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A constitutively active holoenzyme form of the cAMP-dependent protein kinase

(site-directed mutagenesis/subunit interaction/type II regulatory subunit)

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ABSTRACT **The major function of the regulatory (R) subunit of the cAMP-dependent protein kinase is to bind tightly to the catalytic (C) subunit to form an inactive holoenzyme in the absence of cAMP. The hinge region of the R subunit resembles the substrate recognition site for the C subunit and is known to be involved in the R·C subunit interaction. Two arginine residues in this region, Arg-92 and Arg-93, are suggested to be essential for holoenzyme formation. In this study, a mutant in which Arg-92 and Arg-93 of type II regulatory subunit (RII) were replaced with alanine was constructed. Formation of the holoenzyme from mutant RII and C subunits was analyzed by gel-filtration and cation-exchange chromatography. Mutant RII in its cAMP-free form formed a stable holoenzyme with the C subunit, which dissociated in the presence of cAMP. Interestingly, the holoenzyme formed from mutant RII and C subunits retained full enzymatic activity even in the absence of cAMP. Although mutant RII could no longer be phosphorylated by the C subunit, the rate of [^{3}H]cAMP release from mutant RII·cAMP was increased by addition of the C subunit, indicating that C-induced cAMP release is not the result of the interaction of the C subunit with the hinge region. These results demonstrate that Arg-92 and Arg-93 are not essential for holoenzyme formation but are critical for inhibiting kinase activity in the holoenzyme probably by occupying the substrate binding site. The results suggest that, in addition to the hinge region, a second site on the RII subunit may interact with the C subunit in a cAMP-dependent manner.**

The cAMP-dependent protein kinase (PKA) consists of regulatory (R) and catalytic (C) subunits that associate to form an inactive tetrameric holoenzyme (R_2C_2). The binding of cAMP to the R subunit leads to dissociation of the inactive holoenzyme and the release of the active C subunit (1).

Two major types of PKA have been classified based on the isozymic identity of the R subunit, which exists in two forms, RI and RII (2, 3). Both forms of the R subunit share the general domain structure, which has been defined by limited proteolysis and affinity labeling (4). The 50 residues at the amino terminus of the R subunit are essential for dimerization to R_2 (5). Two tandem cAMP-binding domains are located at the carboxyl terminus of the R subunit (6, 7). The sequence of these cAMP binding domains is homologous to that of the catabolite gene activator protein (CAP) from *Escherichia coli*, and a β-barrel structural model of cAMP-binding sites has been proposed, based on the crystallographic coordinates of CAP (8–10). The two cAMP binding sites (sites A and B) of the R subunit have different preferences for cAMP analogs and display different dissociation rates for cAMP (11, 12).

A proteolytically sensitive "hinge" region (residues 90–100, see Fig. 1) has been emphasized thus far as the site of interaction with the C subunit. The hinge region in RII contains a substrate consensus sequence, Arg-Arg-Xaa-Ser, while that in RI has a pseudosubstrate sequence, Arg-Arg-Xaa-Ala (4). It is well established that a pair of basic residues located one residue amino terminal to a serine or threonine is a strong recognition determinant for phosphorylation by the C subunit (for review, see ref. 13). Thus, it is believed that the hinge region of the R subunit occupies the substrate binding site of the C subunit in the holoenzyme, which is supported by the observation that the R subunit is a competitive inhibitor of PKA and that Ser-95 in the Arg-Arg-Xaa-Ser sequence of RII is autophosphorylated in the holoenzyme (14–16). In addition, Cys-97 in RII is selectively protected from alkylation in the holoenzyme, indicating that this residue is probably in close contact with the C subunit (17).

The important role of Arg-92 and Arg-93 at the hinge region in the interaction of the R with the C subunits was first noted by Corbin *et al.* (14), who showed that the chemical modification of these arginine residues led to activation of the holoenzyme and abolished autophosphorylation of RII. Weber and Hilz (18, 19) demonstrated that an RII fragment generated by chymotryptic cleavage at the hinge region, which still contained the arginine residues, was capable of inhibiting the C subunit and undergoing autophosphorylation but that a similar fragment lacking the arginine residues obtained after tryptic digestion lost the ability to undergo autophosphorylation and to inhibit the C subunit. By using a monoclonal antibody to detect the stable R·C complex, Weldon and Taylor (20) showed that removal of the first 90 residues by chymotryptic digestion did not affect the ability of the remaining RII fragment to form a stable complex with the C subunit, whereas an RII fragment, in which amino-terminal residues 1–93 were removed by thermolysin digestion, lost the ability to form the complex. These experiments were interpreted as supporting the hypothesis that Arg-92 and Arg-93 at the hinge region are essential for the formation of holoenzyme.

In a recent study, we showed that substitution of the hinge region of RII with the inhibitory domain of the heat-stable inhibitor of PKA blocked holoenzyme dissociation and kinase activation in response to cAMP (21). However, this did not eliminate C-induced cAMP release from the mutant RII. This observation suggests that the effect of the C subunit on the rate of cAMP dissociation from RII may have been due to direct contact of the C subunit with the R subunit at a site other than the hinge region.

To test the hypothesis that regions of RII besides the hinge region may be important in R·C interaction, in the present study we have constructed a mutant form, in which Arg-92 and Arg-93 in the hinge region are replaced with alanine residues. This mutant RII still has the ability to form a stable complex with the C subunit, which dissociates on addition of

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Abbreviations: R, regulatory; C, catalytic; PKA, cAMP-dependent protein kinase.

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cAMP. Furthermore, the C subunit promotes dissociation of cAMP from the mutant RII·cAMP complex. However, the holoenzyme containing the mutant form of RII is fully active even in the absence of cAMP. These results show that a region in RII besides the hinge region must be important in formation of the holoenzyme.

EXPERIMENTAL PROCEDURES

Construction of Bacterial Expression Vectors. The bacterial expression vectors were constructed from a *trp–lac* fusion promoter (*trc*), mouse RII cDNA, and plasmid PUC19. A mouse RII cDNA was obtained in previous study (45). The *trc* promoter fragment was obtained from PKK233-2 vector (Pharmacia) by *Nco* I/*Bam*HI digestion. The PUC19 fragment was generated by *Bam*HI/*Pst* I. Mouse RIIα cDNA was cut with *Pst* I/*Eco*RI to obtain a fragment encoding the carboxyl-terminal portion of RIIα protein. A 47-base-pair oligonucleotide with *Nco* I/*Pst* I sites, encoding the amino-terminal portion of RIIα, was chemically synthesized. These fragments were ligated to generate the wild-type RIIα expression vector. The first amino acid after the initiator methionine was changed to alanine from serine to construct an *Nco* I site at the initiation codon.

The RII mutant was generated by site-directed mutagenesis using the polymerase chain reaction (PCR) described by Higuchi (22). Briefly, two 40-mer synthetic oligonucleotides complementary to each strand of the target sequence, containing the mismatched bases in the middle, were used as primers in primary PCR to generate two DNA fragments with overlapping sequences at the target sequence. After removal of excess primers, secondary PCR was conducted using the overlapped fragments from the primary PCR as a template. The product was digested to generate a fragment with *Sal* I/*Hin*dIII sites, which was substituted for the *Sal* I/*Hin*dIII fragment from the wild-type RII expression vector. DNA sequencing analysis verified the sequence in the mutated region.

Expression and Purification of RII. Wild-type RII and RR9293AA mutant RII were expressed and purified by procedures as described (21). Briefly, *E. coli* (RR1 strain) cells transformed with the RII vector were cultured in 200 ml of L broth medium containing ampicillin for 12–16 hr at 37°C. The suspension was passed through a French pressure cell and then centrifuged at 5000 × *g* for 30 min. The supernatant was frozen at −70°C and then thawed on ice. After centrifugation, the solution was loaded onto a Fast Flow Q column (20-ml bed volume; Pharmacia), equilibrated with 20 mM Tris·HCl, pH 6.8/15 mM 2-mercaptoethanol, and eluted with a linear gradient of NaCl in the same buffer (0–0.5 M). RII was further purified by an analytical Mono Q HR5/5 column with a similar gradient of NaCl as for the Fast Flow Q column.

Preparation of cAMP-Free RII. Mutant RII protein in a cAMP-free form was obtained using a modification of the procedure of Cadd *et al.* (23). RII purified as described above was incubated with 10 mM cGMP at 4°C for 72 hr to replace bound cAMP with cGMP. The released cAMP and the excess cGMP were then removed by repurification on Mono Q. RII was then dialyzed against 20 mM Tris·HCl, pH 6.8/15 mM 2-mercaptoethanol for 72 hr to remove bound cGMP.

Protein Kinase Assays. The protein kinase activity of the sample was determined by a phosphocellulose paper assay (24), using a synthetic peptide (Arg-Arg-Leu-Ser-Ser-Leu-Ser-Ala or RRLSSLSA) as substrate. The reaction mixtures contained 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP (≈200 cpm/pmol), 150 μM peptide substrate, 2 nM C subunit, and the indicated amount of RII. For activation of the holoenzyme, the activity was measured in the presence of various concentrations of cAMP (0–100 μM). The reaction was carried out at 30°C for 10 min at which time 40 μl of the reaction mixture was spotted onto a phosphocellulose paper square (P81; Whatman). The paper was washed several times with 150 mM phosphoric acid and then radioactivity was measured in aqueous scintillant (Ecolume; ICN).

Formation of Holoenzyme. Purified RII was mixed with a 2-fold molar excess of the C subunit and then dialyzed against 50 mM potassium phosphate, pH 6.8/15 mM 2-mercaptoethanol overnight at 4°C. The holoenzyme was separated from free C subunit on two Superose 12 HR10/30 gel-filtration columns in tandem. The flow rate was 0.3 ml/min and 0.3-ml fractions were collected. Kinase activity in each fraction was measured in the presence and absence of 2 μM cAMP.

cAMP Binding Assay. The cAMP binding capacity of RII was measured with a Millipore filtration method described by Døskeland and Ogreid (25). The reaction mixture contained 2 μM [^{3}H]cAMP (25–30 Ci/mmol; 1 Ci = GBq), 50 mM potassium phosphate (pH 6.8), 1 mM EDTA, type IIA histone (Sigma) at 0.5 mg/ml, 2 M NaCl, and 1 μg of RII. The binding reaction was carried out at 30°C for 30 min and terminated by adding ice-cold 95% (wt/vol) ammonium sulfate solution. The sample was then filtered through a nitrocellulose filter (type HA; Millipore) and radioactivity bound to the filter was measured. In measurements of the dissociation rate of cAMP from RII, the sample was incubated with 1 μM [^{3}H]cAMP at 37°C for 20 min, and then a 100-fold excess unlabeled cAMP and the indicated amounts of the C subunit were added. The sample was diluted immediately with 50 mM potassium phosphate (pH 6.8) containing bovine serum albumin at 50 mg/ml to a final RII concentration of 20 nM. At various time intervals, 200 μl was taken to determine the amounts of bound [^{3}H]cAMP.

Phosphorylation of RII. Purified RII (2 μg) was incubated in a final volume of 50 μl with 100 μM [γ-^{32}P]ATP (1000 cpm/pmol), 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, and 15 ng of the C subunit for 5 min at 30°C. Bovine serum albumin (10 μg) was added, and the proteins were precipitated by addition of ice-cold trichloroacetic acid to a final concentration of 20% (wt/vol). The precipitate was washed with 5% trichloroacetic acid to remove ATP and the radioactivity in the pellet was measured.

Other Methods. The native C subunit was purified from bovine heart as described by Beavo *et al.* (26). Protein concentrations were determined by the method of Bradford (27). SDS/polyacrylamide gel electrophoresis was performed according to Laemmli (28). Radioimmunoassay of cAMP was conducted using a kit from DuPont/NEN according to the manufacturer's specifications. Amino acid sequence determination was performed using an Applied Biosystem gas-phase sequencer (model 470A).

Materials. All chemicals were purchased from Sigma unless stated otherwise. Protease inhibitors were obtained from Boehringer Mannheim and the enzymes used in DNA manipulations were from New England Biolabs. [2,8-^{3}H]cAMP was purchased from ICN. CM-Sephadex C-50 was from Pharmacia. The peptide and oligonucleotides were synthesized in the Peptide and Oligonucleotide Synthesis Facility of the Howard Hughes Medical Institute, University of Washington.

RESULTS

To evaluate the role of Arg-92 and Arg-93 in the formation of the holoenzyme form of PKA, these residues were converted to alanine residues in a mutant RII by using site-directed mutagenesis. The domain structure and the sites of mutation are indicated in Fig. 1. The mutant RII was expressed in *E. coli* (strain RR1) using methods comparable to those reported for wild-type RII (21). The expressed protein made up 2–5% of total soluble protein in crude extracts obtained after French press treatment, as judged by SDS/PAGE and cAMP

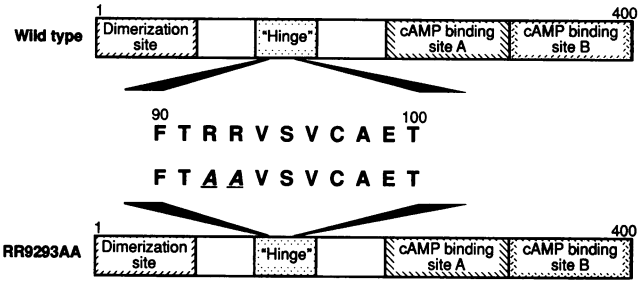

FIG. 1. Domain structure of RII and localization of mutation. RII is represented as a linear chain. Wild-type RII contains two arginine residues within the hinge region. Mutant RII (RR9293) was created by replacing the arginine residues with alanine residues.

binding activity, and was not degraded significantly in the crude extract, based on Western blot analysis. After purification to homogeneity by ion-exchange chromatography, amino acid sequence analysis confirmed that no proteolysis had occurred during purification in the amino-terminal region. The mutant RII had a slightly slower mobility than the wild-type RII on SDS/PAGE (see below), which was consistent with our previous observation that mutations in the hinge region alter the mobility (unpublished observations).

Purified mutant RII as isolated contained ≈2 mol of cAMP per mol of RII monomer, as determined by radioimmunoassay (data not shown). Therefore, cGMP was used to replace the bound cAMP, which could then be removed by extensive dialysis. More than 95% of the bound cAMP was eliminated by this procedure (data not shown). The mutant RII was found to bind 1.7–1.8 mol of cAMP per mol of RII monomer by using the Millipore filtration method, and the dissociation of cAMP displayed a biphasic time course (data not shown). These data indicated that the two cAMP binding sites on each monomer were functionally intact.

To examine properties of subunit association, the mutant RII was incubated with a 2-fold molar excess of the C subunit under conditions in which the wild-type RII and C subunits form a holoenzyme, and the mixtures were analyzed by gel filtration. Initial experiments were attempted using mutant RII saturated with cAMP. Under these conditions, no holoenzyme formation was detected, and all of the kinase activity eluted as free C subunit at a position corresponding to a molecular mass of 40 kDa (Fig. 2*A*). When cAMP-free mutant RII was incubated with the C subunit, however, activity was distributed in two peaks eluting at positions corresponding to 200 kDa and 40 kDa (Fig. 2*B*). Kinase activities of the 200-kDa peak as well as that of the 40-kDa peak, measured in the absence of cAMP, were the same as those measured in the presence of cAMP. The enzyme eluting at 200 kDa contained approximately equal molar amount of R and C subunits (Fig. 3), indicating that the peak most likely represents a holoenzyme with an R_2C_2 structure. Finally, mutant RII in its cAMP-free form preincubated with the C subunit was exposed to 100 μM cAMP for 20 min prior to gel filtration. The activity at 200 kDa disappeared, and the activity at 40 kDa doubled, consistent with the dissociation of holoenzyme to free C subunit (Fig. 2*C*). The results indicate that the holoenzyme containing mutant RII is sensitive to dissociation in the presence of cAMP.

To further demonstrate the formation of holoenzyme from the C and mutant RII subunits, the mutant enzyme from the 200-kDa peak was absorbed onto CM-Sephadex resin (C-50) and then eluted with a high-salt buffer. CM-Sephadex is commonly employed to separate free C subunit from holoenzyme since the latter does not bind to this resin (26). As would be expected for the holoenzyme, which is known not to bind to this type of resin (26), most of the kinase activity failed to bind to the resin and was present in the flow-through

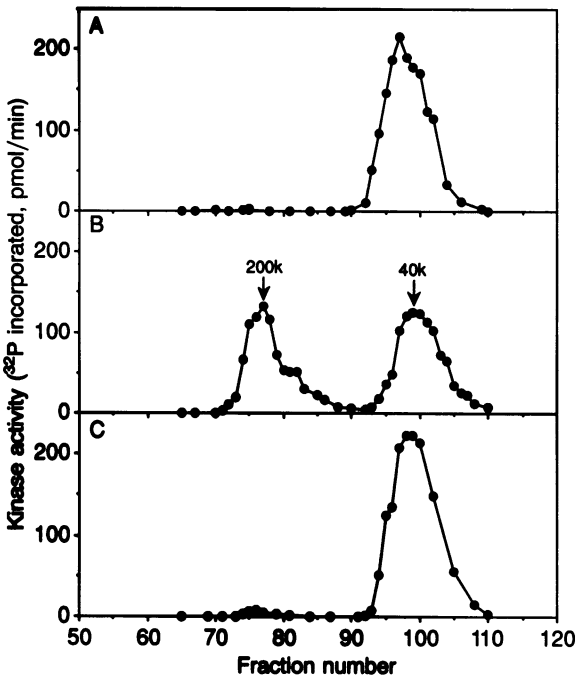

FIG. 2. Analysis of holoenzyme formation by gel filtration. Purified mutant RII (0.3 mg) was incubated with a 2-fold molar excess of the C subunit overnight at 4°C. The sample was then loaded on two Superose 12 columns arranged in tandem (5 × 60 cm) to separate the holoenzyme from free C subunit, and the kinase activity in each fraction (0.3 ml) was measured using the peptide RRLSSLSA as substrate in the presence and absence of 2 μM cAMP. (*A*) Purified mutant RII containing bound cAMP. (*B*) cAMP-free mutant RII. (*C*) cAMP-free mutant RII preincubated with the C subunit followed by addition of cAMP to 100 μM for 20 min before loading. The arrows indicate the molecular mass of each peak calibrated with a set of molecular mass markers.

fraction (Fig. 4). When the enzyme was exposed to 100 μM cAMP for 20 min before absorbing onto CM-Sephadex, the kinase activity was retained on the resin and could only be eluted with high salt, consistent with formation of free C subunit. These results confirm that mutant RII can associate with the C subunit to form a holoenzyme that dissociates in the presence of cAMP.

The activity of mutant and wild-type holoenzyme in the presence of various concentrations of cAMP was examined using the synthetic peptide as substrate. As anticipated (see above), the mutant holoenzyme was fully active even in the absence of cAMP whereas the wild-type holoenzyme was inactive under this condition but exhibited a K_a value about 40 nM for activation in response to cAMP (Fig. 5). This result suggests that Arg-92 and Arg-93 are essential for inhibition for the C subunit in the holoenzyme complex.

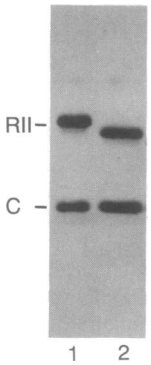

FIG. 3. Analysis of holoenzyme on SDS/polyacrylamide gel. A Coomassie blue-stained gel is shown. The holoenzyme formed with mutant RII (RR9293AA; lane 1) and wild-type RII (lane 2) was obtained from the Superose 12 column (see legend of Fig. 2) and analyzed on a 10% polyacrylamide gel containing SDS.

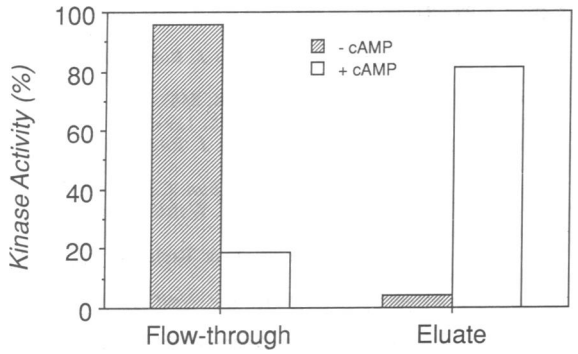

FIG. 4. Analysis of holoenzyme formation by cation-exchange chromatography. The mutant holoenzyme from the gel-filtration column (100 μg) was applied batchwise to CM-Sephadex (0.3 ml) for 30 min at 4°C. The flow-through fraction was collected and the material was then eluted with 3 vol of a buffer containing 0.4 M NaCl. The kinase activity was analyzed in the flow-through and the eluate. Hatched bars represent experiments performed using enzyme applied directly to CM-Sephadex and open bars represent enzyme preincubated with 100 μM cAMP for 20 min at 4°C before being applied to CM-Sephadex.

Since mutant RII remains attached to the C subunit, although it lacks the recognition site for C in the hinge region, there was a possibility that this latter region might still be in close proximity to the substrate binding site of the C subunit and Ser-95 might be phosphorylated. Under conditions that led to fully stoichiometric phosphorylation of wild-type RII, however, there was essentially no phosphorylation of mutant RII by the C subunit (Fig. 6), indicating that interaction of the hinge region of mutant RII with the active site of the C subunit was apparently abolished.

It is known that addition of the C subunit causes the release of cAMP from the R·cAMP complex with either RI and RII (22, 29, 30). Mutant RII provided a suitable means for testing whether or not the effect of the C subunit on cAMP dissociation is due to the interaction of the C subunit with the hinge region. To examine this aspect, mutant RII was allowed to bind [^{3}H]cAMP. Free C subunit and unlabeled cAMP were then added and the amount of [^{3}H]cAMP still bound to RII was determined with time. The rate of [^{3}H]cAMP dissociation was doubled in the presence of 100 nM C subunit relative to that measured in the absence of the C subunit (Fig. 7). This result suggests that the effect of the C subunit on cAMP release is not due to a direct interaction of the C subunit with the hinge region since the interaction of the C subunit with the hinge region of mutant RII was apparently eliminated. Therefore, the interaction of the C subunit with another region of RII must be responsible for the effect of the C subunit on the dissociation of RII·cAMP complex.

FIG. 6. Phosphorylation of RII subunit. Mutant RII (RR9293AA; lane 2) and wild-type RII (lane 1) (2 μg) were phosphorylated by the C subunit. The phosphorylation stoichiometry was 1 mol of P per mol of RII monomer for wild-type RII and 0.02 mol of P per mol of RII monomer for mutant RII.

DISCUSSION

In this study, site-directed mutagenesis of RII has been used to assess the roles of Arg-92 and Arg-93 on holoenzyme formation and inhibition of the C subunit. Mutant RII, in which the two arginine residues were replaced by alanine residues, retained the ability to form a holoenzyme with the C subunit but this holoenzyme was fully active even in the absence of cAMP. These results demonstrate that Arg-92 and Arg-93 are not essential for the formation of holoenzyme but are critical for inhibiting activity of the C subunit. The lack of inhibition of the C subunit by mutant RII in the holoenzyme suggests that the hinge region no longer occupies the active site. This conclusion is supported by the inability of the C subunit to phosphorylate Ser-95 in the hinge region of mutant RII. Furthermore, the effect of the C subunit on cAMP dissociation is still present in mutant RII. Thus the results from this study support the hypothesis that RII contains a region that interacts with the C subunit in addition to the hinge region.

The hinge region of the R subunit was initially identified by its susceptibility to limited proteolysis (for review, see ref. 31). Interaction of this region with the active site of the C subunit was suggested by the presence of two positively charged arginine residues that are ordinarily present in substrates for the C subunit. Therefore, a substrate/pseudosubstrate autoinhibitor hypothesis was suggested (14). The importance of

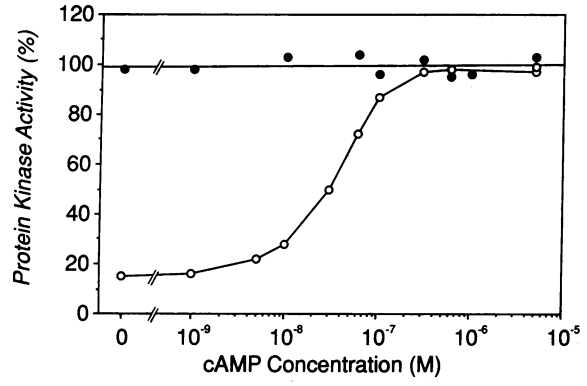

FIG. 5. Activation of holoenzyme by cAMP. The holoenzyme concentration was 2 nM. Solid circles represent the mutant holoenzyme (RR9293AA) and open circles represent wild-type holoenzyme. Activity was measured using synthetic peptide (RRLSSLRA) as substrate and is expressed as a fraction of the maximal activity.

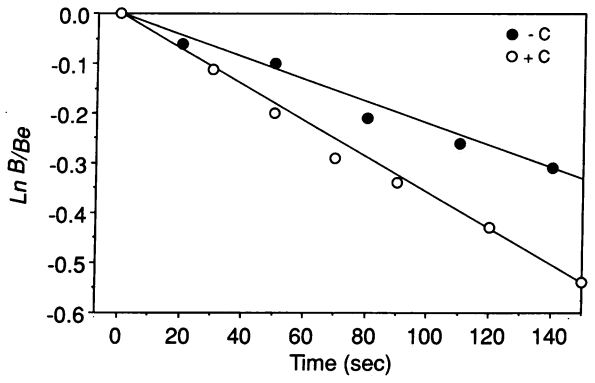

FIG. 7. Dissociation of cAMP from the mutant RII·cAMP complex. The mutant RII concentration was 20 nM. Dissociation was measured in the absence of the C subunit (solid circles) and in the presence of 100 nM C subunit (open circles), LnB/Be; natural logarithm of [^{3}H]cAMP bound/[^{3}H]cAMP bound at time 0.

arginine residues in this region was supported by studies on the inhibitory domain sequence of the heat-stable PKA inhibitor in which changes in arginine residues resulted in a decrease in its inhibitory potency (32, 33). The substrate/pseudosubstrate autoinhibitor concept has been extended to other kinases including cGMP-dependent protein kinase (34), protein kinase C (35), smooth muscle myosin light chain kinase (36), skeletal muscle myosin light chain kinase (37), and Ca^{2+}-calmodulin-dependent protein kinase II (38). However, the strong interaction between R and C subunits cannot be due entirely to the substrate-like sequence of the hinge region since the R subunit binds to the C subunit with an affinity in the nanomolar range whereas most of the substrates for the C subunit have an affinity in the micromolar range (39, 40). Furthermore, we have been unable to generate a potent peptide inhibitor of the C subunit based on the hinge region sequence (unpublished observations). Flockhart *et al.* (41) showed that heat treatment of RII destroys its cAMP-binding activity and its ability to inhibit the C subunit but not its ability to be phosphorylated, implying that structural elements other than the hinge region might contribute to the strong interaction of RII and C subunits. Levin *et al.* (42) reported that a mutation in the yeast C subunit decreased its affinity for the yeast R subunit without altering its affinity for substrate, suggesting that a region in the C subunit other than the substrate binding site may participate in forming the R·C interaction.

The results from this study do not support the conclusion from a previous study that Arg-92 and Arg-93 are essential for formation of a stable complex between RII and C subunits (20). In our studies we observed that mutant RII retained the ability to form holoenzyme only in a cAMP-free form. This might explain why no stable complex was detected in the previous study because RII purified from cAMP affinity columns is likely to contain bound cAMP.

Inhibition of C subunit activity by the R subunit has been employed to assess R·C interaction in many studies. Since replacement of arginine with alanine abolished inhibition of the C subunit but did not affect holoenzyme formation, it is obvious that measurements of kinase activity may be sensitive to the localized interaction between the substrate binding site of the C subunit and the hinge region of the R subunit but do not always reflect all aspects of the interaction between R and C subunits.

The results from this study raise interesting questions about the possibility of a second R·C interaction region in RII and the mechanism of holoenzyme activation by cAMP. Although the precise location of this region in RII remains to be determined, we speculate that it may be located in the cAMP binding domains themselves. Several lines of observation favor this speculation. (*i*) Formation of the holoenzyme from mutant RII is still affected by cAMP. (*ii*) The C subunit promotes the release of cAMP from mutant RII, an effect that can be distinguished from the direct interaction of the hinge region with the substrate binding site of the C subunit. Deletion of the amino partion of RI did not abolish this effect (43). (*iii*) Mutations in the cAMP-binding domains of RI decrease the stability of the holoenzyme (44). (*iv*) Based on an analysis of the predicted structure of the cAMP binding domains of the R subunit, it has been suggested that the C-helix, which is analogous to the intersubunit interaction region of catabolite gene activator protein, might interact with the C subunit (9). Further studies using mutated or truncated forms of RII and C subunits may help to determine the cAMP-regulated R·C interacting regions.

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1. Krebs, E. G. & Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48**, 923–959.
2. Corbin, J. D., Keely, S. L. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 218–225.
3. Rosen, O. M. & Ehrlichman, J. (1975) *J. Biol. Chem.* **250**, 7788–7794.
4. Taylor, S. S. (1989) *J. Biol. Chem.* **264**, 8443–8446.
5. Reimann, E. M. (1986) *Biochemistry* **25**, 119–125.
6. Rannels, S. R. & Corbin, J. D. (1979) *J. Biol. Chem.* **254**, 8605–8610.
7. Potter, R. L. & Taylor, S. S. (1979) *J. Biol. Chem.* **254**, 9000–9005.
8. Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A. & Titani, K. (1984) *Biochemistry* **23**, 4200–4206.
9. Weber, I. T., Seitz, T. A., Bubis, J. & Taylor, S. S. (1987) *Biochemistry* **26**, 343–351.
10. Weber, I. T. & Seitz, T. A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3972–3973.
11. Rannels, S. R. & Corbin, J. D. (1980) *J. Biol. Chem.* **255**, 7085–7088.
12. Rannels, S. R. & Corbin, J. D. (1981) *J. Biol. Chem.* **256**, 7871–7876.
13. Casnellie, J. E. & Krebs, E. G. (1984) *Adv. Enzyme Regul.* **22**, 501–515.
14. Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M. & McCarthy, D. (1978) *J. Biol. Chem.* **253**, 3997–4003.
15. Granot, J., Midvan, A. S., Hiyama, K., Kondo, H. & Kaiser, E. T. (1980) *J. Biol. Chem.* **255**, 4569–4573.
16. Rangel-Aldao, R. & Rosen, O. M. (1976) *J. Biol. Chem.* **251**, 7526–7529.
17. Nelson, N. C. & Taylor, S. S. (1983) *J. Biol. Chem.* **258**, 10981–10987.
18. Weber, W. & Hilz, H. (1978) *Eur. J. Biochem.* **83**, 215–225.
19. Weber, W. & Hilz, H. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1073–1081.
20. Weldon, S. L. & Taylor, S. S. (1985) *J. Biol. Chem.* **260**, 4203–4209.
21. Wang, Y., Scott, J. D., Wecker, M. & Krebs, E. G. (1990) *FASEB J.* **4**, A2074 (abstr.).
22. Higuchi, R. (1990) in *PCR Protocols: A Guide to Methods and Applications*, eds. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (Academic, New York), pp. 177–183.
23. Cadd, G. G., Uhler, M. D. & McKnight, G. S. (1990) *J. Biol. Chem.* **265**, 19502–19506.
24. Roskoski, R. (1983) *Methods Enzymol.* **99**, 3–6.
25. Døskeland, S. O. & Ogreid, D. (1988) *Methods Enzymol.* **159**, 147–150.
26. Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1974) *Methods Enzymol.* **38C**, 299–308.
27. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
28. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
29. Chau, V., Huang, C., Romero, G., Biltonen, R. L. & Huang, C.-H. (1980) *Biochemistry* **19**, 924–928.
30. Ogreid, D. & Døskeland, S. O. (1983) *Biochemistry* **22**, 1686–1696.
31. Taylor, S. S., Buechler, J. A. & Yomemoto, W. (1990) *Annu. Rev. Biochem.* **59**, 971–1005.
32. Scott, J. D., Fischer, E. H., Demaille, J. G. & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4379–4383.
33. Cheng, H. C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M. & Walsh, D. A. (1986) *J. Biol. Chem.* **261**, 989–992.
34. Lincoln, T. M., Flockhart, D. A. & Corbin, J. D. (1978) *J. Biol. Chem.* **253**, 6002–6009.
35. House, C. & Kemp, B. E. (1987) *Science* **238**, 1726–1728.
36. Kemp, B. E., Pearson, R. B., Guerriero, V., Bagchi, I. C. & Means, A. R. (1987) *J. Biol. Chem.* **262**, 11958–11963.
37. Kennelly, P. J., Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *J. Biol. Chem.* **262**, 11958–11963.
38. Schworer, C. M., Colbran, R. J., Keefer, J. R. & Soderling, T. R. (1988) *J. Biol. Chem.* **263**, 13486–13489.
39. Kemp, B. E., Graves, D. J., Benjamini, E. & Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 4888–4894.
40. Flockhart, D. A. & Corbin, J. D. (1982) *CRC Rev. Biochem.* **13**, 133–186.
41. Flockhart, D. A., Watterson, D. M. & Corbin, J. D. (1980) *J. Biol. Chem.* **255**, 4435–4440.
42. Levin, L. R., Kuret, J., Johnson, K. E., Powers, S., Cameron, S., Michaeli, T., Wigler, M. & Zoller, M. J. (1988) *Science* **240**, 68–70.
43. Ringheim, G. E. & Taylor, S. S. (1990) *J. Biol. Chem.* **265**, 4800–4808.
44. Woodford, T. A., Correll, L. A., McKnight, G. S. & Corbin, J. D. (1989) *J. Biol. Chem.* **264**, 13321–13328.
45. Scott, J. D., Glaccum, M. B., Zoller, M., Uhler, M. D., Helfman, D. M., McKnight, G. S. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5192–5196.