclase complexes were solubilized by extraction of the membrane fraction (approximately 1 mg of protein) with 1 ml of buffer C {50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6.2), 0.1 mM $MgCl_2$, 0.1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 1 mM 2-mercaptoethanol} containing 1% Lubrol PX, 0.04% sodium dodecyl sulfate (SDS), 0.5 M NaCl, and 0.5 mM phenylmethylsulfonyl fluoride and by subsequent centrifugation at 100,000 × *g* for 1 h. The supernatant was precleaned by passage through Sepharose CL-4B columns (200 μl) twice and then incubated with 2 μg of anti-CYR1HP and 20 μl of protein A-Sepharose (Pharmacia) at 4°C for 2 h with gentle swirling. After thorough washing with the same buffer, the immunoprecipitated material was dissolved in an SDS-dye solution, fractionated by SDS–6% polyacrylamide gel electrophoresis (PAGE) (28), and electroblotted onto a nitrocellulose filter (50). The filter was incubated first with biotinylated anti-CAP and then with alkaline phosphatase-conjugated streptavidin (DAKO Japan, Kyoto, Japan) to visualize CAP as a purple band by color development with 4-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Subsequently, adenylyl cyclase on the same filter was visualized by incubation with anti-CYR1NS1 and the horseradish peroxidase-conjugated $F(ab')_2$ fragment of anti-mouse immunoglobulin G (Amersham, Amersham, Buckinghamshire, United Kingdom) and then red color development with aminoethyl carbazol. Immunoprecipitation of adenylyl cyclase complexes with anti-CAP was performed similarly, and the resulting immunoprecipitates bound to protein A-Sepharose beads were assayed for adenylyl cyclase activity as described below.

Purification of the C-terminal fragments of adenylyl cyclase. Plasmids pAD-GST and pAD-GST-CYR1(Δ1-1878) were constructed as described in the legend to Fig. 1. The plasmids were used to overexpress the C-terminal segment of adenylyl cyclase in yeast cells as a GST fusion protein under control of the *ADC1* promoter. The fusion protein was extracted with buffer C containing 1% Lubrol PX and 0.5 M NaCl from membranes of yeast cells harboring the plasmids and purified by affinity chromatography on glutathione-Sepharose (42). The purified material was examined for CAP and GST-CYR1 protein by Western immunoblotting with anti-CAP, anti-GST, and anti-CYR1CT.

cAMP determination. Glucose-stimulated increase of cAMP concentration was measured essentially as previously described (33, 35). Yeast cells were cultured in YPD at 30°C to a density of 2 × 10^7/ml, harvested by centrifugation, washed twice with 50 mM MES (pH 6.2)–0.1 mM EDTA, and suspended in the same buffer at a density of 2 × 10^8/ml. After incubation for 2 h at 30°C under shaking, glucose was added to a final concentration of 30 mM and 500-μl aliquots of the culture were withdrawn at various time intervals, directly poured into test tubes containing 0.5 ml of 10% trichloroacetic acid and 0.8 ml of glass beads, and quickly frozen in a dry ice-ethanol bath. After thawing, the yeast

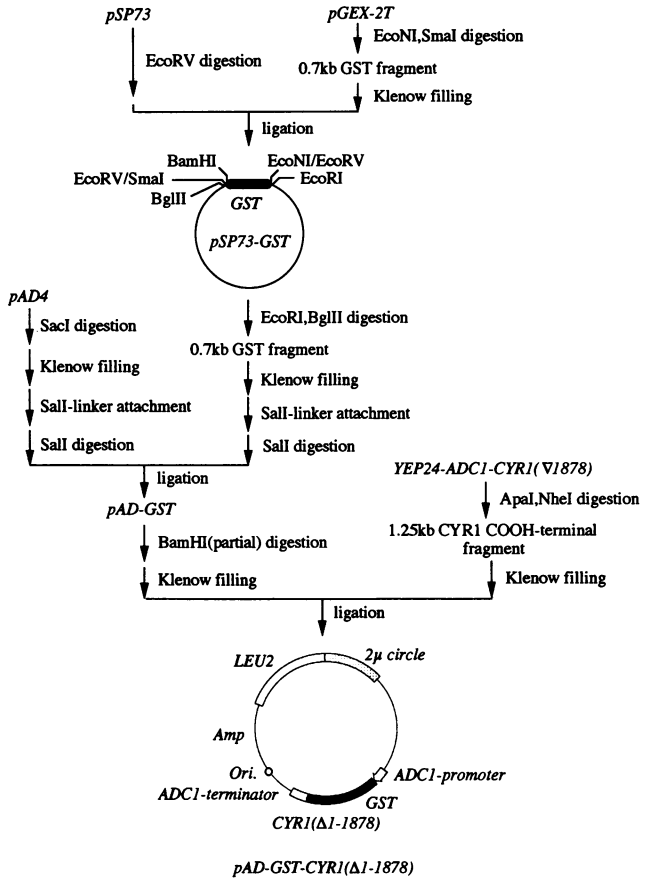

FIG. 1. Construction of pAD-GST-CYR1(Δ1-1878). A 0.7-kb fragment containing the GST coding sequence was cut out of pGEX-2T (42) and cloned into pSP73 (Promega, Madison, Wis.) to produce pSP73-GST by cleavages with the indicated restriction endonucleases, filling of the cohesive termini with the large (Klenow) fragment of *E. coli* DNA polymerase I, and ligation with T4 DNA ligase. Similarly, an *Eco*RI-*Bgl*II GST fragment of pSP73-GST was cloned into pAD4, which contained the 2μm circle origin of replication, the *S. cerevisiae LEU2* gene, and the transcription promoter and terminator sequences of the *S. cerevisiae ADC1* gene (1). The resulting plasmid, pAD-GST, was cleaved with *Bam*HI at the COOH terminus of GST, where an *Apa*I-*Nhe*I fragment of YEP24-ADC1-CYR1(∇1878), corresponding to amino acids 1879 to 2026 of adenylyl cyclase and the 3′-flanking sequence, was cloned to produce pAD-GST-CYR1(Δ1-1878). Subsequently, a *Cla*I-*Nhe*I fragment of YEP24-ADC1-CYR1(∇2011), corresponding to amino acids 1960 to 2026 and the 3′-flanking sequence of CYR1(∇2011), was tranferred to pAD-GST-CYR1 to produce pAD-GST-CYR1(Δ1-1878, ∇2011).

cells were disrupted by shaking with a mini-Beads Beater (Biospec Products, Bartlesville, Okla.) and subjected to cAMP determination by using the [^{3}H]cAMP assay kit from Amersham. The cAMP concentration of yeast cells growing exponentially in YPD was measured similarly after thorough washing of the cells with H_2O. Total protein contents of yeast cells were determined by the method of Lowry et al. (30) after cells were treated with 1 M NaOH.

Fractionation of adenylyl cyclase complexes and adenylyl cyclase assay. Size fractionation of adenylyl cyclase complexes solubilized from yeast membranes was performed by high-performance liquid chromatography on a Superose 6 HR 10/30 column (10 mm by 30 cm; Pharmacia) as described

previously (52). Adenylyl cyclase activities were measured under various conditions essentially as previously described (6, 44, 53). Before the assay, the crude membrane fraction was treated with 1% Lubrol PX–0.5 M NaCl for 1 h at 0°C in 10 μl of buffer C containing 10% glycerol and then added to the 90-μl reaction mixture. For assay of the immunoprecipitates, materials bound to protein A-Sepharose were assayed without dissociation from the gel matrix. For column fractions, 40-μl aliquots were used for the assays. Where indicated, purified RAS2 protein, produced in *E. coli* (6), was incubated with 0.1 mM guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) for 30 min at 30°C in 20 mM Tris-HCl (pH 7.4)–10 mM EDTA–1 mM dithiothreitol–5 mM $MgCl_2$ and added to the reaction mixture.

Other methods and materials. Preparation of yeast genomic DNA and Southern blot hybridization were performed as described previously (26). Commercial sources of other reagents were provided elsewhere (44, 53).

RESULTS

Mutational mapping of a region of adenylyl cyclase whose overexpression suppressed heat shock sensitivity. Field et al. (15) showed that overexpression of the leucine-rich repeat region (amino acids 733 to 1301) of adenylyl cyclase suppressed the heat shock sensitivity of yeast strain TK161-R2V cells carrying the *RAS2*$^{Val-19}$ gene. While confirming their data and examining other regions, we found that another region of adenylyl cyclase, the catalytically inactive C-terminal 258 amino acid residues, overexpressed from plasmid YEP24-ADC1-CYR1(Δ41-1768), displayed the ability to suppress the heat shock sensitivity of TK161-R2V cells (Fig. 2A). To localize the region responsible for the suppression more precisely, we introduced two-amino-acid insertions into various locations between amino acids 1769 and 2026. A 6-bp oligonucleotide, GGGCCC, was inserted into every possible cleavage site by restriction endonucleases *Alu*I, *Hae*III, *Hin*cII, and *Ssp*I after partial digestion of pUC-CYR1NS with the respective enzyme. Similarly, 6-mer pCGGGCC was used to make insertions into every possible cleavage site by *Taq*I and *Aha*II. Both of the linkers introduced *Apa*I cleavage sites at the insertion sites. The resulting mutant *CYR1* fragments were used to construct YEP24-ADC1-CYR1(Δ41-1768) plasmids bearing the corresponding mutations for overexpression in yeast cells as described in Materials and Methods. In addition, some of the resulting insertion mutant *CYR1* genes were used to make further deletions in the C-terminal segments. Heat shock suppression experiments utilizing transformation of YEP24-ADC1-CYR1 with the various deletion mutations enabled us to localize the region required for suppression to the C-terminal 148 residues (amino acids 1879 to 2026) (Fig. 2A). Subsequently, YEP24-ADC1-CYR1(Δ41-1768) plasmids possessing the linker insertions or small deletions originating from the *Cla*I site at amino acid 1959 were examined similarly to analyze requirements for particular amino acid residues within the C-terminal region (Fig. 2A and B). As summarized in Fig. 2C, two-amino-acid insertions at amino acids 1976 and 2011 abolished the suppression activity, whereas those at other locations, including 1958, 2003, and 2006, did not. A deletion between amino acids 1950 and 1959 had no effect, but a deletion further upstream, to 1942, destroyed the suppression activity. The C-terminal 60 residues were also required for suppression.

The amounts of the mutant C-terminal polypeptides in the soluble and membrane fractions of TK161-R2V were measured by Western immunoblotting with anti-CYR1CT. As shown in Fig. 3, the COOH-terminal segments with the expected molecular sizes were overproduced without significant degradation and most of them were recovered in the membrane fractions. The extra bands around 50 to 60 kDa in the membrane fractions appeared to be cross-reacting proteins, and a band corresponding to endogenous wild-type adenylyl cyclase in strain TK161-R2V was undetectable under the conditions used. The result indicated that neither the amounts of the mutant proteins produced nor their

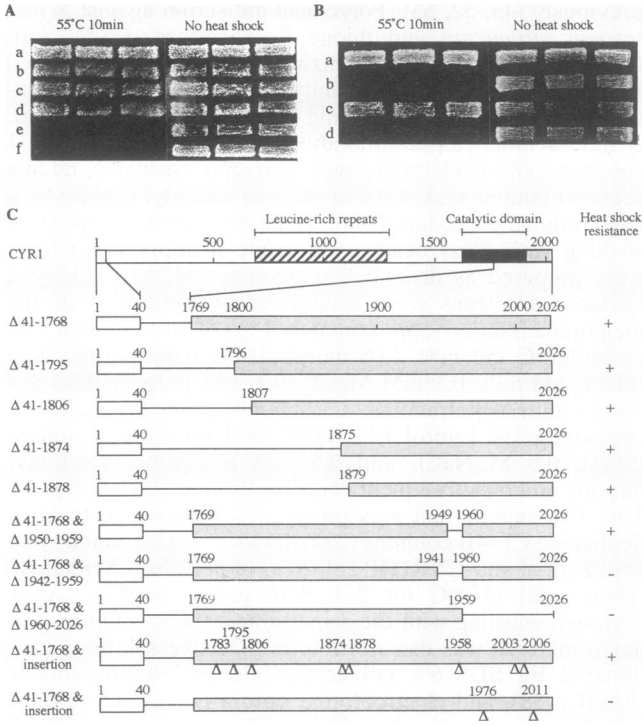

FIG. 2. Suppression of heat shock sensitivity by overproduction of the C-terminal segments of CYR1. (A) TK161-R2V cells were transformed with YEP24-ADC1-CYR1 plasmids possessing the following deletion mutant *CYR1* genes: *CYR1*(*Δ41-1768*) (a), *CYR1*(*Δ41-1806*) (b), *CYR1*(*Δ41-1878*) (c), *CYR1*(*Δ41-1768*, *Δ1950-1959*) (d), *CYR1*(*Δ41-1768*, *Δ1942-1959*) (e), and *CYR1*(*Δ41-1768*, *Δ1960-2026*) (f). Three independent Ura$^+$ colonies were isolated from each transformation and streaked in a row on a plate of yeast synthetic medium lacking uracil. Heat shock sensitivity was measured by the replica plating method as described in Materials and Methods. Shown are photographs of the two replica plates, one subjected to 55°C heat shock for 10 min (left) and the other kept at 30°C (right), after 2 days of growth at 30°C. (B) A similar heat shock experiment was performed on TK161-R2V cells transformed with YEP24-ADC1-CYR1(Δ41-1768) bearing the two-amino-acid insertion at amino acid 2003 (a), 1976 (b), 2006 (c), or 2011 (d). (C) Results of the heat shock experiments shown schematically. Acquirement of heat shock resistance indicates that heat shock sensitivity has been suppressed. The top bar represents the structure of adenylyl cyclase and approximate locations of the leucine-rich repeats and the catalytic domain bearing the Mn^{2+}-dependent activity (24, 44, 53). The numbers on the left indicate the natures of mutations in the overproduced CYR1 proteins in amino acid numbers. Horizontal bars represent the region contained in the mutant proteins with shading over the C-terminal subregions, while lines indicate the ranges of deletions. Numbers on the bars indicate amino acid numbers at borders of the deletions or at the insertion sites. Triangles under the bars indicate locations of the two-amino-acid insertions.

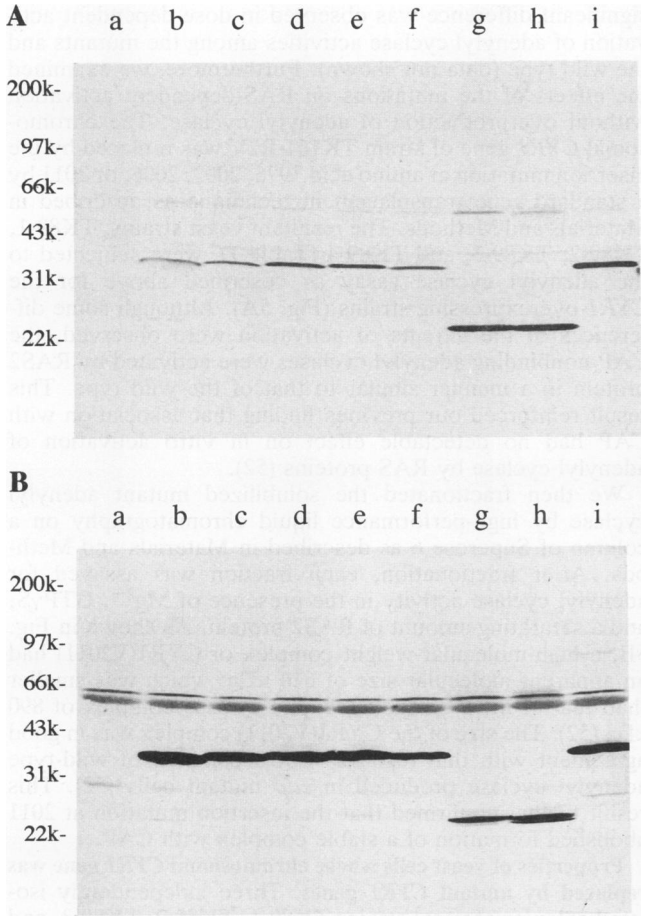

FIG. 3. Immunoblotting analysis of the C-terminal segments bearing various mutations. TK161-R2V cells harboring the expression plasmids of various mutant C-terminal segments were disrupted by shaking with glass beads and centrifuged at 27,000 × *g* for 90 min. The resulting supernatants and the precipitates were concentrated by precipitation with acetone, fractionated by SDS-PAGE (6 to 20% linear gradient gel), and analyzed by immunoblotting with anti-CYR1CT as described in Materials and Methods. Shown are the results of the immunoblotting analyses of the supernatants (approximately 45 μg of protein) (A) and the precipitates (30 μg of protein) (B) obtained from about 3×10^7 cells each of the yeast strains harboring forms of YEP24-ADC1-CYR1 possessing various deletion and insertion mutant *CYR1* genes. Lanes: a, *YEP24* only; b, *CYR1*(Δ*41-1768*, Δ*1942-1959*); c, *CYR1*(Δ*41-1768*, Δ*1950-1959*); d, *CYR1*(Δ*41-1768*, ∇*2011*); e, *CYR1*(Δ*41-1768*, ∇*2006*); f, *CYR1*(Δ*41-1768*, ∇*1976*); g, *CYR1*(Δ*41-1874*); h, *CYR1*(Δ*41-1878*); i, *CYR1*(Δ*41-1768*). Molecular size markers were the myosin heavy chain (200 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (43 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (22 kDa).

extents of membrane localization correlated with the ability to suppress heat shock sensitivity. Therefore, we concluded that the loss of suppression could not be explained by preferential degradation, lower production, or loss of membrane attachment of the mutant proteins.

Measurement of the amounts of CAP bound to mutant adenylyl cyclases. We postulated that the mechanism of suppression of heat shock sensitivity by the overproduced C-terminal region might be competitive sequestration of a protein somehow involved in maintenance of adenylyl cyclase activity. Because overproduction of the leucine-rich repeat region was proposed to sequester RAS proteins (15), we assumed that the best candidate sequestered by the C terminus would be 70-kDa CAP. To test this possibility, we made yeast expression plasmids of the full-length adenylyl cyclases (YEP24-ADC1-CYR1) possessing the same C-terminal mutations as used for the heat shock experiments (see Materials and Methods). The mutant and wild-type adenylyl cyclases were overexpressed in yeast strain TK35-1 cells deficient in CYR1, solubilized from the membranes, and examined for immunoprecipitability of their RAS2 protein-dependent activities by anti-CAP. Only the mutations downstream of amino acid 1878 were examined because the upstream mutations destroyed the catalytic activity of adenylyl cyclase and because the C-terminal 148 residues had been shown to retain the suppression activity. As shown in Fig. 4A, the wild type and one of the C-terminal deletion mutants had about 40 to 50% of their RAS2-GTPγS-dependent activities precipitated by anti-CAP but activities of the other mutants were almost unprecipitable by the same antibody. A notable correlation was observed between the ability of a particular mutant to suppress heat shock sensitivity and the percentage of its adenylyl cyclase activity precipitable by anti-CAP. The mutations that abolished the suppression activity brought about the loss of reactivity with anti-CAP, which probably meant dissociation of CAP from the mutant adenylyl cyclases.

To demonstrate this further, we measured the amount of CAP coimmunoprecipitable with an antibody against adenylyl cyclase. The adenylyl cyclase complexes were immunoprecipitated with anti-CYR1HP, and CAP present in the immunoprecipitates was detected as a 70-kDa band by Western immunoblotting with anti-CAP as described in Materials and Methods. Subsequently, the same blot was probed with a monoclonal antibody against adenylyl cyclase, anti-CYR1NS1, to visualize the bands of adenylyl cyclase as 220- and 250-kDa bands. The nature of the faint 250-kDa band which has always been observed (52) is unknown. The band of CAP and that of adenylyl cyclase were distinguishable from each other by their different colors (see Materials and Methods). As shown in Fig. 4B, CAP was not detectably bound to the mutant adenylyl cyclases whose activities could not be immunoprecipitated with anti-CAP. This implied that the C-terminal 148 residues of adenylyl cyclase contained a CAP-binding site and that the mechanism of suppression of heat shock sensitivity upon their overexpression was competitive sequestration of CAP from endogenous adenylyl cyclase. This was further demonstrated by measuring the amount of CAP bound to the C-terminal 148-residue segment, CYR1(Δ1-1878), that was produced as a fusion protein with GST in yeast strain TK161-R2V cells and purified by glutathione-Sepharose chromatography as described in Materials and Methods (Fig. 4C). CAP binding to this segment was also abolished by the insertion mutation at amino acid 2011. No significant difference was observed in the amounts of CAP expressed in the three strains used (data not shown). We confirmed that the overexpressed *GST-CYR1*(Δ1-1878) gene retained the ability to suppress the heat shock sensitivity of $RAS2^{Val\text{-}19}$. This activity was also abolished by the mutation at amino acid 2011 (data not shown).

RAS activation and molecular sizes of mutant adenylyl cyclases. Adenylyl cyclase having the insertion at amino acid 1976, 2003, 2006, or 2011, overproduced in TK35-1 cells, was solubilized by extraction with 1% Lubrol PX and 0.5 M NaCl and assayed for adenylyl cyclase activity in the presence of Mg^{2+}, GTPγS, and various amounts of RAS2 protein. No

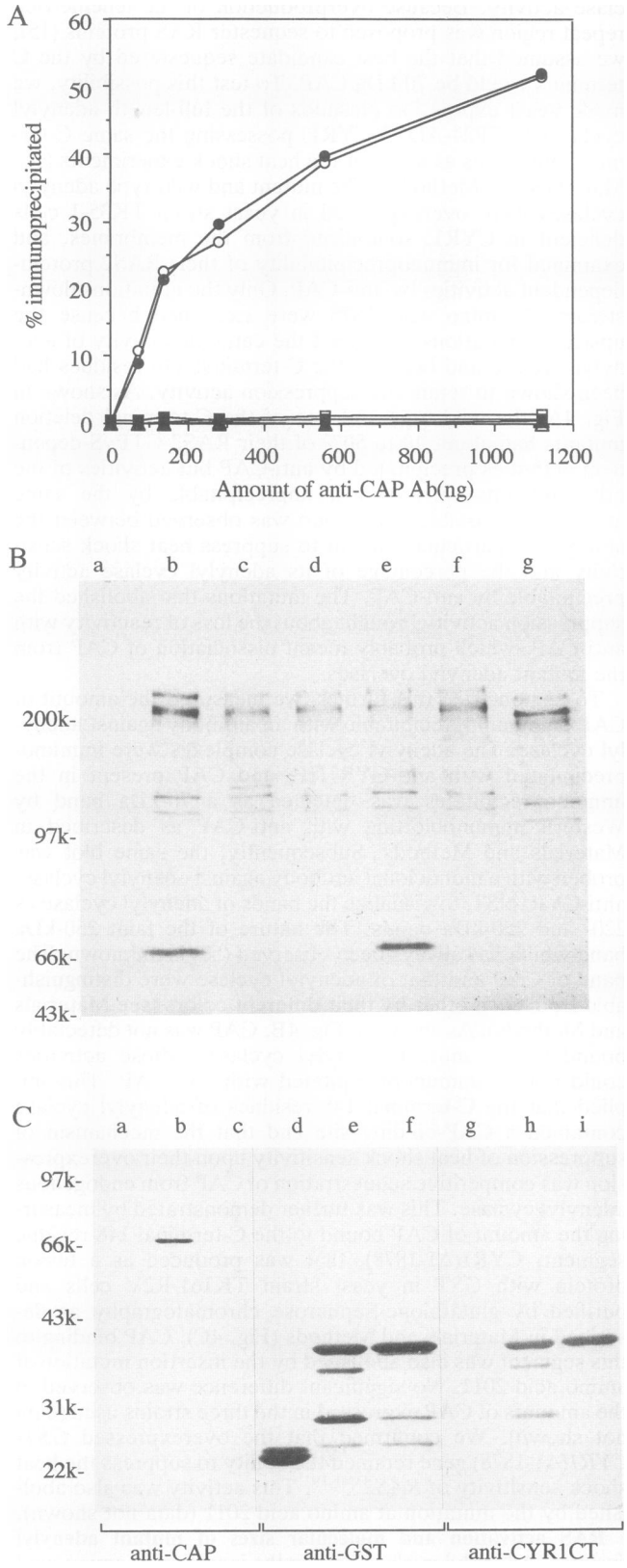

FIG. 4. Measurement of association of CAP with adenylyl cyclase mutants. (A) RAS2- and GTPγS-dependent adenylyl cyclase activities were measured as described in Materials and Methods for immunoprecipitates, with increasing amounts of anti-CAP, of extracts from TK35-1 cells harboring YEP24-ADC1-CYR1 with the wild type (○) and the following forms of mutant *CYR1*: *CYR1*(Δ*1942-1959*) (□), *CYR1*(Δ*1950-1959*) (●), *CYR1*(Δ*1960-2026*) (△), *CYR1*(∇*1976*) (▲), and *CYR1*(∇*2011*) (■). The vertical axis represents percentages of the original activities recovered in the precipitates. (B) Amounts of CAP and adenylyl cyclase in immunoprecipitates of extracts from various yeast strains with anti-CYR1HP were measured by Western immunoblotting as described in Materials and Methods. The extracts were prepared from TK35-1 possessing plasmid YRP7-TPK1 (lane a) or forms of plasmid YEP24-ADC1-CYR1 with wild-type *CYR1* (b) and mutant *CYR1* gene *CYR1*(∇*1976*) (c), *CYR1*(∇*2011*) (d), *CYR1*(Δ*1950-1959*) (e), *CYR1*(Δ*1942-1959*) (f), or *CYR1*(Δ*1960-2026*) (g). Although not visible in the black and white photograph, the 70-kDa band was produced by anti-CAP and the other bands by anti-CYR1NS1 as judged by their colors (see Materials and Methods). (C) The proteins purified by glutathione-Sepharose chromatography were fractionated by SDS-PAGE (8 to 15% exponential gradient gel) and analyzed by Western immunoblotting with anti-CAP (lanes a, b, and c), anti-GST (lanes d, e, and f), and anti-CYR1CT (lanes g, h, and i). Approximately 100 ng of the proteins purified from TK161-R2V harboring pAD-GST (lanes a, d, and g), pAD-GST-CYR1(Δ1-1878) (lanes b, e, and h), or pAD-GST-CYR1(Δ1-1878, ∇2011) (lanes c, f, and i) was applied to each lane.

significant difference was observed in dose-dependent activation of adenylyl cyclase activities among the mutants and the wild type (data not shown). Furthermore, we examined the effects of the mutations on RAS-dependent activation without overproduction of adenylyl cyclase. The chromosomal *CYR1* gene of strain TK161-R2V was replaced by the insertion mutation at amino acid 1976, 2003, 2006, or 2011 by a standard gene transplacement technique as described in Materials and Methods. The resultant yeast strains, TK89-1, TK89-2, TK89-3, and TK89-4 (Table 1), were subjected to the adenylyl cyclase assay as described above for the *CYR1*-overexpressing strains (Fig. 5A). Although some differences in the extents of activation were observed, the CAP-nonbinding adenylyl cyclases were activated by RAS2 protein in a manner similar to that of the wild type. This result reinforced our previous finding that association with CAP had no detectable effect on in vitro activation of adenylyl cyclase by RAS proteins (52).

We then fractionated the solubilized mutant adenylyl cyclase by high-performance liquid chromatography on a column of Superose 6 as described in Materials and Methods. After fractionation, each fraction was assayed for adenylyl cyclase activity in the presence of Mg^{2+}, GTPγS, and a saturating amount of RAS2 protein. As shown in Fig. 5B, a high-molecular-weight complex of CYR1(∇2011) had an apparent molecular size of 610 kDa, which was smaller than that of the wild-type adenylyl cyclase complex of 890 kDa (52). The size of the CYR1(∇2011) complex was in good agreement with that (610 kDa) of a complex of wild-type adenylyl cyclase produced in *cap* mutant cells (52). This result further confirmed that the insertion mutation at 2011 abolished formation of a stable complex with CAP.

Properties of yeast cells whose chromosomal *CYR1* gene was replaced by mutant *CYR1* genes. Three independently isolated colonies of yeast strains TK89-1, TK89-2, TK89-3, and TK89-4 were examined for sensitivity to heat shock as shown in Fig. 6A. Only yeast cells possessing the CAP-nonbinding mutant forms of *CYR1*, i.e., *CYR1*(∇1976) and *CYR1*(∇2011), lost the sensitivity. This result finally demonstrated that the association of CAP with adenylyl cyclase is required for induction of a cellular response that results in heat shock sensitivity in the presence of $RAS2^{Val-19}$.

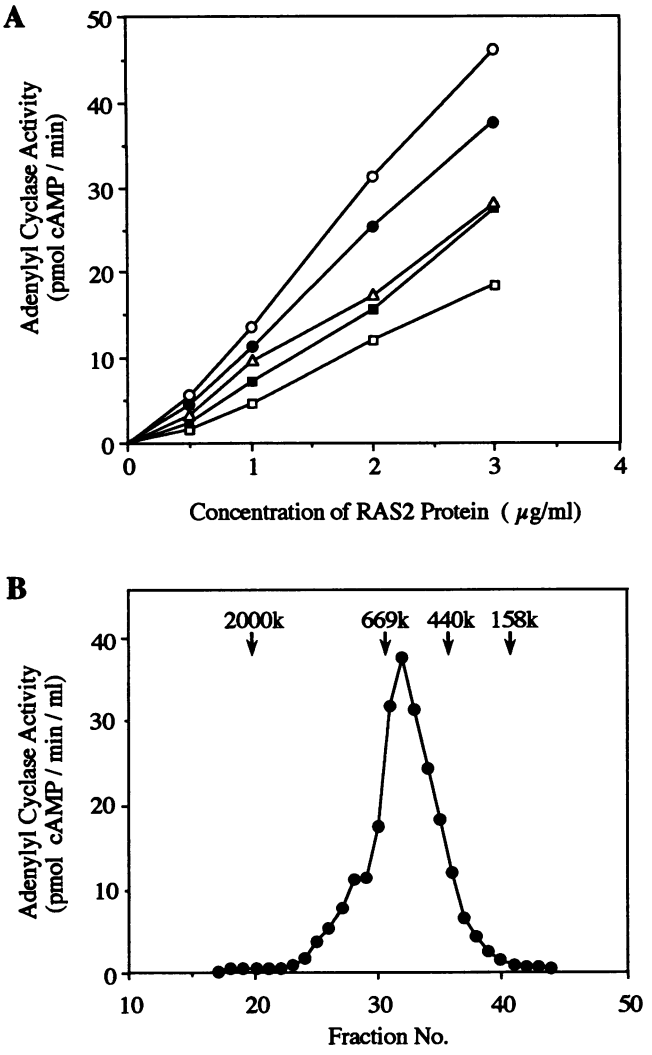

FIG. 5. RAS activation and molecular sizes of mutant adenylyl cyclases. (A) Crude membrane fractions of various yeast strains were treated with 1% Lubrol PX and 0.5 M NaCl and assayed for adenylyl cyclase activity in the presence of Mg^{2+}, GTPγS, and various concentrations of RAS2 protein as described in Materials and Methods. Membranes of TK89-1 (41 μg of protein) (□), TK89-2 (30 μg) (●), TK89-3 (25 μg) (■), TK89-4 (26 μg) (○), and TK161-R2V (39 μg) (△), all of which possessed approximately 30 U of the Mn^{2+}-dependent activities, were used for the assay. One unit of activity is defined as 1 pmol of cAMP formed in 1 min of incubation with 1 mg of membrane protein at 32°C under standard assay conditions. (B) An extract (2 mg of protein) from TK35-1 cells harboring YEP24-ADC1-CYR1(∇2011) was fractionated by gel exclusion chromatography on a Superose 6 column, and an aliquot of each fraction (400 μl) was assayed for adenylyl cyclase activity in the presence of 2.5 mM $MgCl_2$, 50 μM GTPγS, and 2.0 μg of RAS2 protein as described in Materials and Methods. The apparent molecular mass of the adenylyl cyclase complex was calculated by assuming a globular conformation as described previously (2). The molecular size markers used to calibrate the column were blue dextran (~2,000 kDa), bovine thyroglobulin (669 kDa), equine ferritin (440 kDa), and rabbit muscle aldolase (158 kDa).

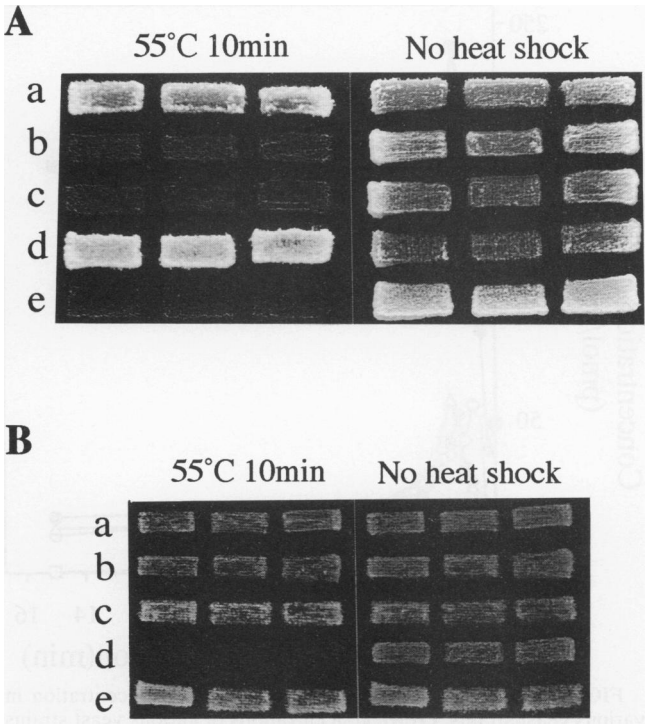

FIG. 6. Heat shock sensitivity of *RAS2*$^{Val-19}$ mutant yeast cells carrying mutations in the *CYR1* gene. (A) Three independent isolates of yeast strains TK89-1 (a), TK89-2 (b), TK89-3 (c), and TK89-4 (d) and three colonies of TK161-R2V (e) were examined for heat shock sensitivity as described in the legend to Fig. 2. (B) TK89-4 cells were transformed with pAD-RAS2^{Val-19} (a), pAD-CDC25 (b), pADH-CAP (c), or pAD4 (e), and the resulting three independent Leu$^+$ colonies from each transformation were examined for heat shock sensitivity. TK161-R2V cells transformed with pAD4 (d) were included as a control.

We next examined the effects of overexpression of CAP, RAS2^{Val-19}, and Cdc25 on the heat shock sensitivity of TK161-R2V cells bearing the mutant adenylyl cyclases. Plasmids pADH-CAP, pAD-RAS2^{Val-19}, and pAD-CDC25 were tranformed into TK89-1 and TK89-4 cells, and the resulting Leu$^+$ transformants were examined for heat shock sensitivity. Overexpression of CAP from the *ADC1* promoter, which resulted in about a 10-fold increase of CAP (data not shown), failed to restore heat shock sensitivity in TK89-4 (Fig. 6B) and TK89-1 (data not shown) cells, suggesting that the affinities of CYR1(∇2011) and CYR1(∇1976) for CAP were severely impaired or even totally lost. Similarly, heat shock sensitivity was not restored by overproduction of RAS2^{Val-19} or Cdc25 protein (Fig. 6B). About 30- and 50-fold overproduction was achieved for RAS2^{Val-19} and Cdc25, respectively (data not shown). Thus, it is unlikely that CAP plays a facilitative role in the interaction of adenylyl cyclase with RAS proteins in a manner that can be compensated by overproduction of RAS2^{Val-19} or Cdc25 protein. Transformation of low-copy-number plasmid pCYR1-2 (24), which contained the genomic wild-type *CYR1* gene, the *URA3* marker, *CEN3*, and *ARS1*, into TK89-1 or TK89-4 restored heat shock sensitivity (data not shown). This indicated that other genes involved in the heat shock response were not affected by the genetic manipulation for *CYR1* gene replacement.

Glucose-induced cAMP accumulation in yeast cells having mutant *CYR1* genes. To analyze the mechanism of the disappearance of heat shock sensitivity, we measured time courses of changes in cAMP concentrations in various strains of yeast cells which were induced by addition of

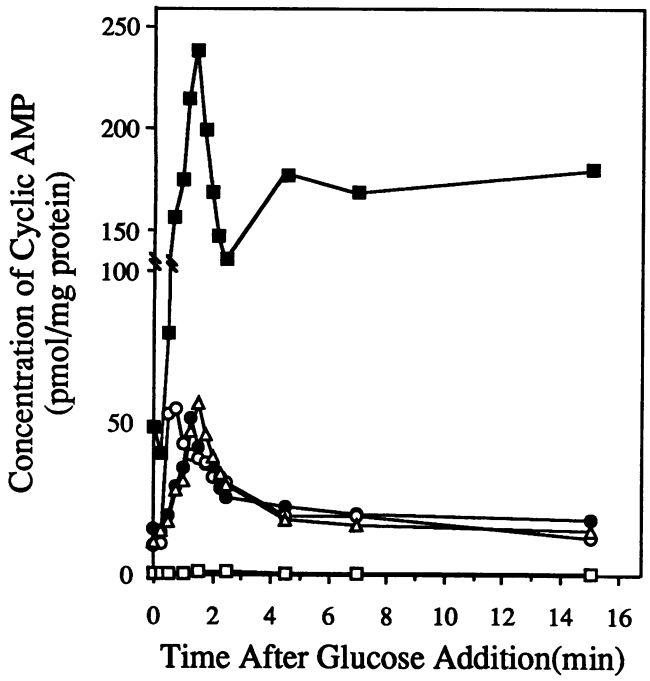

FIG. 7. Glucose-stimulated increase of cAMP concentration in various yeast strains. cAMP concentrations of various yeast strains were determined, as described in Materials and Methods, at the indicated time intervals after addition of glucose. Shown are the time courses of changes in cAMP concentration of yeast strains SP1 (○), TK89-4 (●), TK90-1 (△), TK161-R2V (■), and T50-3A with YRp7-TPK1 (□). For TK89-4 and TK90-1, each cAMP concentration value represents the mean of two determinations each for three independent isolates. For the other strains, each value represents the mean of three determinations. The standard deviation of each point was less than 13, 20, 13, 3, and 31% of the corresponding mean value for SP1, TK89-4, TK90-1, TK161-R2V, and T50-3A with YRp7-TPK1, respectively.

glucose to the culture medium after the cells had been starved for glucose for 2 h. As shown in Fig. 7, wild-type yeast SP1 underwent a transient sixfold rise in the intracellular cAMP concentration that peaked at about 50 pmol/mg of protein around 30 to 45 s after glucose addition. After the peak, the cAMP concentration returned to a steady-state level of about 10 pmol/mg of protein. These data are similar to those reported previously (33–35, 39). In contrast, TK161-R2V, possessing *RAS2*$^{Val-19}$ and wild-type *CYR1*, gave a transient peak of a much higher cAMP concentration of 230 pmol/mg of protein around 75 s after glucose addition, which was followed by a persistent elevation at a concentration of about 170 pmol/mg of protein. The relative rise of cAMP from the zero-time value at the initial peak (about fivefold) was not so much different from that in SP1. This pattern was quite different from that reported by another group (33), who reported disappearance of cAMP accumulation in the *RAS2*$^{Val-19}$ mutant. We do not know the reason for this discrepancy. When TK89-4 cells possessing *RAS2*$^{Val-19}$ and *CYR1*(∇2011) were examined similarly, a pattern strikingly different from that of its parent, TK161-R2V, was observed (Fig. 7). The prolonged elevation and high peak level of the cAMP concentration characteristic of TK161-R2V were totally lost in TK89-4 cells. Moreover, the time course and the level of cAMP accumulation in TK89-4 cells were apparently indistinguishable from those in SP1 except that the transient rise reached a peak at around 75 s, about 30 s later than in SP1. TK89-1 cells were also examined and gave a result essentially similar to those of TK89-4 (data not shown). This indicated that the CAP-nonbinding adenylyl cyclases could respond to RAS proteins in vivo but lost the exaggerated response to RAS2^{Val-19}. To determine whether the effect of the mutations was specific to RAS2^{Val-19}, we constructed TK90-1, a yeast strain carrying the *CYR1*(∇2011) gene in the wild-type *RAS2* background, as described in Materials and Methods, and examined the time course of glucose-stimulated cAMP accumulation (Fig. 7). TK90-1 cells exhibited a response pattern almost indistinguishable from that of TK89-4, with a peak cAMP level that was almost the same and an about 30- to 45-s delay in reaching the peak compared with wild-type SP1. This suggested that the dissociation of CAP specifically abolished the exaggerated response of adenylyl cyclase to activated RAS without affecting the normal response to wild-type RAS2. As expected, T50-3A cells, which were deficient in CYR1 and made viable by transformation with YRp7-TPK1 (48), did not accumulate detectable levels of cAMP throughout the time course. In addition, we also measured the cAMP concentrations of yeast strains growing exponentially in YPD (Table 2). TK89-1, TK89-4, and TK90-1 cells had steady-state cAMP concentrations of about 13, 14, and 11 pmol/mg of protein, respectively, which were close to that of SP1. TK161-R2V had a much higher value of about 79 pmol of cAMP per mg of protein.

DISCUSSION

We found that overexpression of the C-terminal segment of adenylyl cyclase could suppress the heat shock sensitivity of yeast cells bearing the *RAS2*$^{Val-19}$ gene. Structural requirements of the C-terminal region for suppression were analyzed further by in vitro mutagenesis, and measurement

TABLE 2. Steady-state cAMP levels of various yeast strains

Strain[a]	Genotype		Plasmid	cAMP level[b] (pmol/mg of protein)
	RAS2	*CYR1*		
TK89-1	*RAS2*$^{Val-19}$	*CYR1*(∇*1976*)		12.6 ± 0.9
TK89-4	*RAS2*$^{Val-19}$	*CYR1*(∇*2011*)		14.2 ± 0.8
TK161-R2V	*RAS2*$^{Val-19}$	Wild type		78.9 ± 2.5
SP1	Wild type	Wild type		10.7 ± 1.1
TK90-1	Wild type	*CYR1*(∇2011)		11.4 ± 0.9
T50-3A	Wild type	*cyr1-2*[c]	YRP7-TPK1	0.7 ± 0.2

[a] Complete genotypes of the yeast strains are given in Table 1.
[b] Values represent the mean ± the standard deviation of three determinations.
[c] Encodes temperature-sensitive adenylyl cyclase inactive at 30°C.

of the association of CAP with the resulting mutants revealed a perfect correlation between the activity to suppress heat shock sensitivity and the ability to bind CAP. This was further confirmed by determination of the size of an adenylyl cyclase complex carrying the mutant adenylyl cyclase. These data enabled us to conclude that the suppression is caused by competitive sequestration of CAP from endogenous adenylyl cyclase and to assess some structural requirements for CAP binding in the C-terminal region. The resultant CAP-nonbinding mutant *CYR1* genes were used for construction of yeast strains whose chromosomal *CYR1* genes were replaced by them. The mutant strains were used to assess the function of CAP in the RAS-adenylyl cyclase pathway. Although the extent of activation by RAS proteins, measured both in vivo and in vitro, appeared to be unaffected by the mutations, heat shock sensitivity in the *RAS2*$^{Val-19}$ background was abolished in the CAP-nonbinding mutant strains. At the same time, the time courses of cAMP accumulation and the peak and steady-state cAMP levels in these strains lost the typical *RAS2*$^{Val-19}$ pattern and became almost indistinguishable from those of the wild-type yeast cells. Thus, it seems reasonable to assume that the loss of heat shock sensitivity resulted from the change in the cAMP accumulation pattern.

Studies of the function of CAP have revealed that CAP is a bifunctional protein (14, 17). Gerst et al. (17) have shown that the N-terminal 168 amino acid residues of CAP are required for acquirement of heat shock sensitivity in the *RAS2*$^{Val-19}$ background and the C-terminal 158 residues are required for normal cell morphology and responsiveness to nutrient deprivation and excess. Loss of the latter function appears to be compensated by overexpression of profilin (51), an actin-sequestering protein (37, 43), or of SNC1, a yeast homolog of synaptobrevin (18). In addition to binding to an actin monomer, profilin binds to polyphosphoinositides, which results in its dissociation from actin and protection of polyphosphoinositides from hydrolysis by phospholipase C (20, 29). Recently, homologs of CAP have been identified in the fission yeast *Schizosaccharomyces pombe* and in humans (27, 32). The C-terminal domains of the CAP proteins of the three species are functionally, as well as structurally, conserved (27, 32). Comparison of the amino acid sequences has revealed that human CAP may be a human counterpart of porcine ASP-56, a protein possessing an actin-sequestering function (19, 32). From these data, it has been proposed that the C-terminal domain of CAP might be involved in regulation of actin filament formation and/or in modulation of phosphoinositide metabolism (19, 32).

In contrast, the N-terminal domain is not functionally conserved, although some structural homology exists. *S. pombe* CAP binds to adenylyl cyclase of the same species but not to that of *S. cerevisiae* and is unable to replace the function of the N-terminal domain of *S. cerevisiae* CAP (27). In these studies, disappearance of *RAS2*$^{Val-19}$-dependent heat shock sensitivity was used as a phenotypic marker ascribed to loss of the N-terminal function of CAP. Although previous reports on disruption of the *CAP* gene suggested a critical role of the N-terminal domain for interaction of adenylyl cyclase with RAS proteins (14), we have demonstrated that CAP is not required for RAS-dependent in vitro activation of adenylyl cyclase (52). Our present data further confirm this and, in addition, suggest that the in vivo RAS response also is not significantly affected by the dissociation of CAP. Furthermore, our data indicate that adenylyl cyclase is the only target of the N-terminal domain of CAP that is critical in a pathway leading to *RAS2*$^{Val-19}$-dependent heat shock sensitivity. Considering the result of measurement of cAMP concentrations, this pathway is presumably the RAS-adenylyl cyclase pathway.

Our data suggest that the observed effect of association of CAP with adenylyl cyclase is ascribable to a function of CAP which is not directly involved in the RAS protein-adenylyl cyclase interaction. This is further supported by the fact that *S. pombe* adenylyl cyclase, which is not regulated by its Ras protein (16), is associated with CAP. The cAMP level in yeast cells is known to be subject to rigorous feedback control. The glucose-triggered rapid rise in the intracellular cAMP level, which is dependent on Cdc25 and RAS (33, 35), is immediately followed by return to the steady-state level (33–35, 39; Fig. 7), and the return appears to require the activity of cAMP-dependent protein kinases (34). Recently, phosphorylation of protein Cdc25 by cAMP-dependent protein kinases has been implicated in this feedback downregulation of the cAMP level following the rapid rise (21). Similarly, the steady-state cAMP level is controlled by a rigorous feedback mechanism which also appears to depend upon phosphorylation of Cdc25 or adenylyl cyclase by cAMP-dependent protein kinases (36). In either case, the feedback regulations appear to be overridden by the *RAS2*$^{Val-19}$ mutation (36; this report), although the underlying mechanism remains unclear. Our data suggest that the suppressive effect of *RAS2*$^{Val-19}$ on feedback regulation, which results in the typical *RAS2*$^{Val-19}$ pattern of cAMP accumulation, is lost as a result of dissociation of CAP from adenylyl cyclase. However, the dissociation of CAP does not appear to affect the cAMP accumulation pattern of wild-type *RAS2*-containing cells. Thus, it is tempting to speculate that the association of CAP with adenylyl cyclase is specifically involved in a mechanism by which RAS2^{Val-19} inhibits the feedback downregulation of adenylyl cyclase. However, it is still possible that the loss of association with CAP may simply cause attenuation of the activity of adenylyl cyclase in a manner that is undetectable in our in vitro and in vivo assay system. In either case, the C-terminal function of CAP, which is possibly related to regulation of the actin cytoskeleton, may be involved. One interesting possibility is that CAP mediates interaction of adenylyl cyclase with actin filaments, which might affect the subcellular localization of or lateral movement on the membrane of adenylyl cyclase. Because association with CAP does not appear to be necessary for membrane attachment of adenylyl cyclase (Fig. 3), it might be spatial distribution on the membrane that is affected. Such a change might cause altered accessibility of adenylyl cyclase to RAS proteins or to other key factors in the RAS-adenylyl cyclase system.

We have mapped a CAP-binding site of adenylyl cyclase. Specifically, amino acid residues around positions 1976 and 2011 and between 1942 and 1950 appear to be critical for CAP binding. They are interspersed with nonessential residues around positions 1958, 2003, and 2006 and between 1950 and 1959. However, it is impossible to deduce any structural feature essential for association with CAP from the present study. *S. pombe* adenylyl cyclase has little amino acid sequence homology with the C-terminal region of *S. cerevisiae* CYR1 (53). This may explain why the N-terminal domains of the CAP proteins in the two species are not functionally interchangeable.

Finally, we have shown that overexpression of the C-terminal CAP-binding site of *S. cerevisiae* adenylyl cyclase results in suppression of the phenotypes attributable to the activated RAS2 protein. The resultant yeast cells grow normally. Because the suppressive effect appears to result

from competitive sequestration of CAP from endogenous adenylyl cyclase, it is expected to be specific on activated $RAS2^{Val-19}$ in view of the specificity of the effects of *CYR1* mutations that abolish CAP binding. Although the mechanism that underlies this phenomenon is unclear, a similar principle might be applicable for suppression of the activity of activated Ras in other organisms. At least in the budding yeast, this is superior to the use of overexpression of the leucine-rich repeat region of *S. cerevisiae* adenylyl cyclase, which presumably works by competitive inhibition of interaction with RAS proteins and, therefore, inevitably results in suppression of RAS-dependent signal transduction.

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