Dissecting cAMP Binding Domain A in the R\(^1\)α Subunit of cAMP-dependent Protein Kinase

DISTINCT SUBSITES FOR RECOGNITION OF cAMP AND THE CATALYTIC SUBUNIT*

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The two gene-duplicated cAMP binding domains in the regulatory subunits of cAMP dependent protein kinase are each comprised of an A helix, an eight-stranded \(\beta\)-barrel, and a B and C helix (1). The A domain is required for high affinity binding to C, while the B domain regulates access to the A domain. Using a combination of a yeast two-hybrid screen coupled with deletion analysis, cAMP binding domain A of R\(^1\) was dissected into two structurally and functionally distinct subsites, one that binds cAMP and another that binds the C subunit. The minimum stable subdomain required for binding to C in the 1–3 micromolar range is composed of residues 94–169, while residues 236–244, mapped to the C helix of cAMP binding domain A, were defined as a second surface necessary for high affinity (5–10 nanomolar) binding to C. This portion of the C helix, due to its position directly between the two subsites, serves as a molecular switch for either a cAMP-bound conformation or a C-bound conformation and can thus modulate interactions of cAMP binding domain A with cAMP, with C, and with cAMP binding domain B.

Of fundamental importance for understanding how signal transduction pathways are regulated is to define in molecular terms how various protein kinases interact with inhibitor proteins. cAMP-dependent protein kinase (PKA),\(^1\) probably the best understood protein kinase biochemically, has two known classes of endogenous physiological inhibitors: the regulatory (R) subunits and the heat-stable protein kinase inhibitors (PKIs) (2). Both bind the catalytic (C) subunit with high affinity (<1 nM) in a mutually exclusive manner. The R subunits bind in the absence of cAMP forming an inactive tetrameric holoenzyme (R\(_2\)C\(_2\)). Cooperative binding of cAMP to the R subunit dissociates the complex, thereby unleashing the catalytically active C subunits. PKI binds to free C in a cAMP-independent manner (3).

Both the R subunits and PKIs share a common mechanism for inhibiting C. Each contains an inhibitory consensus site sequence, Arg-Arg-Xaa-Ser/Thr/Ala-Yaa, where Xaa is any amino acid and Yaa is a hydrophobic residue (4). This pentapeptide resembles a substrate or inhibitor and binds to the active site cleft of the C subunit (3, 5, 6). PKI and R are thus competitive inhibitors. In addition to serving as inhibitors of C, however, PKI and the R subunits share other common features. Both are modular proteins, and both play roles in subcellular localization; neither is solely an inhibitor of C. PKI, for example, in addition to its inhibitor site at the N terminus (3), has near its C terminus a nuclear export signal that is capable of actively shutting the C-PKI complex out of the nucleus (7).

The R subunits are also modular proteins composed of several distinct, well defined, and stable domains (Fig. 1). At the N terminus is a dimerization/docking domain that not only maintains the R subunits as a stable dimer but also provides a surface that docks to a variety of A kinase anchoring proteins, thereby localizing the enzyme to specific subsites within the cell (8). The “hinge” region that follows the dimerization/docking domain contains the consensus sequence that binds to the active site cleft of C in the absence of cAMP. At the C terminus are two tandem gene-duplicated cAMP binding domains. Although each has a functional cAMP binding site, the two domains serve distinct roles. cAMP binding domain A interacts directly with the C subunit and is essential for high affinity binding to C, whereas cAMP binding domain B is not (9). Instead, its role is to modulate access of cAMP to domain A and to contribute cooperativity to the activation process. In the holoenzyme, only domain B is accessible (10, 11). cAMP thus binds first to domain B, which induces a conformational change. The subsequent binding of cAMP to domain A causes dissociation of C and activation of the holoenzyme (47).

To achieve high affinity binding to C, both PKI and the R subunits utilize a bipartite mechanism. Occupancy of the active site cleft by the small consensus site peptide is not sufficient to convey high affinity binding; additional interaction sites are required. Two additional but different surfaces on the large lobe of the C subunit, peripheral recognition sites 1 and 2 (PRS1 and PRS2), were shown to be essential for high affinity binding of PKI and R, respectively (13). PRS1 lies N-terminal to the region where the consensus site peptide docks (5, 14, 15), while PRS2, essential for the high affinity binding of the R subunits, lies on a surface that follows the consensus site pocket. For PKI, the recognition sequence is linear; residues 5–24, where residues 18–21 constitute the consensus site, are sufficient to achieve nearly full binding affinity (16). An amphipathic helix lies N-terminal to the consensus site, and this helix docks to the mostly hydrophobic PRS1 surface (3, 5, 17).
In contrast to PKI, the consensus site plus the immediate flanking sequences are not sufficient to convey high affinity binding for the R subunits. The binding of R is, therefore, not as simple as PKI and involves regions that are not contiguous to the consensus site. Binding of R to C is also coupled with the release of cAMP.

Limited proteolysis combined with genetic engineering of deletion mutants established previously that the region extending from the consensus site through cAMP binding domain A is sufficient for high affinity binding to C (2, 18, 19). Point mutations also identify a number of specific residues in addition to and not contiguous with the inhibitor site that are important for R binding to C (13, 20, 21). By using a yeast two-hybrid screen with the C subunit as bait and then engineering additional deletion mutants, we have been able to further dissect cAMP binding domain A into two distinct sub-sites: one that recognizes C and complements the PRS2 site and the other that binds cAMP. A critical helix is also identified as a switch that bridges the two sub-sites and modulates interactions of this domain with cAMP, with C, and with cAMP binding domain B.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources: ATP, benzamidine, cAMP, EDTA, MOPS, phenylmethanesulfonyl fluoride, Triton X-100, and GST-agarose resin, from Sigma; nickel-NTA resin from Qiagen; urea, 5-bromo-4-chloro-3-indolyl β-D-galactoside and enzymes used for DNA manipulations from Life Technologies, Inc.; and a DNA sequencing kit from U.S. Biochemical Corp. The peptide substrate Kemptide (60–200 amino acids) was synthesized at the peptide and oligonucleotide facility at the University of California, San Diego. The oligonucleotides were synthesized with a 380B DNA synthesizer from Applied Biosystems at the University of California, San Diego. The oligonucleotides were excised from the rescued prey plasmids of the two-hybrid system and sequenced in the DNA data base (23). Twenty of these clones coded for a protein fragment corresponding to residues 94–235 of RI. To make His<sub>6</sub>RI-(94–169), a NheI site was created at residue 93 and three stop codons were introduced at residue 169 in pRS316-R<sup>i</sup> using site-directed mutagenesis to generate the deletion mutant (24). The DNA corresponding to the N-terminal 93 residues was then excised by digestion with NheI. His<sub>6</sub>RI-(94–260) and His<sub>6</sub>RI-(94–244) were constructed by creating a NheI site at residue 93 and three stop codons at position 261 and 245, respectively, in pRS316-R<sup>i</sup> using site-directed mutagenesis. The DNA corresponding to the N-terminal 93 residues was then excised by digestion with NheI. All constructs were confirmed by DNA sequence analysis (23). All fusion proteins were expressed in Esherichia coli BL21(DE3). The BL21(DE3) cell strain was a gift from Bill Studier (Brookhaven National Laboratory, Upton, NY).

Preparation of Proteins—Recombinant wild type R<sup>i</sup> was expressed in E. coli 222 and purified by DEAE-52 chromatography as described previously (25). GST-R<sup>i</sup>-(18–169) and GST-R<sup>i</sup>-(94–235) were expressed in E. coli BL21(DE3) and purified first on a glutathione-agarose column in 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 0.05% Triton X-100, and 3 mM β-mercaptoethanol. GST-R<sup>i</sup>-(18–169) was further purified on a gel filtration column using Superdex 200. All histidine-tagged proteins were purified on a nickel-NTA column in 10 mM sodium phosphate buffer as described by Huang et al. (26). Untagged R<sup>i</sup>-(94–235) was expressed first as a GST fusion protein, and then the GST was cleaved off of the fusion protein by thrombin. Untagged R<sup>i</sup>-(94–235) was then further purified on a gel filtration column using Superdex 75 column. The C-subunit was purified by phosphocellulose chromatography and then resolved into discrete isoelectric variants on a MonoS HR10/10 column using fast protein liquid chromatography (Amersham Pharmacia Biotech) as described by Herberg et al. (27). Isozymes I and II were used for all experiments.

Preparation of cAMP-free Native and Mutant R<sub>i</sub> Subunits—To obtain cAMP-free R<sub>i</sub> subunit, purified recombinant wild type R<sup>i</sup> was unfolded in 8 M urea as described by Buechler et al. (28). After removing the cAMP by passing the solution over a prepacked Sephadex G-25 column (NAP10 column), proteins were then refolded by dialyzing overnight in 150 mM KCl, 10 mM MOPS, and 5 mM β-mercaptoethanol at pH 7. cAMP-free polyhistidine fusion proteins were obtained by purification on a nickel-NTA resin under 8 M urea denaturing conditions. In summary, cells expressing the fusion proteins were lysed in 10 mM sodium phosphate buffer containing 8 M urea and centrifuged, and the supernatant was then passed through a nickel-NTA column. Polyhistidine fusion R<sub>i</sub> subunits were then eluted from the column with sodium phosphate buffer containing 100 mM imidazole and dialyzed extensively against sodium phosphate buffer, pH 7.4 for refolding.

Activity Assays—All activity assays were performed using the spectrophotometric method described by Cook et al. (29) in an assay mix containing 1 mM ATP and 10 mM MgCl₂. Briefly, 20–50 nM of C was incubated in a 1-ml assay volume containing 1 mM phosphoenolpyruvate, 100 μM NADH, 6 units of lactate dehydrogenase, and 2 units of pyruvate kinase. Kemptide (60–200 μM) was then added to initiate the reaction. The progress of the reaction was monitored continuously by a decrease in absorbance at 340 nm due to the oxidation of NADH in a Hewlett Packard 1587 diode array spectrophotometer. Reaction velocity was constant over 60 s. Values for K<sub>m</sub> and V<sub>max</sub> were determined using Michaelis-Menten kinetics. To determine apparent inhibitory constants, C (20–50 nM) was incubated with different concentrations of R mutants for 1 min at room temperature. The reactions were initiated by the addition of Kemptide. The percentage of inhibition for each reaction was plotted as a function of inhibitor concentration, and the apparent inhibition constant, K<sub>i</sub>(app), was determined by fitting the data to the equation,

\[ f_i = \frac{[I]}{[I] + K_{i(app)}} \]  

(Eq. 1)

where \( f_i \) is the fraction of inhibitor bound, [I] is the inhibitor concentration, and \( K_{i(app)} \) is the apparent dissociation constant for the inhibitor. Since in these studies, various R<sub>i</sub> subunits acted as competitive inhibitors, the calculation of K<sub>i</sub>(app) was performed as described by Buechler et al. (30).

Construction of Expression Vectors—To determine whether the two truncation fragments of R<sub>α</sub> isolated from the two-hybrid screen bind C in vitro, plasmids containing these fragments were engineered, and the resulting proteins were purified as GST fusion proteins. To make GST-R<sup>i</sup>-(94–235) and GST-R<sup>i</sup>-(18–169), a linker containing a NolI site was first ligated into pGEX-KG vector using NolI and HindIII to generate pGEX-KG-NolI. The cDNAs of R<sup>i</sup>-(94–235) and R<sup>i</sup>-(18–169) were then excised from the rescued prey plasmids of the two-hybrid system and then subcloned into the NolI site of pGEX-NolI-R<sup>i</sup>. R<sup>i</sup>-(94–235) was also expressed as a polyhistidine-tagged protein, His<sub>6</sub>R<sup>i</sup>-(94–235). To make His<sub>6</sub>R<sup>i</sup>-(94–235), cDNA of R<sup>i</sup>-(94–235) was excised from pGEX-KG-R<sup>i</sup>-(94–235) and subcloned into pRSETb. A truncation fragment R<sup>i</sup>-(94–169), corresponding to the overlapping region of the two positive clones was engineered and expressed as a polyhistidine-tagged protein.

\[ \frac{V_{app}}{V_{max}} = \frac{1}{1 + [S]/K_{i(app)}} \]  

(Eq. 2)

where [S] is the substrate Kemptide concentration, and \( K_{i(app)} \) is the Michaelis constant of Kemptide. In these studies, the K<sub>i</sub> for Kemptide was determined to be 16 μM since His<sub>6</sub>R<sup>i</sup>-(94–244) and His<sub>6</sub>R<sup>i</sup>-(94–260) inhibited C readily with affinities in the range of the enzyme concentration used in the assay, their inhibitory constants were determined by fitting the inhibition curves to the equation,

\[ \frac{V_{app}}{V_{max}} = \frac{1}{1 + [S]/K_{i(app)}} \]  

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where [S] is the substrate Kemptide concentration, and \( K_{i(app)} \) is the Michaelis constant of Kemptide. In these studies, the K<sub>i</sub> for Kemptide was determined to be 16 μM since His<sub>6</sub>R<sup>i</sup>-(94–244) and His<sub>6</sub>R<sup>i</sup>-(94–260) inhibited C readily with affinities in the range of the enzyme concentration used in the assay, their inhibitory constants were determined by fitting the inhibition curves to the equation.
where \( v \) and \( v_0 \) are the measured kinetic rates of C in the presence and absence of inhibitor, respectively; \( E \) and I are the total enzyme and inhibitor concentrations, respectively. The true \( K_i \) can then be calculated from the \( K_i \) (app) using the Morrison equation. In all inhibition studies, each data point represented triplicate measurements.

**cAMP Activation of Holoenzyme**—Apparent activation constants (\( K_a \)) were measured for holoenzymes formed by C with either wild type R\( ^1 \) or the various deletion mutants, His\( _6 \)R\( ^1-94–244 \) or His\( _6 \)R\( ^1-94–235 \). Holoenzyme for wild type C and R\( ^1 \) was formed by two methods. In method 1, C and R\( ^1 \) were dialyzed for 24 h at room temperature against 20 mM potassium phosphate, 100 mM KCl, 5 mM \( \beta \)-mercaptoethanol, 5% glycerol, 100 \( \mu \)M ATP, and 1 mM MgCl\( _2 \) at pH 6.5. In method 2, 40 nM C and 35 nM cAMP-free R\( ^1 \) were incubated for 30 min in assay mix at room temperature. These two methods were also used to obtain holoenzyme for His\( _6 \)R\( ^1-94–244 \) except that the dialysis was carried out at 4°C instead of at room temperature. Holoenzyme for His\( _6 \)R\( ^1-94–235 \) was obtained by method 2 with 40 nM C and 0.2 \( \mu \)M cAMP-free His\( _6 \)R\( ^1-94–235 \). In all cases, the reaction was initiated by the addition of Kemptide followed by activation with varying concentrations of cAMP. The activity of the free C subunit was then monitored spectrophotometrically. Each data point was measured in triplicate, and the mean was used for calculation.

**RESULTS**

**Isolation of Two R\( ^1 \) Fragments That Interact with C**—A yeast two-hybrid screen was used to identify proteins that associate with the C subunit of PKA. Using LexA-C as the bait, 22 out of 2 \( \times 10^5 \) transformants interacted specifically with the C subunit bait in screening a mouse embryonic library. All 22 positive clones coded for fragments of the R\( ^1 \) subunit isoforms were isolated, nor were any PKIs isolated from the screen.

To determine whether these fragments interact with C in vitro, both fragments were expressed as GST fusion proteins or polyhistidine fusion proteins. GST-R\( ^1-94–235 \) and His\( _6 \)R\( ^1-94–235 \) were expressed in quantities about 10-fold higher than GST-R\( ^1-18–169 \) and were near homogeneity after affinity purification on either glutathione-agarose or nickel-NTA resin. GST-R\( ^1-18–169 \) was purified further by gel filtration to near homogeneity.

In a spectrophotometric assay, a fixed amount of wild-type C subunit was incubated with increasing amounts of GST-R\( ^1-18–169 \), GST-R\( ^1-94–235 \), or His\( _6 \)R\( ^1-94–235 \) for 1 min at 22°C in the presence of MgATP. Kemptide was then added to initiate the kinase reaction. The activity of the C subunit was inhibited by both fusion proteins. To further characterize this
inhibition, the $K_m$ and $V_{max}$ for Kemptide were measured with increasing concentrations of inhibitor His$_6$RI-(94–235). As shown in Fig. 2A, the fusion protein, His$_6$RI-(94–235), was a competitive inhibitor for C with respect to Kemptide. The calculated $K_i$ was 0.3 mM. Based on the fact that all of the constructs had the consensus sequence, we assumed that the other subsequent constructs would behave also as competitive inhibitors to C. By this criterion, all apparent inhibitory constants ($K_i(\text{app})$ values) were calculated by hyperbolic fitting of the binding curves (Fig. 2B).

To demonstrate that tagging the fragment with GST or polyhistidines does not seriously affect the $K_i$ values, different forms of RI-(94–235) were expressed and characterized. The $K_i$ values of GST-RI-(18–169) and GST-RI-(94–235) were 0.25 and 0.3 mM, respectively, indicating that the inhibitory ability was not drastically altered by the presence of the fused protein. These apparent inhibitory constants in the presence of MgATP are summarized in Table I.

A minimal requirement for R$^l$ to Inhibit C—to determine the minimum required sequence for inhibiting C, the overlap piece of the two fragments identified in the two-hybrid screen was constructed as a polyhistidine fusion protein and characterized for its ability to inhibit C. This overlap piece contained residues 94–169 of R$^l$, which includes the consensus site and part of cAMP binding domain A. His$_6$R$^l$-(94–169) inhibited the C subunit with a $K_i$ of 0.2 mM. This inhibitory constant was still 100-fold lower than that of an inhibitor peptide where the Ser residue of Kemptide (LRRASLG) is replaced with Ala (30). A peptide corresponding to residues 89–111 of R$^l$ was not able to compete with Kemptide in the assay (data not shown). Therefore, residues 94–169 of R$^l$ were defined as the minimum required sequence for RI to inhibit C in the low micromolar (1–3 mM) range. All apparent $K_i$ values with respect to the different deletion constructs are summarized in Fig. 3.

**Importance of the C Helix of cAMP Binding Site A for R-C Interaction**—As described by Herberg et al. (19) and by other proteolysis experiments (9, 18), the N-terminal 91 residues do...
not contribute to high affinity binding of R to the C subunit; (Δ1–91)R is as potent an inhibitor as the wild type R. Both GST-R(94–235) and GST-R(18–169), however, showed a dramatic decrease in affinity for C, compared with the 0.2 nM Kᵢ obtained for cAMP-free full-length R (19). To map the regions on R responsible for this 1000-fold decrease in affinity, His₆R(94–260) was constructed, expressed, and purified on a nickel-NTA affinity column. This mutant, which contains the entire cAMP binding domain A, is monomeric because it lacks the N-terminal dimerization domain. In the spectrophotometric assay, His₆R(94–260) inhibited C rapidly and efficiently with an inhibitory constant of 5–10 nM. Therefore, by comparison to C, the ble for the 2-order of magnitude increase in affinity for binding to cAMP may cause some degree of structural damage in these mutants.

To more specifically map the region on the C helix responsible for the 2-order of magnitude increase in affinity for binding to C, the Kᵢ of His₆R(94–244), which lacks the latter part of the C helix and the connecting loop, was measured. The Kᵢ of this mutant was also 5–10 nM, similar to the Kᵢ of His₆R(94–260). The region between Ser²³⁶ and Tyr²⁴⁴ on the C helix is an inhibitory constant of 5–10 nM. Therefore, by comparison to C, the half-maximum activation of holoenzyme formed with His₆R(94–244) occurred at 763 nM cAMP, similar to wild type R (94–259) at 1.1 μM (17). In contrast to wild type R, activation with both mutants showed no cooperativity, typical of other monomeric forms of R. Since holoenzymes formed with His₆R(94–235) had a Kᵢ 3-fold lower than holoenzymes formed with His₆R(94–244), or His₆R(94–235). This study showed that the two major bonding network that is induced by cAMP binding and interacts weakly with C. This study showed that the two major functions of the R subunits, high affinity binding to C versus high affinity binding to cAMP, are mediated by structurally distinct subsites within the cAMP binding domain A (cA:A).

**DISCUSSION**

As summarized in Table II, the modular domains of the regulatory subunit all interact with multiple protein partners, whether it is another domain or a separate molecule. For example, the dimerization/docking domain at the amino terminus not only interacts with the other R protomer in the dimer; the dimeric domain also serves as a docking surface for A kinase anchoring proteins (31–33). In some cases, such as cAMP binding site A (the cA:A domain), the partners also differ depending on the activation state of the enzyme. In vitro, the regulatory subunit exists in two major and structurally distinct conformations. In the absence of cAMP, it binds tightly to the C subunit, while the cAMP-bound conformation has an extended hydrogen bonding network that is induced by cAMP binding and interacts weakly with C. This study showed that the two major functions of the R subunits, high affinity binding to C versus high affinity binding to cAMP, are mediated by structurally distinct subsites within the cAMP binding domain A (cA:A).

Evolution has yielded many proteins that contain tandem...
gene-duplicated sequences. Examples of repeated catalytic units are the mammalian hexokinases (34) and the protein-tyrosine phosphatases (35). Examples of repeated binding domains are also common, one of the most extreme being the 36 repeats of the epidermal growth factor domain in Notch (36). Typically, one domain serves a primary catalytic or ligand

**FIG. 6. Subdomains of cAMP binding domain A.** The residues preceding domain A are shown in white, domain A with the exception of the C helix (residues 141–235) is shown in blue, and the C helix is shown in red. cAMP is in yellow. Residues important for binding to C, Asp^{140} and Glu^{143}, are shown in red. A, domain A through residue 235. B, complete A domain. C, residues 141–244 of domain A comprise two distinct subdomains, with residues 236–244 in the C helix serving as a bridge between the cAMP binding site and the C binding site.
binding role, while the other serves more of a regulatory role. In the case of PKA, cAMP binding domain A plays a primary role in mediating direct interactions with C, while domain B modulates the cAMP binding access to domain A.

The cAMP binding domain found in the R subunit, defined initially on the basis of sequence similarities, is found in a number of proteins (37). It was first described structurally when the catabolite gene activator protein was crystallized (38). This structure was then used as a model for the cAMP binding domains found in the R subunits (39, 40) and for the cGMP binding domains in cGMP-dependent protein kinase (41). The same motif is found in the cyclic nucleotide gated channels (42). The structural similarity of these domains was confirmed when the crystal structure of a deletion mutant of the R1a subunit of PKA was solved (1).

The generic cAMP binding domain consists of three helices and an eight-stranded \( \beta \)-barrel. Most attention in these cAMP binding domains has focused so far on a number of conserved residues that interact with the cyclic phosphate portion of cAMP and sequester it completely from solvent. Genetic and mutational analyses have confirmed the importance of these residues for cAMP binding (43, 44). The results described here, however, focus on cAMP binding domain A of the R1a subunit and dissect it into two subunits that are structurally and functionally distinct. The structure of domain A, highlighting some of the key residues that are important for function, is summarized in Fig. 5.

From a yeast two-hybrid screen using the C subunit as the bait, only clones corresponding to two fragments of the R1 subunit, R1-(18–169) and R1-(94–235), were isolated. Somewhat surprisingly, no clones were found that corresponded to other R-subunit isoforms, to PKI, or to PKA substrates. Both fragments contained the consensus inhibitor site, RRGAI, from residue 94 to 98, and portions of domain A but lacked the entire B domain, consistent with the finding that domain B does not contribute to binding to the C subunit (18). Further deletion analysis identified the overlap piece, residues 94–169 (Fig. 6, A and B), as a minimum required sequence for inhibiting C in the low micromolar range. R1-(94–169) inhibited the C subunit with a \( K_i \) of 2 \( \mu \)M, an affinity that is at least 100-fold higher than the Ala-Kemptide. This minimal fragment contained the stretch of acidic residues Glu106-Glu108-Asp107 as well as Asp140 and Glu143 that were all protected from chemical modification with a water-soluble carbodiimide in the holoenzyme (13, 45). Mutagenesis confirmed that Asp140 and Glu143 were critical for binding to R. In addition, Glu143 was predicted to pair with Lys213 in the C subunit in the holoenzyme complex (46).

In addition to this minimal binding fragment, residues in the C helix of domain A were identified as being necessary not only for binding cAMP as predicted by the crystal structure but also for conveying high affinity binding to C. In cAMP binding domain A, the C helix (residues 234–260) is a bent helix with an extended turn at the C terminus that folds over and positions a critical tryptophan, Trp260, so that it stacks against the adenine ring of cAMP. We showed here that R1-(94–260) had a \( K_i \) of 5–10 nm, only 10-fold greater than the \( K_d \) of the full-length R-subunit, whereas R1-(94–235) had a \( K_i \) of 300 nm. Surprisingly, R1-(94–244) also had a \( K_i \) of 5–10 nm, similar to R1-(94–260). Thus, residues 236–244, but not 245–260, are essential for tight binding to C. This relatively short segment of the C helix, highlighted in Figs. 5 and 6, thus serves as a critical switch that regulates the circuitry of the domain such that it is in either a cAMP-bound conformation or a C-bound conformation.

This helical switch region of the C helix contains several residues that are known to be important. Arg241, for example, is critical for the cooperative binding of cAMP (12). It is part of the network that links domain A and B by forming hydrogen bonds to residues in both domains (Glu267 in domain A and Asp267 in domain B) and is conserved in every R subunit. This network allows cAMP binding domain A, which is masked in the holoenzyme, to be “opened up” in response to cAMP binding to domain B. The opposite surface of this helix faces toward the C binding site, where several residues important for binding to C are localized, in particular Asp140 and Glu143. Arg239 on the opposite surface of the C helix from Arg241 is conserved in every R1 subunit but is a Lys in every R1 subunit. This may define a

![Subdomains of cAMP binding site A.](Image)

**Fig. 7.** Subdomains of cAMP binding site A. **Left,** residues 236–244 on the C helix of cAMP binding domain A are highlighted in red on the crystal structure model of (Δ1–91)R1. The two cAMP molecules are shown in yellow, domain A is shown in dark blue, and domain B is shown in light blue. Residues 113–140, corresponding to the segment before domain A is shown in white. A close up of residues 236–244 on the C helix of cAMP binding domain A. The communicating networking among cAMP, Glu200, Arg241, and Asp267 is shown by hydrogen bonds. **Right,** residues 236–244 on the C helix of cAMP binding domain A are shown on the whole structure of (Δ1–91)R1. Structurally, residues 245–260 on the latter part of the C helix of domain A belong more to domain B then domain A.
critical difference in the interaction of the RII and RII subunits with the C subunit.

Residues that complete the C helix and form the turn that leads into domain B are indicated in Fig. 7. In this figure, it can be seen that residues 245–260 at the end of cAMP binding domain A are structurally more a part of domain B than domain A. This segment and the B domain to which it is anchored, primarily by hydrophobic contact, clearly modulate access of ATP to site A. It is the A helix of domain B that is docked directly to the A domain by two electrostatic contacts: Asp267, which pairs with Arg241 in the C helix switch, and Trp260, which stacks against cAMP bound to domain A.

As seen in Fig. 1, cAMP binding domain A is sandwiched between domain B and an additional helix that precedes the domain. As seen in Figs. 5 and 6, the β-barrel is the primary motif for binding of cAMP. This is consistent with the result that holoenzyme formed with RII(194–235) can still be activated motif for binding of cAMP. This is consistent with the result that holoenzyme formed with RI-(94–169) was cAMP-insensitive. Based on the results to undergo significant conformational changes compared with the cAMP-bound form that exists free in solution. Understand-

In conclusion, we have defined the functional boundaries of RII for cAMP binding on the molecular and structural level. Two groups of amino acids were identified that contribute to the high affinity binding of RII to C. Residues 94–169, which contain the consensus recognition sequence (Arg241–Ile266), and residues Ser236–Tyr244 on the C helix of cAMP binding domain A. These two regions are highlighted on the crystallographic coordinates these various functions.

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