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Effect of Association with Adenylyl Cyclase-Associated Protein on the Interaction of Yeast Adenylyl Cyclase with Ras Protein

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Posttranslational modification of Ras protein has been shown to be critical for interaction with its effector molecules, including *Saccharomyces cerevisiae* adenylyl cyclase. However, the mechanism of its action was unknown. In this study, we used a reconstituted system with purified adenylyl cyclase and Ras proteins carrying various degrees of the modification to show that the posttranslational modification, especially the farnesylation step, is responsible for 5- to 10-fold increase in Ras-dependent activation of adenylyl cyclase activity even though it has no significant effect on their binding affinity. The stimulatory effect of farnesylation is found to depend on the association of adenylyl cyclase with 70-kDa adenylyl cyclase-associated protein (CAP), which was known to be required for proper in vivo response of adenylyl cyclase to Ras protein, by comparing the levels of Ras-dependent activation of purified adenylyl cyclase with and without bound CAP. The region of CAP required for this effect is mapped to its N-terminal segment of 168 amino acid residues, which coincides with the region required for the in vivo effect. Furthermore, the stimulatory effect is successfully reconstituted by in vitro association of CAP with the purified adenylyl cyclase molecule lacking the bound CAP. These results indicate that the association of adenylyl cyclase with CAP is responsible for the stimulatory effect of posttranslational modification of Ras on its activity and that this may be the mechanism underlying its requirement for the proper in vivo cyclic AMP response.

The budding yeast *Saccharomyces cerevisiae* has two *RAS* genes, *RAS1* and *RAS2*, whose protein products are structurally, functionally, and biochemically similar to mammalian Ras proto-oncoproteins (for reviews, see references 1 and 13). The yeast Ras proteins are essential regulatory elements of adenylyl cyclase (2, 38), which catalyzes the production of cyclic AMP (cAMP), a second messenger vital for yeast cell growth. The Ras-adenylyl cyclase pathway has been implicated in transduction of a glucose-triggered signal to an intracellular environment where a protein phosphorylation cascade is induced by cAMP. Yeast cells bearing the activated *RAS2* gene, $RAS2^{Val\text{-}19}$, exhibit an elevated level of intracellular cAMP and display abnormal phenotypes, including sensitivity to heat shock, sensitivity to nutritional starvation, and failure to sporulate (21, 38).

Yeast adenylyl cyclase, a product of the *CYR1* gene (28), consists of 2,026 amino acid residues that comprise at least four domains: N terminal, middle repetitive, catalytic, and C terminal (20, 47). The middle repetitive domain is composed of a repetition of 23-amino-acid amphipathic leucine-rich motifs that have homology to the leucine-rich repeat (LRR) family proteins found in a wide variety of organisms (for a review, see reference 24). Genetic and biochemical studies showed that the LRR domain of adenylyl cyclase contains a binding site for Ras proteins (5, 29, 37, 41). Adenylyl cyclase forms a complex with the 70-kDa adenylyl cyclase-associated protein (CAP). CAP was identified biochemically as the only protein associated with adenylyl cyclase (7). It was also identified independently by genetic screening of the gene whose mutation abolished the $RAS2^{Val\text{-}19}$-dependent heat shock sensitivity (6, 7). CAP is a bifunctional protein (11). Its C-terminal region binds to actin monomer and appears to be involved in cytoskeletal regulation (8, 14). The N-terminal region of CAP binds to the C-terminal region of adenylyl cyclase (44), and this association appears to be required for proper in vivo response of adenylyl cyclase to Ras because its loss by mutation of either CAP or adenylyl cyclase resulted in disappearance of the $RAS2^{Val\text{-}19}$-dependent heat shock sensitivity (11, 44). However, the mechanism by which CAP is involved in the Ras-adenylyl cyclase interaction was unknown. By using an in vitro reconstituted system, we have shown that CAP is not essential for activation of adenylyl cyclase by purified unmodified Ras proteins produced in *Escherichia coli* (43, 44).

Ras proteins undergo a series of posttranslational modifications in their unique C-terminal region, called a CAAX motif (C, cysteine; A, aliphatic; and X, any amino acid), which are essential for their biological activities (for reviews, see references 3 and 4). The first stage of the processing consists of three successive modifications of the CAAX motif: (i) farnesylation of the cysteine residue, (ii) proteolytic cleavage of the amino acids AAX, and (iii) methyl esterification of the new C-terminal cysteine. Mammalian H-Ras and yeast Ras are further modified by acylation with palmitic acid on cysteine residues (Cys-181 and Cys-184 for H-Ras) immediately upstream of the CAAX motif, finally yielding the posttranslationally fully modified forms. Although it is established that these modifications are essential for anchoring Ras proteins to the plasma membrane (15, 45), we have shown by using a membrane-free in vitro pure reconstituted system that the modifications of yeast Ras, especially the farnesylation step, are also critical for activation of its effector, yeast adenylyl cyclase (25). A similar finding was obtained for in vitro activation of B-Raf, a mammalian effector of Ras, by Ras proteins (30, 46), and it was further shown that the modification did not detectably affect the binding affinity of B-Raf for Ras (30). These results prompted us to examine the molecular mechanism by which the posttranslational modification enhances Ras-dependent

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TABLE 1. Yeast strains used in this study

Strain[a]	Genotype	Extrachromosomal plasmid
SP1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1*	
TK36-1	*MAT*α *his3 leu2 trp1 ura3 ade8 cyr1-2 ras2::LEU2*	YEP24-ADC1-CYR1
FS1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1 cap::*pCAPΔN-1	
TM5-1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1*	pAD4-GST-CYR1(606-1380)
NS1-1	*MAT*α *his3 leu2 trp1 ura3 ade8 cyr1-2 ras2::URA3*	pAD4-GST-CYR1(606-2026)
FS1-1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1 cap::*pCAPΔN-1	pAD4-GST-CYR1(606-2026)
FS2-1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1 cap::*pCAPΔN-1	pAD4-GST-CYR1(606-2026) YEP-HIS3-ADC1-CAP
FS3-1	*MAT*α *his3 leu2 trp1 ura3 ade8 cyr1-2 ras2::URA3*	pAD4-GST-CYR1(606-2026) YEP-HIS3-ADC1-CAP
FS10-1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1 cap::*pCAPΔN-1	pAD4-GST-CYR1(606-2026) YEP-HIS3-ADC1-CAP(1-368)
FS12-1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1 cap::*pCAPΔN-1	pAD4-GST-CYR1(606-2026) YEP-HIS3-ADC1-CAP(1-168, 369-526)
FS14-1	*MAT*α *his3 leu2 trp1 ura3 ade8 cyr1-2 ras2::URA3*	YEP-HIS3-ADC1-CAP

[a] Strains SP1, TK36-1, TM5-1, and NS1-1 were described previously (21, 29, 37). FS1 was constructed by replacement of the chromosomal CAP gene by the N-terminal deletion mutant gene of pCAPΔN-1 as described in Materials and Methods. Other strains were prepared by transformation of the indicated plasmids into suitable host strains.

adenylyl cyclase activity and its relationship with the requirement of CAP for the in vivo cAMP response.

MATERIALS AND METHODS

Cell strains and growth media. The *S. cerevisiae* strains used are listed in Table 1. Yeast cells were 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. Yeast cells bearing the *cyr1-2* mutation were cultured at 30°C in the presence of 1 mM cAMP as described previously (28). Genetic manipulations of yeast cells were performed as previously described (32). Transformation into yeast cells was carried out with lithium acetate (19).

Construction of expression plasmids. Constructions and structures of pAD4-GST-CYR1(*x-x*)s, which overexpressed adenylyl cyclase fragments corresponding to amino acid positions *x* to *x* as fusions with *Schistosoma japonicum* glutathione *S*-transferase (GST) (36) under control of the yeast alcohol dehydrogenase I (*ADC1*) promoter, were described before (25, 29). YEP24-ADC1-CYR1, which moderately overexpressed the full-length adenylyl cyclase under control of the *ADC1* promoter, is described elsewhere (37). A DNA fragment bearing the full-length *CAP* cDNA sandwiched between the *ADC1* promoter and terminator was cut out from pADH-CAP (7) and inserted into YEP-HIS3, which had been created by replacing the *URA3* gene of YEP24 with the *HIS3* gene fragment. The resulting plasmid, YEP-HIS3-ADC1-CAP, was subjected to internal deletions by partial cleavage with suitable pairs of restriction endonucleases *Stu*I, *Eco*47III, and *Ssp*I and by subsequent resealing with T4 DNA ligase. The plasmid produced was designated YEP-HIS3-ADC1-CAP(*y-y*), where *y-y* represents the range of the expressed CAP polypeptide in amino acid positions.

Replacement of the chromosomal *CAP* gene with an N-terminal deletion mutant. A 107-bp *CAP* fragment corresponding to amino acids 2 to 36, *CAP(2-36)*, was amplified by PCR (34) using the primers 5′-CGGGATCCAGCTGAC TCTAAGTACACAATG-3′ and 5′-GCGGATCCAAGCTTAACCTTCTTGAT AGATGGTG-3′ and cleaved with *Bam*HI in the primer sequences. A *CAP* fragment corresponding to amino acids 369 to 526, *CAP(369-526)*, was amplified by PCR using oligonucleotide primers 5′-ACGCGTCGACCATGCCTAGAA AGGAATTGGTAG-3′ and 5′-GGGGTACCTCGCAATATTAACCAGC-3′ and, after cleavage with *Sal*I and *Kpn*I in the primer sequences, inserted downstream of the *ADC1* promoter of pAD4. The *ADC1-CAP(369-526)* fragment was isolated from the resulting plasmid by cleavage with *Bam*HI and *Kpn*I. The primers were designed to insert a termination codon immediately downstream of amino acid residue 36 of CAP(2-36) and an initiator ATG immediately preceding residue 369 of CAP(369-526). The *CAP(2-36)* and *ADC1-CAP(369-526)* fragments were inserted into pSP73 with the *URA3* selectable marker in between to produce pCAPΔN-1. The insert of pCAPΔN-1 was cut out by digestion with *Pvu*II and *Eco*RI and transformed into SP1 for one-step gene replacement (32). Successful gene replacement of the resulting Ura^+ transformant, designated FS1, was verified by Southern blot analysis (data not shown).

Purification of recombinant proteins. pAD4-GST-CYR1(*x-x*) plasmid was transformed into a suitable yeast strain alone or in combination with an YEP-HIS3-ADC1-CAP(*y-y*) construct. The resulting transformants were grown to the density of 10^7 cells/ml, harvested by centrifugation, disrupted by shaking with glass beads in buffer C (50 mM 2-(*N*-morpholino)ethanesulfonic acid [pH 6.2], 0.1 mM $MgCl_2$, 0.1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 1 mM β-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and a crude membrane fraction was prepared as described previously (37, 47). GST-CYR1(*x-x*) protein was solubilized from the crude membrane fraction with buffer C containing 1% Lubrol PX, 0.5 M NaCl, 1 mM dithiothreitol, and 1 mM PMSF as described before (25, 43). After centrifugation at 100,000 × *g* for 1 h, the supernatant was incubated with glutathione-Sepharose resin (Pharmacia) in buffer A (20 mM Tris-HCl [pH 7.4], 40 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Lubrol PX) by continuous mixing for 4 h at 4°C. After extensive washing with buffer A containing 0.15 M NaCl, the resin with GST-CYR1(*x-x*) protein attached was used for the Ras protein-binding assay, or GST-CYR1(*x-x*) protein was eluted from the resin with buffer A containing 20 mM glutathione and subjected to the adenylyl cyclase assay. The posttranslationally fully modified and unmodified forms of human H-Ras protein were purified from the membrane and cytosol fractions, respectively, of *Spodoptera frugiperda* Sf9 cells, which had been infected with the recombinant baculovirus expressing the human H-*ras* cDNA, as described previously (25, 30). Similarly, the mutant $H\text{-}Ras^{Ser\text{-}181,184}$, which lacks two cysteine residues to be palmitoylated and therefore is farnesylated but not palmitoylated, and the fully modified form of the effecter mutant $H\text{-}Ras^{Asn\text{-}38}$ were produced in Sf9 cells and purified as described previously (30).

Ras protein-binding assays. A 40-μl aliquot of the resin with 0.8 to 1.0 μg each of GST-CYR1(*x-x*) attached was incubated with 15 pmol each of various forms of H-Ras, which had been loaded with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) or guanosine 5′-*O*-(2-thiodiphosphate), in 60 μl of buffer A for 30 min with continuous mixing at 30°C and subsequently washed three times with 20 bed volumes each of buffer A. The bound H-Ras was eluted from the resin along with the GST-CYR1 with buffer A containing 20 mM glutathione, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel), and detected by Western immunoblotting with the anti-H-Ras monoclonal antibody F235 (Oncogene Science Inc., Manhasset, N.Y.). The Amersham ECL immunodetection system or an alkaline phosphatase immunodetection system (ProtoBlot Western Blot AP system; Promega) was used for signal development.

Adenylyl cyclase assays. GST-CYR1(606-2026) protein (0.05 pmol), produced in various yeast strains and purified by glutathione-Sepharose chromatography, was subjected to the measurement of adenylyl cyclase activity in the presence of 2.5 mM $MnCl_2$ (Mn^{2+}-dependent activity) or of various concentrations of the GTPγS-bound form of H-Ras and 2.5 mM $MgCl_2$, using [α-^{32}P]ATP (300 cpm/pmol) as a substrate, as described before (25, 47). In some experiments, a crude 1% Lubrol PX–0.5 M NaCl extract (10 μg of protein) of yeast strain TK36-1 replaced the purified adenylyl cyclase. The activation constants (K_a) for the various forms of Ras were determined from double-reciprocal plot analyses of the adenylyl cyclase activities and Ras concentrations. For determination of the dissociation constant (K_d) of adenylyl cyclase for the posttranslationally unmodified H-Ras, we measured the pattern of inhibition of the fully modified H-Ras-dependent adenylyl cyclase activity by various concentrations of the GTPγS-bound unmodified form. The K_d value was derived from the inhibition profile as described in Results.

In vitro reconstitution. Yeast FS14-1 cells carrying YEP-HIS3-ADC1-CAP were disrupted with glass beads in buffer C containing 1 mM PMSF and centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting supernatant was used as a crude cytoplasmic extract containing CAP. GST-CYR1(606-2026) protein without the bound CAP was purified by adsorption onto glutathione-Sepharose resin from FS1-1 as described above. Subsequently, the resin with about 20 pmol of GST-CYR1(606-2026) attached was incubated with the crude cytoplasmic extract (0.7 mg of protein) of FS14-1 containing about 300 pmol of CAP for 1 h at 4°C. The resin was washed extensively and subjected to elution by 20 mM glutathione. Aliquots of the eluted protein were analyzed by Western immunoblotting and by adenylyl cyclase assays in the presence of various forms of H-Ras as described above.

Other methods. The polyclonal antisera for the full-length CAP and for GST were described previously (43, 44). SDS-PAGE and Western immunoblot analysis were performed as described elsewhere (26, 39).

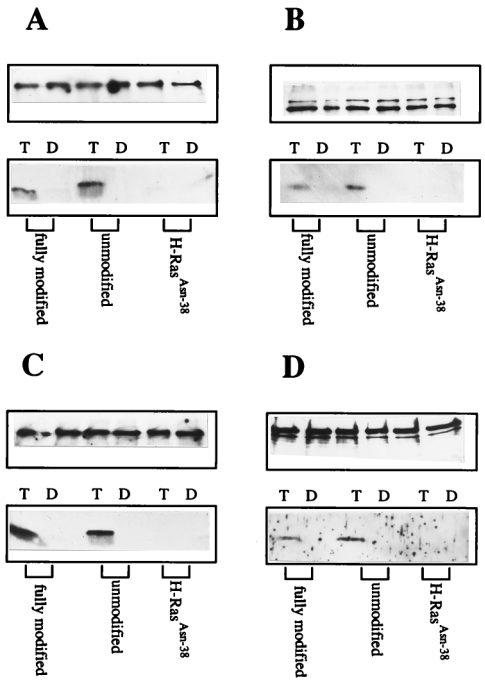

FIG. 1. In vitro binding of the fully modified and unmodified forms of H-Ras to adenylyl cyclase. The fully modified and unmodified forms of H-Ras and the fully modified form of the effector mutant H-Ras^{Asn-38} were loaded with GTPγS (lanes T) or GDPβS (lanes D) and incubated at the concentration of 150 nM with GST-CYR1(606-2026) purified from NS1-1 (A), GST-CYR1(606-1380) purified from TM5-1 (B), GST-CYR1(606-2026) purified from FS3-1 (C), or GST-CYR1(606-2026) purified from FS1-1 (D) (see Table 1 for genotypes) as described in Materials and Methods. After incubation, the GST-CYR1 proteins were eluted with glutathione along with the bound H-Ras, and the eluted proteins were subjected to detection by Western immunoblotting with either the anti-GST polyclonal antibody (upper panels) or the anti-H-Ras monoclonal antibody F235 (lower panels). The signals were developed by using the Amersham ECL immunodetection system. The results shown are representative of two experiments performed with different preparations of each purified GST-CYR1 protein.

RESULTS

Effect of posttranslational modifications on association between adenylyl cyclase and H-Ras. A strong stimulatory effect of posttranslational modification of yeast Ras2 (25) or of human H- and K-Ras (17) was observed on activation of the full-length adenylyl cyclase that was moderately overexpressed in yeast cells. The farnesylation step, not the palmitoylation step, was mainly responsible for this effect (25). In this study, we tried to determine what kinetic parameter was affected by the modifications. To analyze the binding reaction, GST-CYR1(606-2026), a GST fusion protein of the C-terminal 1,421 amino acid residues of adenylyl cyclase, was overexpressed in yeast strain NS1-1 (Table 1) and purified by adsorption onto glutathione-Sepharose resin. The N-terminal 605 residues of adenylyl cyclase deleted in this construct had been shown to be dispensable for the response to Ras (47). The resin with GST-CYR1(606-2026) attached was examined in vitro for direct association with either the posttranslationally fully modified or the unmodified form of H-Ras as described in Materials and Methods. Both forms bound equally to GST-CYR1(606-2026) in a GTP-dependent manner (Fig. 1A). No binding was observed for the posttranslationally fully modified form of the effector mutant H-Ras^{Asn-38}. We also examined the association of the LRR domain of adenylyl cyclase, expressed as GST-CYR1(606-1380) and purified, with H-Ras and found that this domain exhibited binding with various forms of H-Ras similar to that of GST-CYR1(606-2026) (Fig. 1B).

To analyze the binding reaction more quantitatively and exclude the possibility that the observed specificity is an artifact caused by the use of GST-CYR1(606-2026), the full-length adenylyl cyclase was moderately overproduced in yeast strain TK36-1 carrying plasmid YEP24-ADC1-CYR1 and examined for activation by the three forms of H-Ras protein, i.e., the fully modified and unmodified forms of H-Ras and the farnesylated but not palmitoylated intermediate form H-Ras$^{Ser-181,184}$, all in the GTPγS-bound state (Fig. 2A). The full-length adenylyl cyclase in the crude extract was preferentially stimulated by either the fully modified or intermediate form compared to the unmodified form. The maximal level of activity attained was

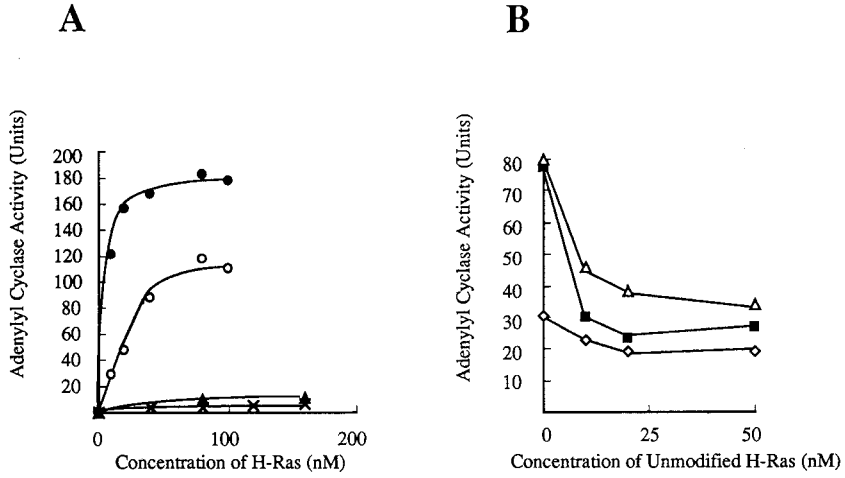

FIG. 2. Determination of K_d values of the full-length adenylyl cyclase for various forms of H-Ras. (A) The full-length adenylyl cyclase moderately overexpressed in TK36-1 was solubilized from the crude membrane fraction and assayed for activation by various concentrations of the fully modified (●), intermediate (○), and unmodified (▲) forms of H-Ras and of the fully modified form of H-Ras^{Asn-38} (×), all in the GTPγS-bound state, as described in Materials and Methods. One unit of activity is defined as 1 pmol of cAMP formed in 1 min of incubation with 1 mg of protein at 30°C. (B) Adenylyl cyclase activities obtained with fully modified H-Ras at concentrations of 5 (◇), 10 (■), and 20 (△) nM were subjected to inhibition by various concentrations of unmodified H-Ras. Kinetic analyses of the inhibition pattern were carried out to derive the K_d value for unmodified H-Ras as described in Results. The activities were expressed as described for panel A. Similar experiments performed on two occasions with different preparations of adenylyl cyclase and H-Ras yielded equivalent results.

about nine times higher by the intermediate form or about 15 times higher by the fully modified form than that attained by the unmodified form and reached a level comparable to the Mn^{2+}-dependent activity. According to the steady-state enzyme kinetics, the K_d for H-Ras can be determined as K_a, the H-Ras concentration giving a half-maximal activity of adenylyl cyclase. The K_a values for all three forms of H-Ras were calculated by double-reciprocal plots of the data in Fig. 2A and found to be almost equal (approximately 10 nM).

To further prove that the observed K_a value for the unmodified H-Ras, which gave a very low extent of activation, faithfully reflects its binding affinity, we performed the following kinetic analysis. Adenylyl cyclase activity obtained with a fixed concentration (C_m) of the GTPγS-bound fully modified H-Ras was subjected to competitive inhibition by inclusion of various concentrations (C_u) of the GTPγS-bound unmodified form (Fig. 2B). In the reaction mixture, the total amount of adenylyl cyclase (E_0) comprised three populations: that bound to the fully modified H-Ras (E_m), that bound to the unmodified form (E_u), and that which was free. Adenylyl cyclase available for interaction with the fully modified form is $E_0 - E_u$, while that available for interaction with the unmodified form is $E_0 - E_m$. Assuming Michaelis-Menten-type kinetics,

$$E_m = \frac{(E_0 - E_u)\, C_m}{K_m + C_m} \text{ and } E_u = \frac{(E_0 - E_m)\, C_u}{K_u + C_u}$$

where K_m and K_u represent dissociation constants for the fully modified and unmodified forms of H-Ras, respectively. The total adenylyl cyclase activity obtained (V) is the sum of those from E_m and E_u and thus is formulated as

$$\frac{(E_0 - E_u)\, C_m\, V_{\max(m)}}{E_0\,(K_m + C_m)} + \frac{(E_0 - E_m)\, C_u\, V_{\max(u)}}{E_0\,(K_u + C_u)}$$

where $V_{\max(m)}$ and $V_{\max(u)}$ represent the maximal activities attained by the fully modified or the unmodified form alone, respectively. Simplifying this equation yielded

$$V = \frac{V_{\max(m)}\, K_u\, C_m + V_{\max(u)}\, K_m\, C_u}{K_m\, K_u + K_u\, C_m + K_m\, C_u}$$

Taking account of the values observed in Fig. 2A, $V_{\max(u)} = 0.2V_{\max(m)}$ and $K_m = 10$ nM, the K_u value could be estimated as that which gave the best fit for the observed inhibition curves, which were obtained for the C_m values of 5, 10, and 20 nM (Fig. 2B), and was determined to be about 8 nM, which was very close to the estimated K_a value for this form. Thus, it is clear that no appreciable difference exists in the affinities for the three forms of H-Ras, indicating that the posttranslational modification, especially the farnesylation step, is responsible for 5- to 10-fold increase in Ras-dependent activation of adenylyl cyclase activity even though it has no significant effect on Ras binding affinity.

Effect of association with CAP on adenylyl cyclase activation. Next, we examined the in vitro activation of the purified GST-CYR1(606-2026) by the three forms of H-Ras protein as described in Materials and Methods. Unexpectedly, we observed no significant difference in the abilities of the unmodified, intermediate, and fully modified forms to activate adenylyl cyclase (Fig. 3A). The maximal level of activity attained by the three forms of Ras was much lower than that obtained in the presence of Mn^{2+}. This was in striking contrast to the situation obtained with the moderately overexpressed adenylyl cyclase from TK36-1 (Fig. 2A) and prompted us to look for a possible difference in the two preparations of adenylyl cyclase. When the purified GST-CYR1(606-2026) was fractionated by

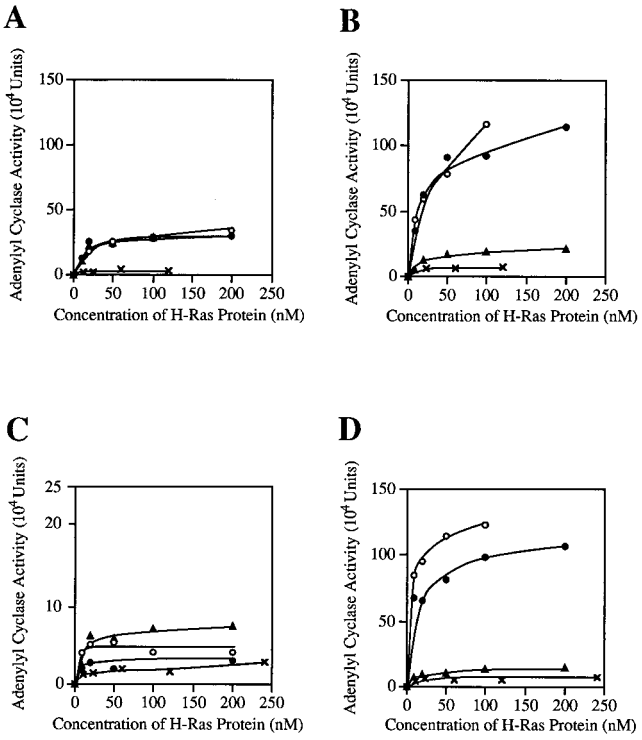

FIG. 3. Activation of adenylyl cyclase with and without bound CAP by various forms of H-Ras. GST-CYR1(606-2026) protein (0.05 pmol) purified from yeast strain NS1-1 (A), FS3-1 (B), FS1-1 (C), or FS2-1 (D) (see Table 1 for genotypes) was assayed for adenylyl cyclase activity in the presence of various concentrations of the fully modified (●), intermediate (○), and unmodified (▲) forms of H-Ras and of the fully modified form of $H\text{-}Ras^{Asn\text{-}38}$ (×), all in the GTPγS-bound state. The Mn^{2+}-dependent activity was constant throughout the experiments (79×10^4 U). One unit of activity is defined as 1 pmol of cAMP formed in 1 min of incubation with 1 mg of the purified GST-CYR1(606-2026) protein at 30°C. The experiments were performed twice with different preparations of each GST-CYR1 protein, yielding equivalent results.

SDS-PAGE, we found that this protein almost lost the association with CAP, which was barely detectable by Coomassie blue staining (Fig. 4A). Because the moderately overexpressed adenylyl cyclase had been shown to be efficiently associated with CAP (7, 44), we speculated that the overexpression of GST-CYR1(606-2026), estimated to be about 10 times higher than that of the full-length protein from YEP24-ADC1-CYR1, might have titrated out the free CAP and caused its limitation. To circumvent this possibility, we simultaneously overexpressed CAP with GST-CYR1(606-2026) in yeast strain FS3-1. As expected, GST-CYR1(606-2026) purified from FS3-1 exhibited restoration of the amount of bound CAP as measured by Coomassie blue staining (Fig. 4A). The great increase of the amount of CAP bound to GST-CYR1(606-2026) was also detected by Western immunoblotting with the anti-CAP antibody (Fig. 4B). When this protein was examined for activation by the various forms of H-Ras, both the fully modified and intermediate forms exhibited four- to fivefold stimulation of the extent of activation compared to the unmodified form (Fig. 3B), which was similar to what was observed for the moderately overexpressed adenylyl cyclase. It was also evident that the K_a values, about 10 nM for all three forms, were not affected by the posttranslational modification. This was confirmed by a measurement of the in vitro association with H-Ras, in which no significant difference was observed for the binding activity of the fully modified and unmodified H-Ras to GST-CYR1(606-2026) with the bound CAP (Fig. 1C). Further,

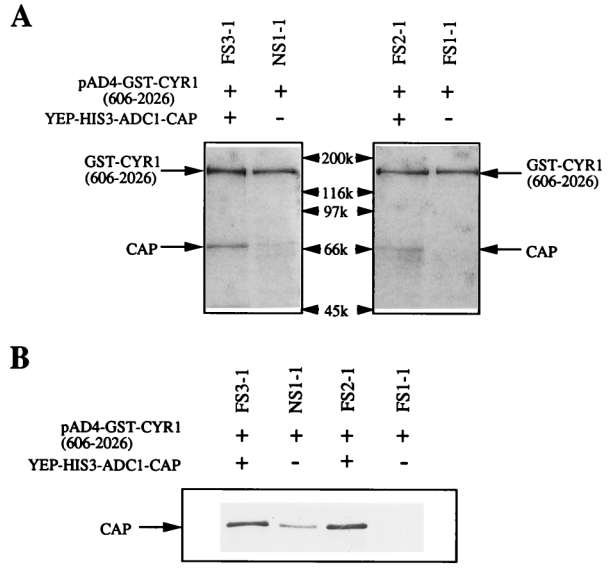

FIG. 4. Measurement of the amount of CAP bound to the purified adenylyl cyclase. (A) GST-CYR1(606-2026) proteins (0.4 μg of each) purified from the indicated yeast strains (see Table 1 for genotypes) were separated by SDS-PAGE (12% gel) and stained with Coomassie brilliant blue. The molecular size markers indicated were myosin heavy chain (200 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and hen egg white ovalbumin (45 kDa). (B) GST-CYR1(606-2026) proteins (0.2 μg of each) purified from the indicated strains were separated by SDS-PAGE (10% gel) and subjected to Western immunoblot detection of the bound CAP with the anti-CAP antibody as described in Materials and Methods. An alkaline phosphatase immunodetection system was used for signal development. The results shown are representative of two experiments performed with different preparations of each purified GST-CYR1 protein.

the K_d value of this protein for the unmodified H-Ras was estimated by the adenylyl cyclase inhibition assay as described above for the full-length adenylyl cyclase and determined to be about 10 nM (data not shown). These results indicated that the in vivo attachment of CAP restored the stimulatory effect of farnesylation on the adenylyl cyclase activation without significantly affecting the binding affinity for H-Ras protein.

Because GST-CYR1(606-2026) purified from NS1-1 still contained a residual, albeit small, amount of CAP, we further examined the effect of CAP association by using the protein devoid of the bound CAP. Since disruption of the entire CAP gene or its C-terminal region resulted in grossly attenuated cells inadequate for propagation, we selectively disrupted the N-terminal region of the chromosomal CAP, which is responsible for its association with adenylyl cyclase (49), by gene replacement with pCAPΔN-1 (see Materials and Methods). The resulting yeast strain, FS1, expressed only the C-terminal 158-amino-acid residue fragment of CAP, which was sufficient to maintain its normal growth. As shown in Fig. 4, GST-CYR1(606-2026) purified from FS1-1 had no detectable level of the bound CAP as measured by both Coomassie blue staining and Western immunoblotting with the anti-CAP antibody. This adenylyl cyclase was activated very weakly by either of the three forms of H-Ras (Fig. 3C). The maximal level of the Ras-dependent activity obtained was even lower than that obtained with GST-CYR1(606-2026) purified from NS1-1, indicating that the latter retained some activity dependent on the residual CAP bound. However, when CAP was simultaneously overexpressed in the same strain, the resulting GST-CYR1 (606-2026) protein, purified from FS2-1, regained a considerable amount of the bound CAP (Fig. 4) and resumed its stimulated activation by both the intermediate and fully modified forms of H-Ras (Fig. 3D). Again, both preparations of adenylyl cyclase, without and with the bound CAP, exhibited no appreciable difference in the K_a values for the three forms of H-Ras, which were determined to be approximately 10 nM by the double-reciprocal plots of the data in Fig. 3C and D. Confirming this result, GST-CYR1(606-2026) devoid of the bound CAP associated almost equally with the fully modified and unmodified forms of H-Ras in vitro (Fig. 1D). These results further supported the idea that the association of adenylyl cyclase with CAP is responsible for its stimulated activation by the farnesylated H-Ras protein. It does not appear to affect the binding affinity of adenylyl cyclase for H-Ras.

Mapping of the region of CAP required for the stimulatory effect of posttranslational modification. Some internal deletions were introduced into the *CAP* gene as described in Materials and Methods. YEP-HIS3-ADC1-CAP(1-368) and YEP-HIS3-ADC1-CAP(1-168, 369-526), which overexpress a CAP fragment lacking its C-terminal 158 amino acid residues and that bearing an internal deletion of 200 residues, respectively, were transformed into FS1-1, and GST-CYR1(606-2026) proteins purified from the resulting transformants, FS10-1 and FS12-1, respectively, were examined for association with the CAP fragments and for activation by the three forms of H-Ras (Fig. 5). Both of the CAP fragments bound efficiently to GST-CYR1(606-2026) (Fig. 5A), and the purified GST-CYR1(606-2026) proteins were activated preferentially by the fully modified and intermediate forms of H-Ras compared to the unmodified form (Fig. 5B). Actually, we observed some difference in the maximal extents of activation between the intermediate and fully modified forms (Fig. 5B), for which we presently do not have a proper explanation. The results indicate that the N-terminal region of CAP shared by the two CAP fragments, corresponding to amino acids 1 to 168, is sufficient to invoke the stimulatory effect of farnesylation on the Ras-dependent adenylyl cyclase activation. This region matched the minimal region of CAP required for its effect on the in vivo cAMP response (11). We tried but were unable to examine the effect of the N-terminal segment CAP(1-168) alone because of its degradation upon overproduction in yeast cells. On the other hand, the C-terminal fragment CAP(369-526) was overproduced from the chromosomal *cap::*pCAPΔN-1 gene in FS1-1, as measured by Western immunoblotting with the anti-CAP antibody (data not shown), and did not exhibit any association with GST-CYR1(606-2026) or any stimulatory effect on activation by the modified H-Ras, as already shown (Fig. 3C and 4).

In vitro reconstitution of the effect of CAP on adenylyl cyclase. We examined whether the stimulatory effect of posttranslational modification could be restored in vitro by association of CAP to purified adenylyl cyclase lacking bound CAP. As shown in Fig. 6A, GST-CYR1(606-2026) carrying no bound CAP (lane b) had its association with CAP reconstituted (lane a) after incubation with a crude cytoplasmic extract of the adenylyl cyclase-deficient yeast cells containing overproduced CAP. The reconstituted GST-CYR1(606-2026) was examined for activation of its activity by the various forms of H-Ras (Fig. 6B). The activation by the fully modified H-Ras was clearly restored to a level comparable to that of the GST-CYR1(606-2026) fully associated in vivo with CAP. The intermediate form also exhibited approximately threefold stimulation of activation compared to the unmodified form, which was clearly distinguishable from the original GST-CYR1(606-2026) before incubation with CAP (Fig. 3C). However, the maximal level of activity was significantly lower than (about 40% of) that attained by the fully modified form. Presently, we do not have an

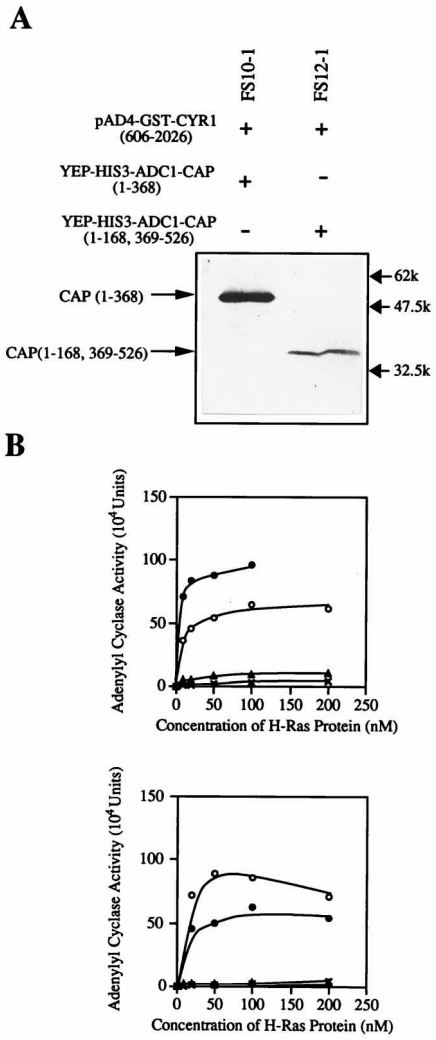

FIG. 5. Mapping of the region of CAP required for the stimulatory effect of posttranslational modification. (A) GST-CYR1(606-2026) was purified by glutathione-Sepharose from FS10-1 harboring YEP-HIS3-ADC1-CAP(1-368) or from FS12-1 harboring YEP-HIS3-ADC1-CAP(1-168, 369-526). The CAP fragments were detected by Western immunoblotting with the anti-CAP polyclonal antibody. Signal development was done with an alkaline phosphatase immunodetection system. The molecular size markers indicated were glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), and triosephosphate isomerase (32.5 kDa). (B) GST-CYR1(606-2026) purified from FS10-1 (upper panel) or from FS12-1 (lower panel) was assayed for adenylyl cyclase activities in the presence of various concentrations of the fully modified (●), intermediate (○), and unmodified (▲) forms of H-Ras and of the fully modified form of H-Ras^{Asn-38} (×). See the legend to Fig. 3 for definition of units. The results shown are representative of two experiments performed with different preparations of each purified GST-CYR1 protein.

explanation for this kind of difference between the intermediate and fully modified forms, which was also observed in other experiments (Fig. 2A and 5).

DISCUSSION

We have shown by using a reconstituted system with purified components that the posttranslational modification, especially the farnesylation, of H-Ras stimulates the Ras-dependent activation of yeast adenylyl cyclase. The mechanism of stimulation appears to involve an enhancement of the extent of activation but not an increase of the binding affinity of H-Ras because no appreciable difference was observed in the K_d values among the three forms of H-Ras bearing various degrees of the modification. A similar observation was made for the in vitro activation of mammalian brain B-Raf by H-Ras (30, 46). These two represent the only systems so far that enable the estimation of the effect of posttranslational modification on Ras-effector interaction separately from the effect on membrane anchoring of Ras, although the requirement of the lipid modification for the activity of Ras has been demonstrated in a number of in vivo systems used for analysis of the biological activities of Ras (15, 31, 35, 45) or activation of the effectors, including mammalian Raf-1 (23). In addition, the LRR domain, which had been shown to be the only major Ras-binding site of adenylyl cyclase, was found to exhibit binding indistinguishable from that of the full-length adenylyl cyclase. This study is the first to demonstrate the GTP-dependent association of the LRR domain with Ras, since previous studies used the yeast two-hybrid assay (41) or the adenylyl cyclase inhibition assay (29), which was in principle unfit for examination of the GTP dependence of the interaction.

Studies on the function of CAP revealed that CAP is a multifunctional protein. It was shown that the N-terminal region, mapped to amino acids 1 to 168 of CAP, is required for acquirement of heat shock sensitivity in the *RAS2*$^{Val-19}$ background, while the C-terminal region, mapped to amino acids 369 to 526, is required for normal cell morphology and respon-

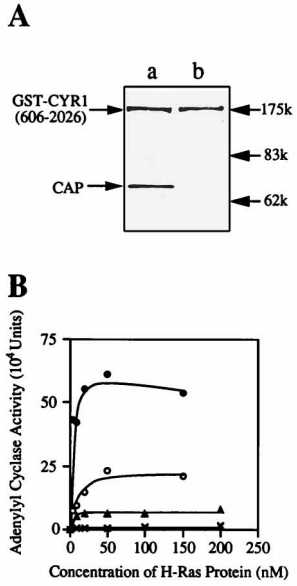

FIG. 6. In vitro reconstitution of the effect of CAP on adenylyl cyclase. Purified GST-CYR1(606-2026) without the bound CAP, immobilized on glutathione-Sepharose resin, was incubated with a crude cytoplasmic extract of FS14-1 overproducing CAP as described in Materials and Methods. Subsequently, the resin was washed extensively and subjected to elution by 20 mM glutathione. (A) About 0.1 μg of purified GST-CYR1(606-2026) protein with (lane a) or without (lane b) incubation with CAP was fractionated by SDS-PAGE (10% gel) and subjected to detection of CAP by Western immunoblotting with the anti-CAP antibody. At the same time, GST-CYR1 protein was visualized by inclusion of the anti-GST antibody. An alkaline phosphatase immunodetection system was used for signal development. The prestained molecular size markers (New England Biolabs, Beverly, Mass.) were maltose-binding protein fusion proteins of *E. coli* β-galactosidase (175 kDa) and paramyosin (83 kDa) and bovine liver glutamic dehydrogenase (62 kDa). (B) The eluted protein (0.05 pmol) was assayed for adenylyl cyclase activity in the presence of various concentrations of the fully modified (●), intermediate (○), and unmodified (▲) forms of H-Ras and of the fully modified form of H-Ras^{Asn-38} (×). The results shown are representative of two experiments performed with different preparations of the purified GST-CYR1 protein, which gave essentially equivalent results.

siveness to nutrient deprivation and excess (11). The C-terminal function appears to be related to regulation of the actin cytoskeleton, as evidenced by complementation of its function by overexpression of profilin or SNC (12, 40) and by demonstration of its direct association with actin monomer and of its actin-sequestering activity (8, 14). In addition, CAP possesses two proline-rich sequences in its middle region with which association of SH3 domain-containing proteins, including yeast actin-binding protein 1, was recently demonstrated (9). However, the function of CAP on the Ras-adenylyl cyclase pathway resides solely in its N-terminal region and is separable from the functions of the other regions as reported previously (11, 22). Although the N-terminal function is required for the exaggerated cAMP response of yeast cells in the *RAS2*$^{Val-19}$ background, we observed that association with CAP has no effect on the in vitro activation of adenylyl cyclase by the unmodified Ras proteins, which were expressed and purified from *E. coli* (43, 44). Here, we found that the purified GST-fusion protein of adenylyl cyclase, vastly overexpressed in yeast, became insensitive to the stimulatory effect of farnesylation of Ras and, at the same time, exhibited very limited association with CAP. This finding prompted us to examine the relationship between the CAP association and the stimulatory effect of farnesylation and resulted in finding that the overexpression of CAP in a CAP-disrupted yeast strain restored both the CAP association with adenylyl cyclase and its stimulated activation by the farnesylated Ras. Furthermore, we demonstrated that the stimulatory effect of the posttranslational modification could also be restored by in vitro association of CAP to the adenylyl cyclase molecule. This finding rules out the possibility that adenylyl cyclase synthesized de novo in the absence of CAP cannot assume its proper conformation and had its activity irreversibly and nonspecifically attenuated. In addition, we showed that the region of CAP responsible for both the cyclase association and the stimulatory effect could be mapped to its N-terminal 168-amino-acid residue region, which coincided with the minimal region mapped for the in vivo N-terminal function (11). Thus, we have so far observed very good correlation among the three phenomena pertinent to CAP: its in vivo function with respect to the cAMP response, its association with adenylyl cyclase, and its requirement for stimulation of the in vitro activation of adenylyl cyclase by the farnesylated Ras. This observation strongly supports the idea that the in vivo function of the N-terminal region of CAP for the proper cAMP response could be explained by its requirement for the stimulatory effect of farnesylation on adenylyl cyclase activity. It must be noted that the possibility exists that the middle or the C-terminal region of CAP exerts a regulatory influence on the N-terminal function through control of the availability of CAP for adenylyl cyclase by, for example, changing its subcellular localization.

The association with CAP affected the extent of activation by the farnesylated Ras protein without significantly changing the binding affinity between adenylyl cyclase and Ras. This is similar to the observation that the posttranslational modification affected the extent of activation without changing the affinity, suggesting that CAP and the modification are involved in the same mechanism for regulation of adenylyl cyclase activity. We can raise some possibilities regarding the mechanism of the CAP action. The most likely possibility favored by the successful in vitro reconstitution is that CAP itself or the complex between CAP and the C terminus of adenylyl cyclase forms an acceptor site for the farnesyl moiety of the modified Ras protein. If this is the case, the binding must be of quite low affinity so that it does not affect the overall affinity of the modified Ras protein for adenylyl cyclase and may work by subtly altering the conformation of the catalytic site of adenylyl cyclase, which is located adjacent to the CAP-binding site (44, 47). The weak association postulated here may be compatible with the observation that the LRR domain makes the principal Ras-binding site and possibly is primarily responsible for the affinity of adenylyl cyclase for Ras protein. Alternatively, the C-terminal farnesylation may induce alteration of the overall conformation of Ras, which is somehow specifically recognized by CAP or the CAP-cyclase complex. It is also possible that CAP itself is not directly involved in physical association with Ras but acts indirectly by altering the conformation of adenylyl cyclase. The existence of potential acceptor sites for the posttranslationally added isoprenyl groups has been postulated before. Geranylgeranylation of Rap1A/K*rev*-1/*smg* p21 was reported to be essential for direct interaction with its GDP-GTP exchange protein, SmgGDS (16). We have shown that farnesylation of Ras is required for direct association between the cysteine-rich region of Raf-1 and the activator domain of Ras protein (18). In addition, isoprenylation of transducin $\beta\gamma$ subunits was reported to be required for association with its α subunit (10). However, recent X-ray crystallographic analysis of the heterotrimeric GTP-binding proteins gave no indication of a particular acceptor site for the isoprenyl group in its α subunit (27, 42). The present study is the first to show that an extraneously bound protein can regulate the response of an effector molecule to the isoprenyl group of regulatory GTP-binding proteins.

Further studies including molecular identification of the hypothetical acceptor site for the farnesyl group will be needed for full elucidation of the CAP function in the Ras-adenylyl cyclase pathway.

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