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PKA-I Holoenzyme Structure Reveals a Mechanism for cAMP-Dependent Activation

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SUMMARY

Protein kinase A (PKA) holoenzyme is one of the major receptors for cyclic adenosine monophosphate (cAMP), where an extracellular stimulus is translated into a signaling response. We report here the structure of a complex between the PKA catalytic subunit and a mutant RI regulatory subunit, RIα(91–379:R333K), containing both cAMP-binding domains. Upon binding to the catalytic subunit, RI undergoes a dramatic conformational change in which the two cAMP-binding domains uncouple and wrap around the large lobe of the catalytic subunit. This large conformational reorganization reveals the concerted mechanism required to bind and inhibit the catalytic subunit. The structure also reveals a holoenzyme-specific salt bridge between two conserved residues, Glu261 and Arg366, that tethers the two adenine capping residues far from their cAMP-binding sites. Mutagenesis of these residues demonstrates their importance for PKA activation. Our structural insights, combined with the mutagenesis results, provide a molecular mechanism for the ordered and cooperative activation of PKA by cAMP.

INTRODUCTION

Cyclic adenosine monophosphate (cAMP) signaling through cAMP-dependent protein kinase A (PKA) is a ubiquitous mammalian signaling pathway conserved in all eukaryotes, with the exception of the plant phyla. While the catalytic (C) subunit has served as a prototype for the protein kinase superfamily, the regulatory (R) subunit defines the mechanism whereby the second messenger, cAMP, translates an extracellular signal into an intracellular biological response. This mechanism of cAMP regulation is conserved from bacteria to man, and the domain that recognizes cAMP is likewise universal.

The crystal structure of the catalytic subunit defined for the first time the conserved structural features of the protein kinase superfamily (Knighton et al., 1991b). The catalytic subunit is a globular bilobal protein with a highly dynamic small lobe that serves as the binding site for ATP, burying the adenine ring in a deep hydrophobic pocket and positioning the γ-phosphate for transfer to a protein substrate. The stable large lobe serves as a framework for the catalytic machinery and also as a docking scaffold for binding to protein partners that act as substrates or inhibitors (Cheng et al., 2001; Johnson et al., 2001; Knighton et al., 1991a, 1991b). The activation loop is a characteristic motif of the protein kinase family that upon phosphorylation optimizes the catalytic machinery for phosphoryl transfer (Adams et al., 1995; Nolen et al., 2004; Steinberg et al., 1993). In PKA, this loop (residues 191–197, VKGRTWT) also functions as a major binding surface for the R subunit (Kim et al., 2005).

In contrast, the R subunit is a highly dynamic and modular protein that serves as one of the major receptors for cAMP in eukaryotic cells. At the N terminus is a helical dimerization/docking (D/D) domain that interacts with scaffold proteins, referred to collectively as A-kinase anchoring proteins (AKAPs) (Kinderman et al., 2006; Newlon et al., 2001). Following this domain is a variable and flexible linker region containing an inhibitor site that docks to the active-site cleft of the C subunit. Two tandem cAMP-binding domains (domain A and domain B) lie at the C terminus. Each cAMP-binding domain consists of a β sandwich and a noncontiguous helical subdomain. Among the four known protein families that bind cyclic nucleotides (PKA/PKG, catabolite activator protein [CAP], hyperpolarization-activated cyclic nucleotide-modulated channel [HCN], and exchange protein directly activated by cAMP [EPAC]), the most conserved feature of each domain is the phosphate-binding cassette (PBC), a helix-loop region where cAMP binds. The C subunit is locked in a dormant state in the absence of cAMP through

formation of a holoenzyme inhibitory complex, where the R subunit dimer binds to two C subunits. Binding of cAMP to the R subunit unleashes the catalytic subunit, thereby allowing phosphorylation of PKA substrates. There are two major classes of R subunits, RI and RII, which are functionally nonredundant; within these classes are α and β subtypes (RIα, RIβ, RIIα, and RIIβ) (Brandon et al., 1997).

While crystal structures for separate catalytic and regulatory subunits of PKA are known, understanding the molecular features of this universal signaling pathway requires a structure of the holoenzyme complex. The recent structure of the C subunit bound to a deletion mutant of RIα containing only domain A provides clues to the dramatic conformational switch that the R subunit must undergo to release the C subunit and bind cAMP (Kim et al., 2005). This complex, however, lacked the second cAMP-binding domain, which is crucial for allosteric activation of PKA by cAMP. Activation of the type I holoenzyme by cAMP is a highly ordered process. In holoenzyme complexes with R subunits containing both cAMP-binding domains, domain A is inaccessible until cAMP occupies domain B (Herberg et al., 1996; Ogreid and Doskeland, 1981a, 1981b). This obligatory activation pathway led to the designation of domain B as a "gatekeeper" for domain A.

Here we report a holoenzyme structure that contains both cAMP-binding domains of RIα (domain A, residues 123–259, and domain B, residues 260–379). The complex reveals an extended R/C interface that protects sites in the C subunit essential for catalysis and substrate binding. It shows an extended interface surrounding the activation loop and defines a novel interaction site between domain B in RIα and an S-shaped loop (residues 276–286) on the large lobe of the catalytic subunit (subsequently referred to as the αH-αI loop). The structure also shows local conformational changes within the helical regions as well as dramatic global rearrangement of the two cAMP-binding domains as the R subunit wraps around the large lobe of the catalytic subunit. Finally, this new structure reveals a highly conserved holoenzyme-specific salt bridge formed in domain B involving two residues (Glu261 and Arg366) that are solvent exposed in the cAMP-bound conformation. In the holoenzyme, this salt bridge tethers the two adenine capping residues (Trp260 and Tyr371). The importance of this salt bridge and the two capping residues in facilitating PKA activation is confirmed by mutagenesis. Taken together, the molecular features revealed by this structure allow understanding of the communication pathway between the two cAMP-binding domains and provide a mechanism for the ordered and cooperative activation of PKA by cAMP.

RESULTS AND DISCUSSION

Structural Overview of the RIα(91–379):C Complex

The holoenzyme crystal structure of a mutant RIα(91–379:R333K) in complex with the C subunit, AMP-PNP, and two Mn^{2+} ions was solved to 2.3 Å resolution using the RIα(91–244):C complex as a molecular replacement probe (Kim et al., 2005). We attempted to crystallize three deletion mutants of RIα (RIα(91–379), RIα(91–379:R209K), and RIα(91–379:R333K)) in complex with the C subunit (for rationale, see the Supplemental Results and Discussion in the Supplemental Data available with this article online), but only the holoenzyme formed with RIα(91–379:R333K) (subsequently referred to as RIα*) produced crystals that diffracted. The RIα*:C structure was crystallized in a $P3_221$ space group with 73% solvent and shows minimum contact between symmetrically related molecules (Table S1). The surface area on the catalytic subunit that is masked by binding of RIα is approximately 3800 $Å^2$ (Figure 1). As with the previous RIα(91–244) holoenzyme structure, the C subunit adopts a closed conformation with its active site bound to AMP-PNP, two Mn^{2+}ions, and the inhibitor site of the R subunit. The previous RIα(91–244):C structure showed major reorganization of domain A upon binding to the C subunit (Kim et al., 2005). Our new holoenzyme structure defines the full extent of the conformational change in the R subunit that must occur to accommodate the C subunit. As RIα adopts an extended dumbbell shape that complements the large lobe of the C subunit, the two cAMP-binding domains become uncoupled. The largest binding interface lies between the C subunit and domain A of the R subunit, while domain B extends the interaction surface and makes a contact to the αH-αI loop in the C subunit.

Domain B of the Regulatory Subunit Provides an Additional Docking Surface for the Activation Loop and Presents a Novel Interaction Site on the Catalytic Subunit

As defined previously (Kim et al., 2005), an extended surface on the C subunit is utilized for binding to the R subunit: (1) site 1, the predominantly acidic active site (Figure S1); (2) site 2, the substrate binding loop (P+1 loop, residues 198–205) and the hydrophobic αG helix (Figure S2); and (3) site 3, the activation loop (Figure 2B). The new RIα*:C structure reveals expanded interaction surfaces at sites 1 and 3 and defines a fourth site, the αH-αI loop, that interacts uniquely with domain B in the R subunit (Figure 2C), supporting previous hydrogen/deuterium exchange data (Anand et al., 2003). We first discuss new features of site 3 that are revealed by the structure and then describe site 4. Additional information regarding sites 1 and 2 can be found in the Supplemental Data.

At site 3, separation of the RIα cAMP-binding domains provides an additional docking surface that fully encloses the activation loop of the C subunit within the R/C interface (Figures 2A and 2B). The previous RIα(91–244):C complex lacking domain B showed a dramatic extension of the αB/C helix in domain A, which docks against the activation loop and masks the region extending from the P+1 loop to the activation loop (Kim et al., 2005). In the RIα*:C structure, the activation loop is now completely enclosed within the R/C interface and is sandwiched between the two cAMP-binding domains. The additional docking

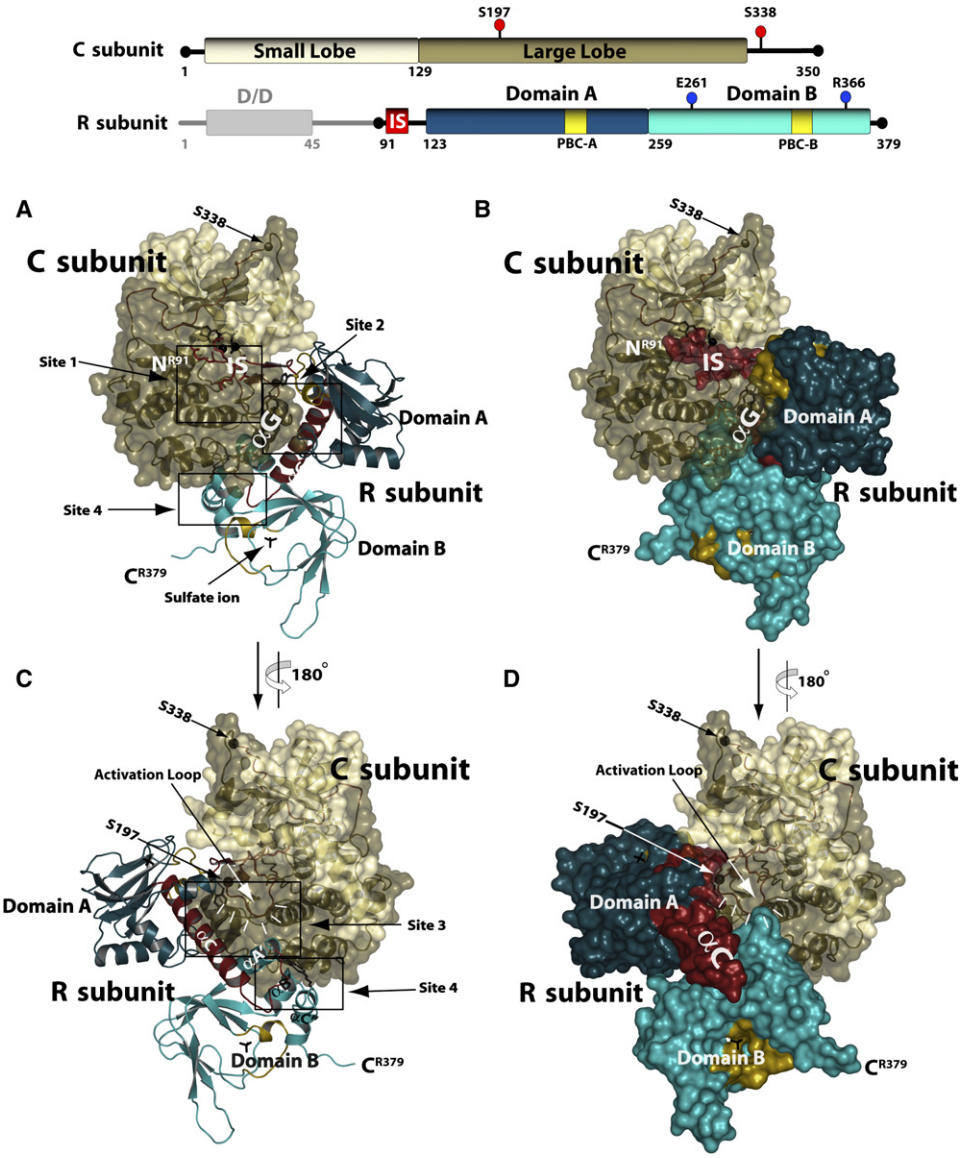


Figure 1. Overview of the PKA RIα(91–379):C Holoenzyme Complex

Top: domain organization of the catalytic and regulatory subunits. The two red spheres indicate the phosphorylation sites Thr197^{C} and Ser338^{C} in the catalytic subunit.

(A and C) (A) shows a view of the inhibitor sequence of the regulatory subunit bound to the active-site cleft of the catalytic subunit. Boxed regions indicate interaction sites between the R and C subunits at the active site (site 1, left) and the αG helix (site 2, right). (C) shows a 180° rotation of the view in (A). Boxed regions indicate the interaction site at the activation loop (site 3, top) and αH-αI loop (site 4, bottom). The regulatory subunit is shown as a cartoon representation with domain A in dark teal, domain B in cyan, the phosphate-binding cassette (PBC) in yellow, and the αB/C helix and inhibitor site in dark red.

(B and D) Surface representation of both subunits in the same view as in (A) and (C), respectively. The catalytic subunit is bound to AMP-PNP (black sticks) and Mn^{2+} (blue spheres) with the small lobe (light tan) and the large lobe (dark tan) in surface rendering.

surface arises from complete extension of the αB/C helix from residues 226 to 250 through to the αA helix in domain B. The entire extension was not observed in the previous structure since the RIα construct terminated at residue 244. Strikingly, the residue used to cap the cAMP-binding site in domain A, Trp260^{R} (hereafter, residues in the regulatory subunit are followed by a superscript R, while residues in the catalytic subunit are followed by a superscript C), packs against the N-terminal tip of the activation loop (Figure 2B, right). As described later, binding of the C subunit causes Trp260^{R} to move nearly 30 Å away from the PBC in domain A.

In addition to the first three sites that surround the C subunit active site, the RIα*:C structure reveals a fourth

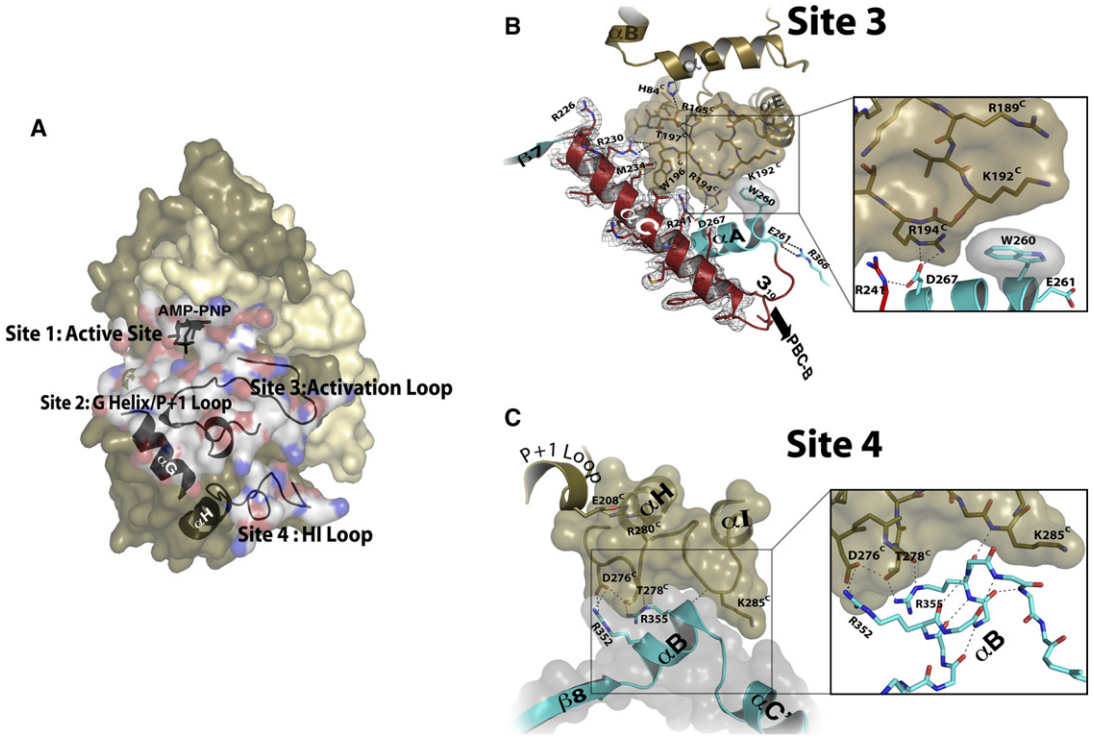


Figure 2. The Regulatory Subunit Provides a Large Docking Surface that Shields the Catalytic Machinery of the C Subunit

(A) Individual sites are mapped on the catalytic subunit, with specific binding regions rendered in cartoon.

(B) Site 3: the activation loop in the catalytic subunit (tan) interacts with the αB/C helix (gold), 3_{10} loop (maroon), and αA helix (cyan). This region of the regulatory subunit is stabilized by a key salt bridge between $Arg241^R$ (in the αC helix of domain A) and $Asp267^R$ (in the αA helix of domain B) and is maintained in both the holoenzyme and cAMP-bound conformations. $Asp267^R$ also interacts with $Arg194^C$, in effect cementing domains A and B of the regulatory subunit to the catalytic subunit activation loop. The $Trp260^R$:$Lys285^C$:$Asn283^C$ hydrophobic stack and the $Arg241^R$:$Asp267^R$:$Arg194^C$ interaction are shown at right.

(C) Site 4: the site between the αB helix of domain B and the αH-αI loop of the catalytic subunit (tan), which contains a segment unique to AGC kinases. A detailed view of the hydrogen bond network between the αB helix in domain B (sticks) and the catalytic subunit is shown at right.

distal site, formed exclusively between domain B of the R subunit and the large lobe of the C subunit. Site 4 consists of the αH-αI loop on the large lobe of the C subunit (residues 276–286) that docks to the αB helix of the R subunit (Figures 2A and 2C). A short segment within the αH-αI loop (residues 282–286) has been found to be an AGC kinase-specific insert (Kannan et al., 2007), and mutagenesis studies suggest that this is an allosteric site that is coupled to peptide recognition (Deminoff et al., 2006). Furthermore, $Arg355^R$ in the R subunit αB helix forms multiple interactions with the catalytic subunit; participation of this residue is noteworthy as it is conserved in both RI and RII isoforms and is likely to be a hotspot for protein:protein interactions for cAMP-binding proteins in general.

Major Conformational Changes Occur in RIα Upon Binding the Catalytic Subunit

Global Changes

The RIα*:C structure containing both cAMP-binding domains shows the major conformational change in the R subunit that must occur to enable binding to the catalytic subunit. In the cAMP-bound conformation, the two cAMP-binding domains, joined by the kinked αB/C helix in domain A, form a compact globular structure where the two domains pack together with a large interface (Figure 3A) (Su et al., 1995). The αB/C helix is anchored to domain B through hydrophobic interactions and is directly linked to $Trp260^R$, the capping residue that stacks with cAMP in domain A. However, upon binding to the C subunit, the two domains separate and the R subunit adopts an extended dumbbell shape. The center of domain B moves over 60 Å away from its position in the cAMP-bound structure due to extension of the αB/C helix. The interface shared between the domains in the cAMP-bound structure is replaced by the C subunit in the holoenzyme. This large domain movement is rarely seen in proteins and arises from rotations at three pivot points along the αB/C helix (Figure 3B).

Each cAMP-binding domain is comprised of two subdomains: a noncontiguous α-helical subdomain and a contiguous β sheet subdomain that contains the PBC. Superposition of the two conformational states of RIα (holoenzyme and cAMP-bound) shows that the conformation of the β sandwich, with the exception of the PBC, does not

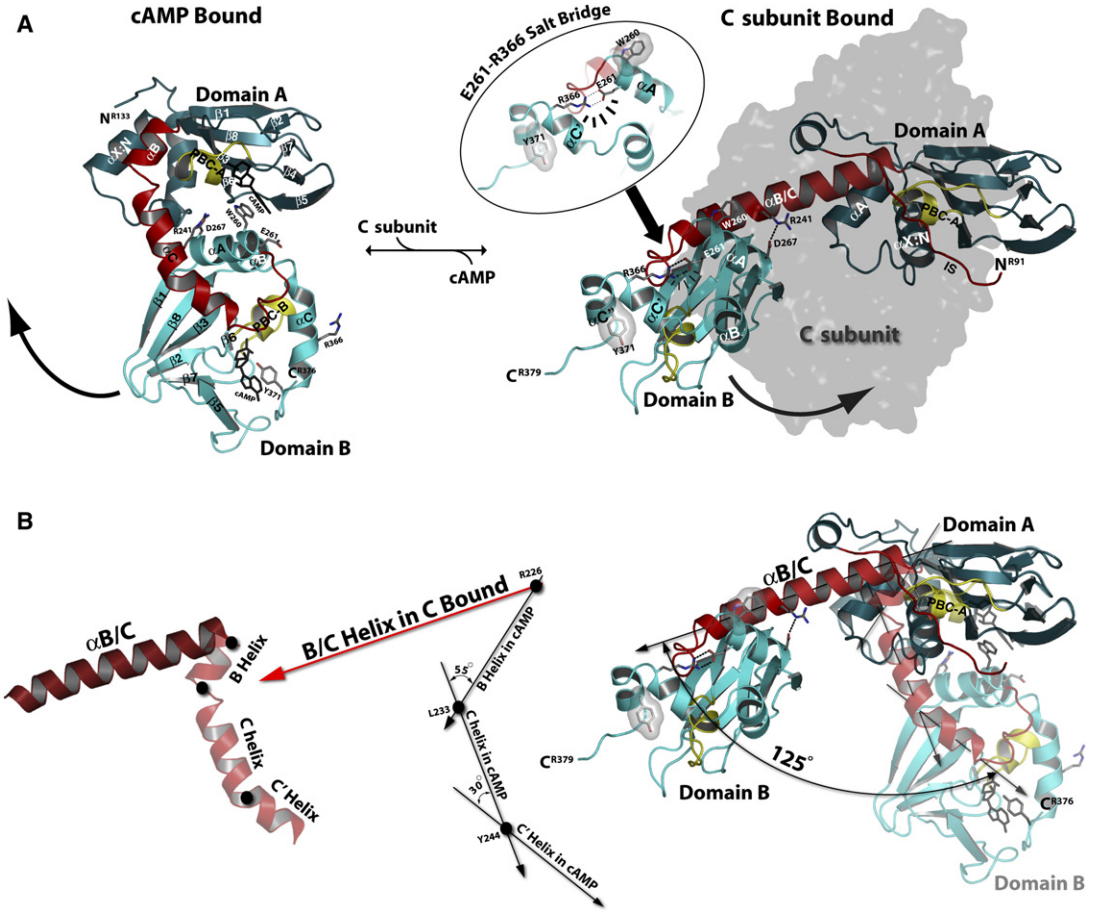


Figure 3. RIα Undergoes Dramatic Conformational Changes Upon Binding the Catalytic Subunit

(A) The regulatory subunit bound to cAMP is shown on the left (PDB ID code 1RGS, in black) and bound to the C subunit (in gray) on the right. The two hydrophobic capping residues important for cAMP binding to the regulatory subunit, $Trp260^R$ and $Tyr371^R$, are shown with a van der Waals surface. (B) Left: the global extension of the regulatory subunit is described by rotations at three pivot points (Arg226, Gly235, and Tyr244) located on the αB/C helix. Right: structural alignment of domain A in the cAMP and catalytic subunit-bound conformations.

change (Figure 4). Removal of cAMP from the cAMP-bound complex creates a more dynamic structure but does not stabilize the open and extended conformation of the αB/C helix (Gullingsrud et al., 2006; Vigil et al., 2006). Instead, the C subunit induces the major conformational change that RIα undergoes, and these changes are associated primarily with the helical regions (Figure 4). For each domain in the R subunit, this includes, in addition to the major changes in the αB/C helices discussed above, reorganization of the PBC and a conserved structural element that bridges the αX:N and αA helices in domain A (residues 123–150) and the αC helix of domain A with the αA helix of domain B (residues 245–267). We define this structural element as the N3A motif (Figure 5; Figure S6). A major consequence of the conformational changes in the R subunit, induced by binding of the catalytic subunit, is that both cAMP-binding sites are essentially destroyed because the phosphate-binding pocket is separated from the adenine-binding pocket.

Local Changes in Domain A

Figure 6 shows the region where the helical motifs (the αB/C helix, PBC, and N3A motif) converge in the holoenzyme structure. For domain A in the cAMP-bound state, the αB/C helix separates the surface formed between the PBC and N3A motif (Figure 6A, left). In the holoenzyme conformation, recruitment of the αB/C helix to the R/C interface and the associated conformational changes allow the remaining helices to reposition (Figure 6A, right). Essentially, the PBC and N3A motif move closer together to create a holoenzyme-specific hydrophobic surface in domain A that then docks onto the hydrophobic surface on the C subunit.

Local Changes in Domain B

The hydrophobic rearrangement associated with the helical regions in domain B is analogous to domain A, except that in domain A, the PBC and the extended αB/C helix are an integral part of the R/C interface (Figure 6B). In contrast to domain A, the PBC in domain B is solvent exposed due to the hydrophobic rearrangement and the docking of αB

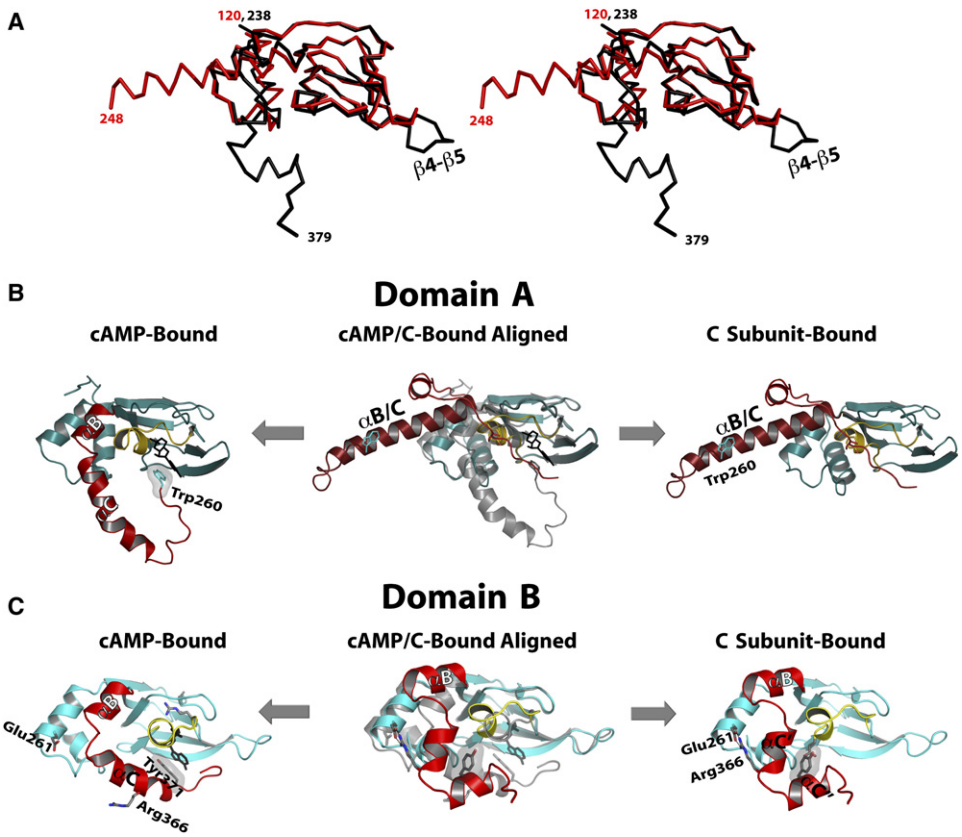


Figure 4. Conformational Changes in the Regulatory Subunit Are a Result of Structural Rearrangements in the Helical Regions

(A) Structural alignment of the regulatory subunit cAMP-binding domains in the holoenzyme conformation. Domains A and B are shown in red and black, respectively. Ninety-two equivalent Cα atoms from the β barrel region overlap with a root-mean-square deviation of 1.1 Å, excluding a short insert between β4-β5.

(B and C) Comparisons between the two cAMP-binding domains in the cAMP and catalytic subunit-bound conformations. The cAMP-bound conformation is shown on the left, and the holoenzyme conformation is shown on the right. The two conformations are superimposed in the center. The αB/C helix is shown in red, and the PBC in yellow. In the two center panels, the cAMP-bound conformation is shown in gray.

helix in domain B to the C subunit (Figure 1). The highly accessible cAMP-binding site in domain B observed in our structure explains kinetic studies showing domain B as the fast association site for cAMP in holoenzyme (Ogreid and Doskeland, 1981a).

Comparison of domains A and B shows that the αB and αC helices do not extend in domain B as they do in domain A. Instead, they remain as distinct helices and form a helix-turn-helix motif that covers the hydrophobic surface (Figure 6C). This helix-turn-helix motif provides the hydrophobic lid (from $Tyr371^R$) for the PBC in the cAMP-bound state.

Residues Required for Stabilizing cAMP in the Regulatory Subunit Are Trapped at a Remote Site in the Holoenzyme Structure

The extended conformation of RIα in the holoenzyme not only partitions the two cAMP-binding domains but also separates many of the key residues that anchor cAMP in the PBC, effectively destroying both cAMP-binding sites. A common feature for cAMP-binding proteins is hydrophobic capping of the cAMP adenine ring (Berman et al., 2005). For CAP, HCN, and domain B of RIα, the hydrophobic capping residue is located in the αC helix of the cAMP-binding domain. For domain A of RIα, the capping residue is $Trp260^R$, located at the beginning of the αA helix of domain B. Thus, for RIα, both capping residues ($Trp260^R$ for domain A and $Tyr371^R$ for domain B) are in domain B (Figure 3A).

In the holoenzyme structure, the capping residues are far removed from their respective PBCs. $Trp260^R$ moves over 30 Å and docks onto the C subunit activation loop (Figure 2B). $Trp260^R$, the only residue from domain B that binds directly to cAMP in domain A, is important for communication between the two cAMP-binding domains (Canaves et al., 2000). As illustrated in Figure 6B, $Tyr371^R$ in the cAMP-bound state has a dual role—aromatic stacking with the adenine base and hydrogen bonding to the conserved $Glu324^R$ in the PBC, which binds to the 2′OH of the ribose ring of cAMP. Mutational studies confirm the importance of this residue for cAMP binding (Bubis et al., 1988a, 1988b; Kapphahn and Shabb, 1997). In

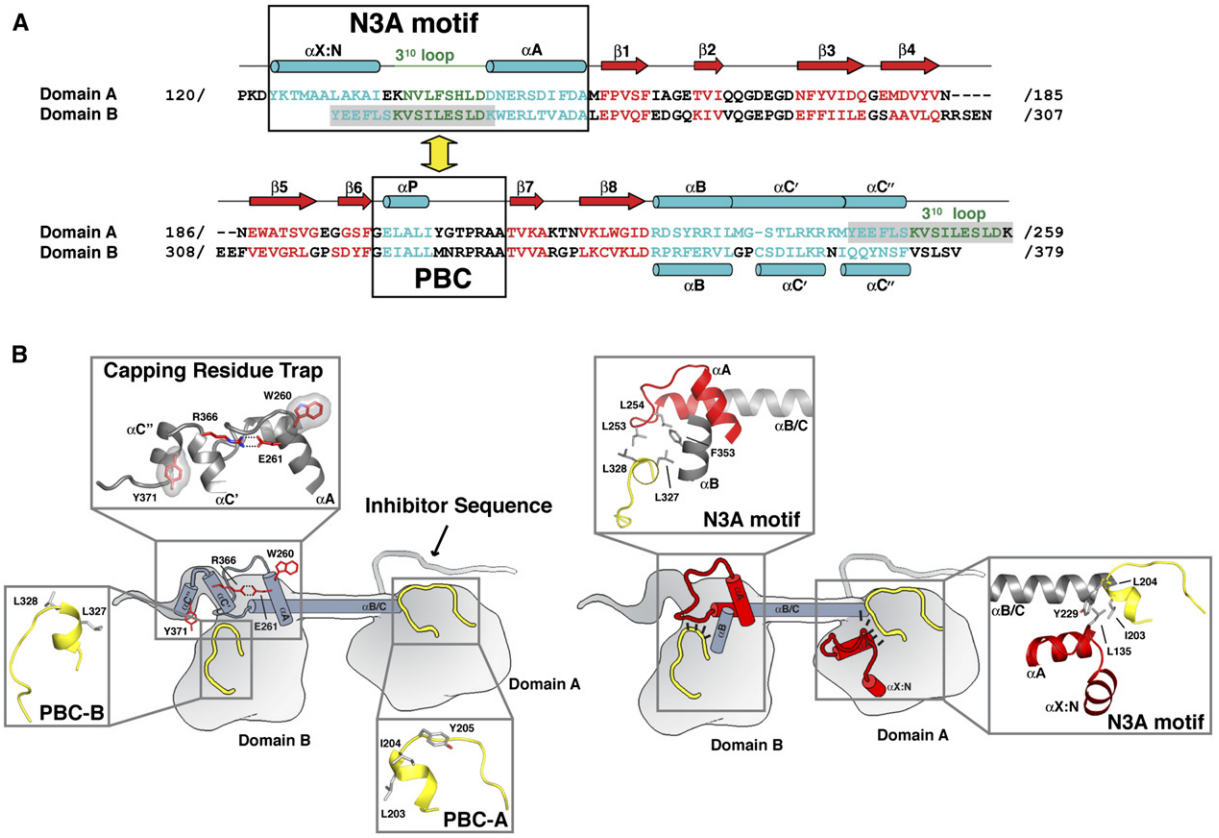


Figure 5. Schematic Diagram of the Structural Motifs in the Regulatory Subunit

(A) Sequence alignment of domains A and B. Residues in the gray boxes belong to domain A but are also aligned as part of the N3A motif of domain B.

(B) Cartoon schematic of the major structural elements of the regulatory subunit in the holoenzyme conformation.

contrast, in the holoenzyme conformation, $Tyr371^R$ is 13 Å away from the PBC. Thus, binding of the catalytic subunit to RIα prohibits many interactions that are needed to stabilize the cAMP-bound structure (see Supplemental Data for additional sites).

The Glu261-Arg366 Salt Bridge Functions to Trap the Two cAMP Capping Residues

The holoenzyme structure reveals a salt bridge formed between $Glu261^R$ and $Arg366^R$. These two residues not only position the RIα C-terminal tail but also sequester the two adenine capping residues ($Trp260^R$ and $Tyr371^R$) away from their cAMP-binding sites. In the cAMP-bound conformation, both of these highly conserved salt bridge residues are 15 Å apart where $Arg366^R$ is exposed to solvent and $Glu261^R$ is near the domain interface. It is only in the holoenzyme conformation that their true function can be appreciated. In effect, the salt bridge traps both hydrophobic residues far away from the cAMP-binding sites and forms a communication path that links the two R subunit cAMP-binding domains. Our holoenzyme structure provides a molecular model explaining the ordered and highly cooperative pathway for the activation of the type I holoenzyme. The biochemical details for this model were first proposed based on kinetic arguments (Ogreid and Doskeland, 1981a, 1981b) and were then confirmed with mutants of the essential arginine residues in the cAMP-binding pocket ($Arg209^R$ for domain A and $Arg333^R$ for domain B) (Herberg et al., 1996).

To test this model and the contribution of the electrostatic trapping of the capping residues, we engineered four RIα(91–379) mutants (W260A, Y371A, E261A, and R366A) and measured the effect of these mutations on PKA activation. For each mutant, the inhibition of the C subunit was not affected (Figure S4). In contrast, there are differences in cAMP-mediated activation of PKA, as measured by a catalytic coupled assay (Cook et al., 1982) and a fluorescence polarization binding assay (Saldanha et al., 2006). Holoenzyme complexes formed with RIα mutants that contain a substitution of either $Trp260^R$ or $Tyr371^R$ with alanine were less sensitive to cAMP compared to the RIα(91–379) holoenzyme. RIα(91–379:W260A) requires 4.6-fold more cAMP, while RIα(91–379:Y371A) requires 9-fold more cAMP (Figure 7A; Figure S5). The difference for the W260A mutation can be attributed to the missing hydrophobic capping abilities of the aromatic side chain. The larger difference observed for the Y371A mutation is most likely due to the absence of

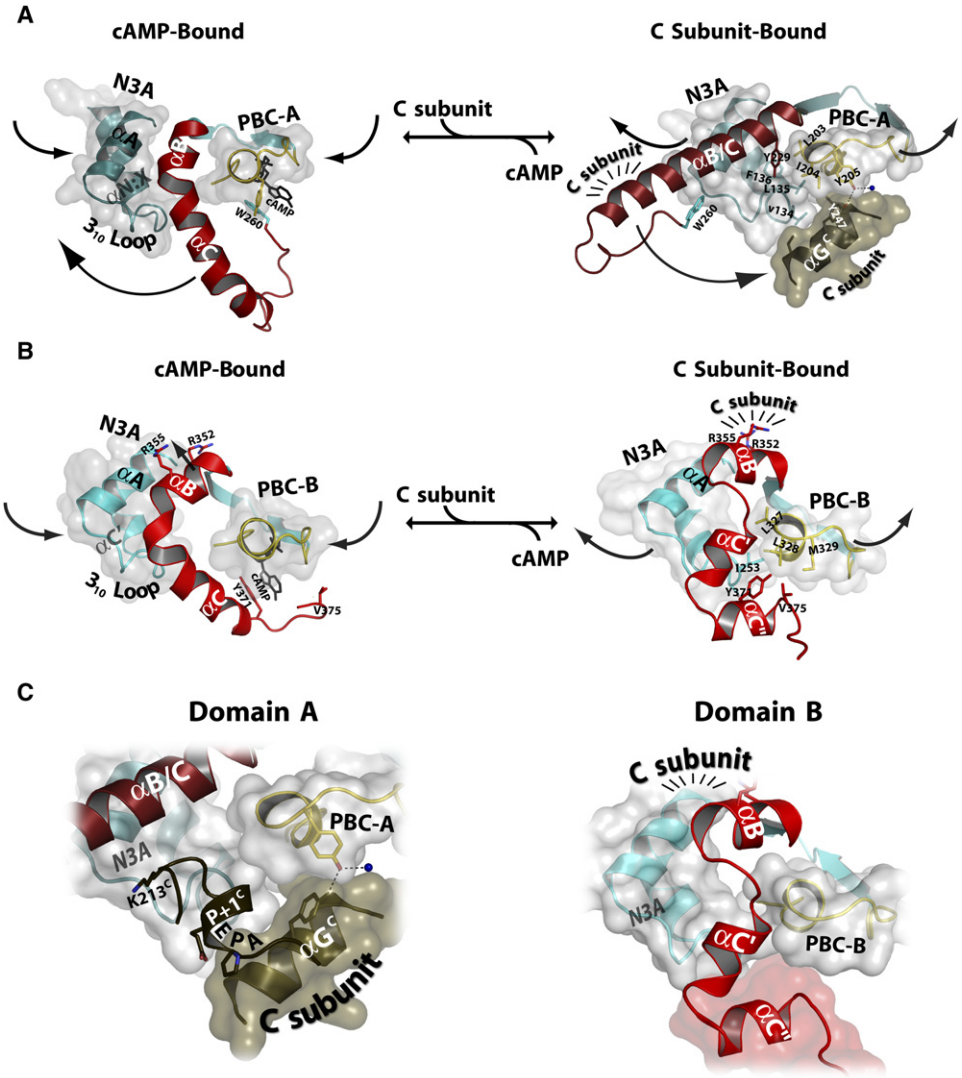


Figure 6. Binding of the Catalytic Subunit Reorganizes the N3A Motif and the Phosphate-Binding Cassette in the Regulatory Subunit to Create a Contiguous Hydrophobic Interface

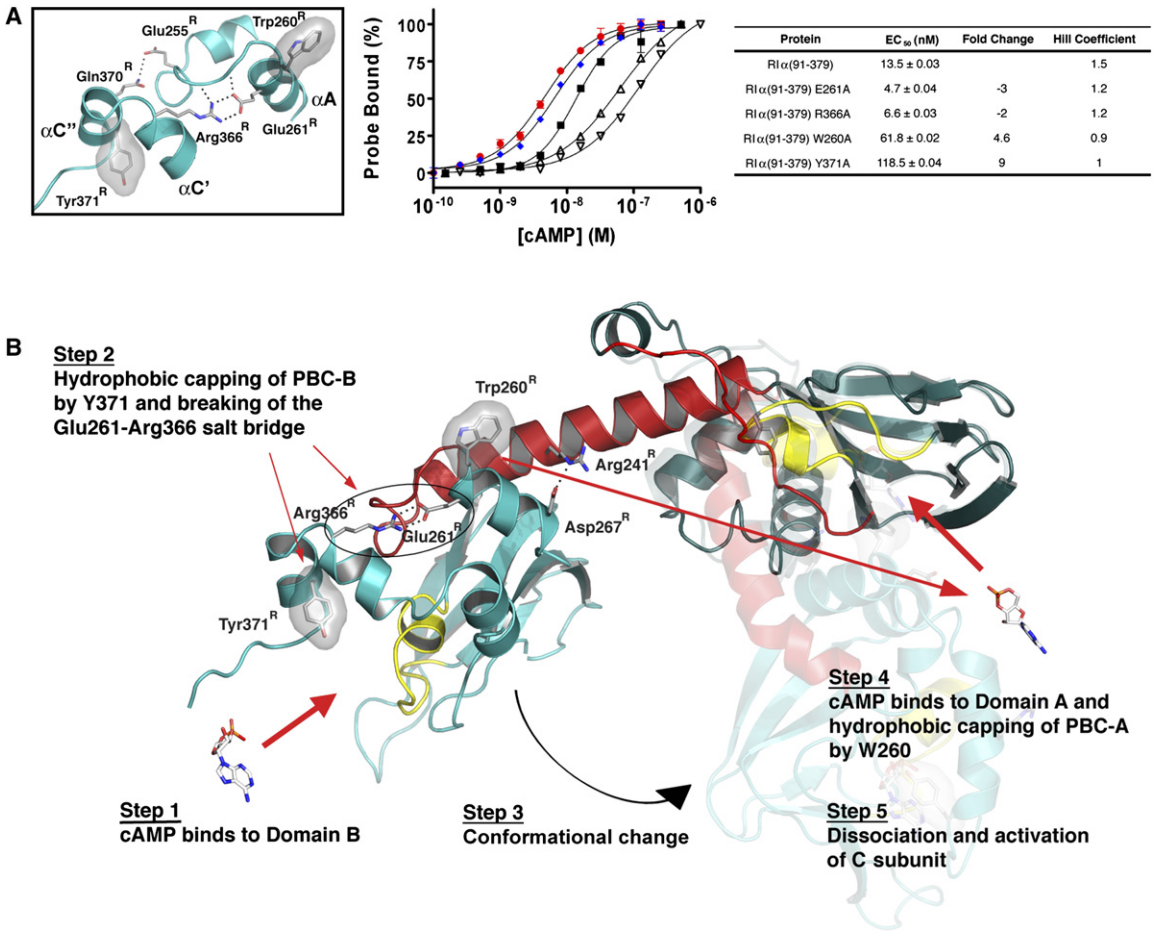
(A) Comparison of the helical regions in domain A between the cAMP (left) and catalytic subunit-bound (right) conformations. Movement of the helical regions is mediated by hydrophobic rearrangement of the hinge residues in the PBC (Ile203^R and Leu204^R), αB helix (Tyr229^R), and 3_{10} loop (Leu135^R).

(B) Comparison of domain B in the cAMP and catalytic subunit-bound conformations, highlighting the C-terminal tail (red). In domain B, the helical rearrangements are similar to domain A where residues in the PBC (Leu327^R and Leu328^R), αB helix (Phe353^R), and 3_{10} loop (Ile253^R and Leu254^R) come together.

(C) Comparison between domains A and B in the holoenzyme conformation. In domain A, the N3A motif (residues 123–150) and PBC come together and serve as a docking surface for the P+1 loop (black) and the αG helix (dark tan) of the catalytic subunit. In domain B, a similar hydrophobic interface is formed between the N3A motif (residues 245–367) and PBC; however, the C-terminal tail (αB, αC′, and αC″ helices) lies on top of the hydrophobic interface.

both the aromatic cap and hydrogen bond, which together help stabilize cAMP in domain B.

In contrast, holoenzyme complexes formed with RIα mutants that contain a substitution of either Glu261^R or Arg366^R with alanine were more sensitive to cAMP activation. The EC_{50} decreased from 13.5 nM for RIα(91–379) to 4.7 and 6.6 nM for RIα(91–379:E261A) and RIα(91–379:R366A) mutants, respectively. The differences in EC_{50} values for these salt bridge-deficient mutants are likely to be greater than 3-fold since 10 nM C subunit was used in our assays. Nevertheless, these results conclusively show that disrupting the salt bridge makes the holoenzyme more sensitive to cAMP and shifts the equilibrium toward a more "activation-prone" state. Not only are Glu261^R and Arg366^R conserved in all regulatory subunit isoforms, their homologous counterparts in domain A



Protein	EC_{50} (nM)	Fold Change	Hill Coefficient
RIα(91-379)	13.5 ± 0.03		1.5
RIα(91-379) E261A	4.7 ± 0.04	-3	1.2
RIα(91-379) R366A	6.6 ± 0.03	-2	1.2
RIα(91-379) W260A	61.8 ± 0.02	4.6	0.9
RIα(91-379) Y371A	118.5 ± 0.04	9	1

Figure 7. A RIα Electrostatic Interaction in the Holoenzyme Conformation Functions as a "Capping Residue Trap" Important for PKA Activation

(A) Left: the salt bridge between $Glu261^R$ and $Arg366^R$ structurally couples the two hydrophobic capping residues for domain A and domain B, $Trp260^R$ and $Tyr371^R$, respectively. Center: the effect of RIα(91–379) (black squares), RIα(91–379:W260A) (upward-pointing triangles), RIα(91–379:Y371A) (downward-pointing triangles), RIα(91–379:E261A) (red circles), and RIα(91–379:R366A) (blue diamonds) on PKA activation by cAMP measured by the fluorescence polarization assay. Right: fold changes are given relative to RIα(91–379) data. Binding curves were fit using GraphPad Prism 4 software; error bars indicate the standard error of the mean.

(B) Stepwise model of PKA activation by cAMP.

($Glu143^R$ and $Arg241^R$, respectively) provide equally important contributions to the molecular architecture of the holoenzyme (Figure S6).

The highly cooperative interaction between the two tandem cAMP-binding domains of RIα allows the enzyme to respond rapidly to the second messenger cAMP. Data from our study and others suggest that several factors contribute to this cooperative process. Comparison of the Hill coefficients in the cAMP activation data for both capping residue mutants relative to wild-type RIα suggests that these residues play an important role in the cooperative cAMP activation process. The Hill coefficient was reduced significantly from 1.5 for wild-type to 0.9 and 1 for W260A and Y371A, respectively (Figure 7A). Mutations that remove the salt bridge between Glu261 and Arg366 also show reductions in the Hill coefficient (1.2 for both E261A and R366A), but the protein concentrations used in our assays may have limited our ability to determine true Hill coefficients since titration effects will also influence these values. Furthermore, previous studies show that $Arg241^R$, which mediates a salt bridge between domain A ($Arg241^R$) and domain B ($Asp267^R$) (Figure 3A; Figure S6), not only disrupts high-affinity cAMP binding but also plays an important role in the cooperative coupling between the two domains (Symcox et al., 1994). In light of both our data and others, it is apparent that cooperativity involves not just one residue, but a number of residues that all contribute to the activation process in a synergistic way.

Model of PKA Activation by cAMP

Previous biochemical data proposed an ordered and sequential pathway of cAMP binding to the type Iα

holoenzyme in which cAMP must first bind to domain B and then to domain A (Herberg et al., 1996). Our structural and mutagenesis data together provide corroboration for this mechanism and allow us to propose a molecular explanation for the highly ordered pathway for activation by cAMP in which domain B serves as a "gatekeeper" for cAMP access to domain A (Figure 7B).

- *Step 1:* cAMP first binds to the PBC in domain B. The PBC in domain B is more accessible than in domain A. The cAMP-binding site in domain A is masked by the R/C interface so that Trp260^R and Arg241^R, key residues that stabilize cAMP binding, are not accessible. In fact, Trp260^R, the hydrophobic capping residue for domain A, not only is 30 Å from the PBC in domain A but also is docked to the activation loop of the C subunit (Figure 2B). Furthermore, the PBC in domain A is partially occluded by the C subunit at the site 2 interface (Figure 1; Figure S2). Specifically, Tyr247^C (in the αG helix of the C subunit) hydrogen bonds to Tyr205^R, and the two subunits are docked through a hydrophobic interface at this site. These structural details are consistent with studies that find domain B to be the fast association site for cAMP in holoenzyme (Ogreid and Doskeland, 1981a).
- *Step 2:* We predict that recruitment of the C-terminal tail to stabilize cAMP in the PBC of domain B will disrupt the Glu261^R-Arg366^R salt bridge. Both our mutational studies and others (Kapphahn and Shabb, 1997) show Tyr371^R to be a critical element that influences PKA activation by cAMP. Mutation of Tyr371^R to alanine results in a 9-fold increase in the level of cAMP needed to activate PKA compared to wild-type RIα, presumably due to removal of the hydrophobic and hydrogen-bonding capabilities of this residue. In addition, single point mutations of either Glu261^R or Arg366^R that disrupt the salt bridge require 3-fold less cAMP to activate PKA, suggesting that positioning of the C-terminal tail is destabilized in the absence of the salt bridge.
- *Step 3:* The R subunit undergoes a large conformational change in response to uncoupling the Glu261^R-Arg366^R salt bridge. Breaking the salt bridge also releases Trp260^R, the capping residue for cAMP binding in domain A. Several observations support this idea. First, as seen in Figure 2C, the αB helix in domain B interacts with the αH-αI loop of C. The movement of the C-terminal tail toward the PBC in domain B weakens the interaction between the C subunit and domain B, thereby facilitating the conformational change. Second, in the holoenzyme complex, Trp260^R is buried in the R/C interface. Since the Glu261^R-Arg366^R interaction structurally couples the two hydrophobic capping residues, Trp260^R and Tyr371^R, docking of cAMP to domain B breaks the salt bridge and pulls Trp260^R away from the C subunit activation loop. These motions collectively destabilize the extended αB/C helix, and the concerted motions of domain B bring Trp260^R toward the PBC in domain A.
- *Step 4:* Binding of a second molecule of cAMP to the PBC in domain A is stabilized by Trp260^R. Mutation of Trp260^R to alanine showed a 4.6-fold decrease in cAMP sensitivity for PKA activation. It is apparent in our holoenzyme structure that a second cAMP molecule can only bind to the PBC in domain A if this domain is dislodged from the C subunit. It remains to be established whether the C subunit dissociates from the pseudosubstrate site in the R subunit before or after trapping cAMP in domain A, or whether these steps are coordinated.
- *Step 5:* In the final step, release of the C subunit from the inhibitor site of the R subunit leads to activation of PKA.

Conclusion

In this report, we describe the structure of the PKA catalytic subunit bound to a deletion mutant of RIα containing both cAMP-binding domains. The structure demonstrates the exceptional mobility of the cAMP-binding domains in RIα and confirms that there is a large movement of domain B relative to domain A as the R subunit shuttles between its binding partners, namely the catalytic subunit and cAMP. The conversion of the globular conformation of the cAMP-bound structure into a dumbbell-shaped holoenzyme complex, in which the two cAMP-binding domains are separated, is mediated by extension of the αB/C helix of domain A. The RIα*:C structure also shows that the αB and αC helices in domain B are equally dynamic, but their conformations are very different from the αB/C helices in domain A. When bound to the C subunit, RIα utilizes a unique set of residues that stabilize the C subunit-bound conformation without directly participating in the R/C interaction. We show through mutagenesis that a conserved salt bridge plays a significant role in cAMP activation of PKA, most likely by trapping the two adenine capping residues in RIα away from their cAMP-binding sites. These hydrophobic capping residues also contribute to the cooperative activation of the enzyme through cAMP. These data provide for the first time a molecular explanation for the highly ordered pathway whereby binding of cAMP to domain B leads to the eventual activation of kinase activity.

EXPERIMENTAL PROCEDURES

Protein Preparation

The catalytic subunit was expressed and purified in *E. coli* as described previously (Gangal et al., 1998). For crystallography, three RIα mutants (RIα(91–379), RIα(91–379:R209K), and RIα(91–379:R333K)) were generated by QuikChange site-directed mutagenesis according to the Stratagene protocol. These mutants lacked the N-terminal dimerization/docking domain (residues 1–90). The essential arginine in the PBC of each cAMP-binding domain was also mutated, Arg209 in domain A and Arg333 in domain B. Four additional mutants (RIα(91–379:E261A), RIα(91–379:R366A), RIα(91–379:W260A), and RIα(91–379:Y371A))

were generated by QuikChange mutagenesis for biochemical analysis. All RIα mutants were expressed in *E. coli* BL21 (DE3) cells (Novagen) and purified as described previously (Su et al., 1995; Wu et al., 2004).

Holoenzyme Formation for Crystallography

Three RIα mutants (RIα(91–379), RIα(91–379:R209K), and RIα(91–379:R333K)) were mixed with wild-type C subunit in a 1:1.2 molar ratio and dialyzed by concentration at 4°C in 10 mM MOPS (pH 7.0), 2 mM $MnCl_2$, 50 mM NaCl, 2 mM EDTA and EGTA, 1 mM TCEP-HCl, 0.2 mM AMP-PNP, and 10% glycerol. Holoenzyme was separated from excess C subunit by gel filtration chromatography as described previously (Wu et al., 2004).

Crystallization and Data Collection

The RIα(91–379:R333K):C complex was crystallized at 25°C in hanging drops using the vapor diffusion method in 2.0 M $(NH_4)_2SO_4$, 0.1 M citrate (pH 5.5). The crystals were transferred to a cryoprotectant solution (mother liquor containing 20% glycerol) and flash cooled in liquid nitrogen. X-ray diffraction data were collected at the SER-CAT insertion device beamline 19ID (Advanced Photon Source, Argonne National Laboratory, Argonne, IL, USA) on SBC2 3k × 3k CCD (ANL). Diffraction data were processed and scaled using HKL2000 (Otwinowski and Minor, 1997). Initial indexing clearly indicated a primitive hexagonal lattice without any ambiguity (distortion index 0.12%). The final data were integrated and scaled in $P3_221$ (a = b = 125.9 Å, c = 141.0 Å) with satisfactory statistics. Data processing statistics are presented in Table S1.

Structure Determination and Refinement

Initial phases of the RIα(91–379:R333K):C complex were generated by molecular replacement using the RIα(91–244):C complex (Protein Data Bank ID code 1U7E) (Kim et al., 2005) as a search model in Phaser (Storoni et al., 2004). Although our initial solvent content analysis predicted that there would be two molecules per asymmetric unit (V_M = 2.2 Å^3/dalton), a Phaser run in single-model mode unambiguously found only one molecule (Z score 24-60) in the asymmetric unit, corresponding to a solvent content of 72.3% (V_M = 4.5 Å^3/dalton). The phases obtained from the Phaser run were improved by solvent flattening using DM (Cowtan, 1994). The resulting F_o map calculated from the improved phases showed a well-defined electron density for RIα domain B. Secondary structure of RIα domain B was built manually using XtalView, followed by iterative cycles of structure refinement using REFMAC (CCP4, 1994). The final refinement implementing TLS refinement (Winn et al., 2001) for each chain converged to R and R_{free} values of 0.192 and 0.212, respectively, with excellent geometry (Table S1). The final model contained residues 13–350 for the C subunit and residues 90–379 for the R subunit and was evaluated using PROCHECK (Table S1) (Laskowski et al., 1993). Water molecules were incorporated using wARP (Murshudov et al., 1997) and manually verified. All figures were made using PyMOL (DeLano Scientific).

cAMP Activation of PKA by Fluorescence Polarization

A new fluorescence polarization assay developed for measuring the apparent activation constant of PKA for cAMP (EC_{50}) was performed in parallel with the standard Cook assay. FAM-IP20 used in this study was synthesized as described previously (Saldanha et al., 2006). Holoenzyme was formed in situ by incubating 7 nM C subunit and 8.4 nM R subunit mutants for 20 min in 25 mM HEPES (pH 7.0), 75 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 0.005% Triton X-100. FAM-IP20 (1.5 nM) was then added and incubated for an additional 10 min. Seventy-five microliters of this holoenzyme solution was aliquoted into each well of a 384-well solid black Fluotrac 200 plate (Greiner Bio-One, part no. 781076). In all cases, 2-fold dilutions of cAMP ranging from 4 to 4096 nM were added to each well and incubated for 60 min at 25°C. The assay was performed using a GENios Pro microplate reader (Tecan) in which fluorescence polarization was measured with 485 nm excitation (20 nm band-pass) and 535 nm emission (20 nm band-pass) filters. Data were analyzed using Prism 4 software (GraphPad). Each protein was tested in quadruplicate.

Supplemental Data

Supplemental Data include Supplemental Results and Discussion, Supplemental Experimental Procedures, Supplemental References, one table, and six figures and can be found with this article online at http://www.cell.com/cgi/content/full/130/6/1032/DC1/.

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Accession Numbers

The coordinates for the structure described herein have been deposited in the Protein Data Bank under the ID code 2QCS.