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Signaling through cAMP and cAMP-dependent Protein Kinase: Diverse Strategies for Drug Design

Susan S. Taylor^{*}, Choel Kim, Cecilia Y. Cheng, Simon H.J. Brown, Jian Wu, and Natarajan Kannan

Department of Chemistry and Biochemistry and Department of Pharmacology, Howard Hughes Medical Institute, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0654, USA

Abstract

The catalytic subunit of cAMP-dependent protein kinase has served as a prototype for the protein kinase superfamily for many years while structures of the cAMP-bound regulatory subunits have defined the conserved cyclic nucleotide binding (CNB) motif. It is only structures of the holoenzymes, however, that enable us to appreciate the molecular features of inhibition by the regulatory subunits as well as activation by cAMP. These structures reveal for the first time the remarkable malleability of the regulatory subunits and the CNB domains. At the same time, they allow us to appreciate that the catalytic subunit is not only a catalyst but also a scaffold that mediates a wide variety of protein:protein interactions. The holoenzyme structures also provide a new paradigm for designing isoform-specific activators and inhibitors of PKA. In addition to binding to the catalytic subunits, the regulatory subunits also use their N-terminal dimerization/docking domain to bind with high affinity to A Kinase Anchoring Proteins using an amphipathic helical motif. This targeting mechanism, which localizes PKA near to its protein substrates, is also a target for therapeutic intervention of PKA signaling.

Keywords

PKA; PKI; Holoenzyme; RI α ; RII α ; Signaling; Allostery; Dynamics; Kinase Inhibitors; A Kinase Anchoring Proteins (AKAPs)

1. Introduction

As we move forward in this genomic era, we have an unprecedented opportunity to understand the molecular basis of disease and to develop new strategies for therapeutic intervention. In no place is this powerful convergence of biochemistry, biophysics, molecular biology, genomics, and structural biology more apparent than in the protein kinase superfamily. The protein kinases constitute one of the largest gene families encoded by the human genome and play a key regulatory role in almost every major pathway in eukaryotic cells including cell division, cell death, growth, differentiation, and memory. Protein kinases also play a key regulatory role in plants and bacteria, including many pathogens. Because they are associated with so many diseases, the protein kinases have also emerged as major therapeutic targets. Without any

^{*}Corresponding author. Tel.: +1 858 534 3677; fax: +1 858 534 8193. E-mail address: staylor@ucsd.edu (S.S. Taylor)

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question the best understood member of the protein kinase superfamily is cAMP-dependent protein kinase (PKA). It was one of the first protein kinases to be discovered [1], it was the first to be sequenced [2] and then cloned [3] and the elucidation of its structure provided the first three dimensional template for this family [4]. It is also the only protein kinase where we have excellent coordinates for a transition state mimetic [5]. Most recently, with the elucidation of holoenzyme complexes, we can also use PKA as a prototype for understanding the macromolecular assembly of protein kinase complexes.

Designing novel kinase-specific inhibitors is now a major international effort. Once again, PKA serves as a prototype for thinking about diverse strategies for designing inhibitors. As indicated in Figure 1, we can not only design inhibitors that target the ATP binding pocket and substrate tethering sites for the catalytic subunit, but also inhibitors that target the activation of the kinase. A third strategy that is proving to be equally effective is to disrupt targeting. For PKA an additional strategy is to target the synthesis and degradation of the second messenger, cAMP. While the structures of the regulatory and catalytic subunits of PKA were elucidated previously [4,6,7], it is only with the structure solution of holoenzyme complexes that the full range of strategies for disrupting PKA signaling can be appreciated [8,9]. We can for the first time begin to fully appreciate the kinase as a scaffold, in addition to its role as a catalyst. Every part of its surface seems committed to some type of protein:protein interaction, and these interactions are as essential to its function as is phosphoryl transfer. Furthermore, with the structure solutions of targeting motifs [10,11], novel mechanisms for disrupting targeting are also being implemented.

PKA is comprised of two types of subunits. Two catalytic (C) subunits bind to a regulatory (R) subunit dimer to form an inactive holoenzyme complex (Figure 1). In response to activation of adenylate cyclase through a G Protein Coupled Receptor, the second messenger, cAMP, is generated. Binding of cAMP to the regulatory subunit causes the catalytic subunit to be unleashed so that it can phosphorylate its protein substrates. These substrates include ion channels and key metabolic enzymes in the cytoplasm as well as transcription factors in the nucleus that regulate gene transcription. In addition to the R and C subunits, there is a diverse set of multivalent scaffold proteins called A Kinase Anchoring Proteins (AKAPs) that localize PKA to specific sites in close proximity to its protein substrates thereby reducing the catalytic event to a two dimensional process [12]. Each of these proteins has diverse properties, and we have begun to understand the regulatory and catalytic subunits as separate proteins for more than a decade where they serve as prototypes for the protein kinase superfamily and for cAMP binding domains, respectively. Now, however, we are beginning to appreciate them as part of larger protein complexes. With these new structures we can for the first time understand how these proteins contribute to the assembly and disassembly of macromolecular signaling complexes.

2. The Catalytic and Regulatory Subunits of PKA are Multifunctional Proteins that Mediate Multiple Protein:Protein Interactions

2.1 Catalytic Subunit

The kinase core, conserved in all members of this superfamily, is comprised of approximately 250 amino acids which include a small and highly dynamic amino-terminal lobe (N-lobe) that is mostly beta strands, a short linker, and a larger mostly helical carboxy-terminal lobe (C-lobe) that contains much of the catalytic machinery as well as the major substrate docking sites. In addition to the core, the C subunit of PKA is flanked by an N-terminal tail (N-tail) and a C-terminal tail (C-tail) (Figure 2). These tails are an integral part of the C-subunit. Both are anchored to the N- and C-lobes of the core and thus can be thought of as *cis*-regulatory elements. Both help to position the core in its active conformation where orientation of the α C helix in

its correct position is essential for catalysis. In mammalian cells there are three isoforms of the C-subunit and the two major isoforms (C α and C β) have multiple splice variants that introduce diversity into the first exon [13]. This isoform diversity is an important mechanism for achieving specificity in PKA signaling

The C-tail (residues 301-350), in particular, is an integral part of the active enzyme. One can classify the tail into three distinct segments. The segments that are tethered to the large and small lobes are designated as the C-lobe Tether (CLT), and the N-lobe Tether (NLT). These two segments are anchored to the α C- β 4 Loop and to the N-terminus of the α C Helix, respectively, as seen in Figure 2, and appear to play an important allosteric role in organizing the active conformation of the enzyme when the Activation Loop is phosphorylated at Thr197. These two segments in the active and phosphorylated enzyme, appear to be quite stable, while the middle segment, termed the Active Site Tether, or AST, is quite dynamic. Phe327, for example, is an essential part of the ATP binding site while Tyr330 is an essential residue for the closed conformation of the enzyme. In the apoenzyme and in the binary complex of the myristylated C-subunit containing only an inhibitory peptide the AST segment is disordered [14,15]. Recent analysis of the AGC subfamily revealed that the C-tail is a conserved feature of all AGC kinases with PDK1 serving as the master activating kinase for most of the AGC family members [16]. The AGC-specific residues in the core have co-evolved with the C-Tail in all of these enzymes. The NLT segment of the tail serves as a common docking motif for recognition by PDK1. Thus the C-Tail is a conserved *cis* regulatory element for all of the AGC kinases and is an integral part of the active enzyme. It is important to recognize that we are thus missing an important part of the active site if the tail is not present or not ordered.

The N-tail (residues 1-40) appears to play a major role in the localization of the AGC kinases overall but, unlike the C-tail, it is not structurally conserved in the AGC protein kinase subfamily. In PKA the novel A Kinase Interacting Protein (AKIP1), for example, binds to the N-terminus of the C-subunit and helps to traffic it into the nucleus [17]. The N-tail can also contribute to interactions with membranes through its N-terminal myristylation site. This site is not exposed in the free subunit, but becomes quite mobile upon association with RII subunits, and in this conformational state, PKA has a high tendency to associate with membranes [18]. Other reversible covalent modifications such as phosphorylation of Ser10 and deamidation of Asn2 also tend to mobilize the N-tail. Deamidation of Asn2, for example, leads to accumulation of the C-subunit in the nucleus [19]. In many ways one can think of the N-terminal tail as “histone-like”. In other AGC kinases the N-terminal regions also contain second messenger binding domains such as C1 and C2 domains for Ca⁺⁺ and diacyl glycerol, respectively, in the case of PKCs and Plextrin Homology Domains for PIP3 in the case of Akt. We do not yet know, however, how these domains interact with the kinase core. They may be functionally conserved in the AGC family but they are not recognized as conserved regulatory elements based on their sequences. So far only the kinase core plus the C-tails of AGC kinases have been crystallized and, as seen in Figure 2, significant portions of the C-tail are often disordered.

2.2 Regulatory Subunits

The regulatory subunits are highly dynamic multi-domain proteins that interact with a variety of proteins in addition to serving as major receptors for cAMP. Although there are multiple isoforms (I α and I β , II α and II β), all retain the same general architectural organization (Figure 3). All have a dimerization/docking (D/D) domain at the N terminus, which is the docking site for AKAPs. The D/D domain is followed by an inhibitor site (a pseudosubstrate for RI subunits and a substrate site for RII subunits) and two cAMP binding domains (CBDs), referred to here as Domains A and B. Structures of the cAMP bound conformations of RI α and RII β revealed that the CBDs were conserved motifs that resemble the catabolite gene activator protein (CAP) in bacteria [8,9]. Each CBD has a non-contiguous alpha subdomain that is linked to an 8-

stranded β sandwich. The hallmark feature of the CBDs is the Phosphate Binding Cassette (PBC), located between $\beta 6$ and $\beta 7$, where the ribose phosphate of cAMP is anchored. Recent NMR studies of Domain A of RI α revealed an extended network of allosteric interactions that radiate out from the bound phosphate [20-22]. A critical residue here is a conserved Arg in each PBC that binds to the phosphate moiety of cAMP. In addition to the PBC and the β sandwich that surrounds the PBC, cAMP is anchored by a hydrophobic residue that binds to the aromatic adenine ring [23]. These features will be discussed in more detail later.

3. Complex of RI α (91-244), C-subunit, and Mg₂ATP reveals the mechanism for inhibition of PKA in the absence of cAMP

While the cAMP-bound structures of RI α and RI β revealed many conserved features of the R subunits, there were other conserved residues that were not explained. These structures also did not explain how the C-subunit is inhibited by the R-subunit, nor did they reveal the mechanism for cAMP-induced activation of the holoenzymes. Finally, they did not explain the fundamental differences between the isoforms. To appreciate these features, it was necessary to solve structures of holoenzyme complexes. While the CBDs are quite stable in their cAMP-bound conformation, as is the D/D domain, the linker region containing the inhibitor site is very mobile in solution [24]. The linker region, for example, was always present but disordered in the crystal structures of cAMP-bound RI α (91-379) and RI β (91-400) where both R-subunits were monomeric and lacked the D/D domain. Crystallization of a full length R-subunit dimer has never been achieved. To obtain a structure of a complex between the R and C subunit required some novel strategies. Previous studies showed that the smallest stable deletion mutant of RI α that bound both cAMP and C-subunit with high affinity was RI α (91-244) [25]. This construct contained the inhibitor site in the linker as well as most of Domain A. Hydrogen/deuterium exchange, coupled with mass spectrometry (H/DMS) allowed us to map the interface between the R and C-subunits [26,27], but this did not provide a detailed picture of the molecular interactions. The structure of this complex, shown in Figure 4, crystallized in the presence of a non-hydrolyzable analog of ATP, AMP-PNP, allowed us for the first time to understand how the C-subunit is actually inhibited by the R-subunit [28]. This holoenzyme complex showed two striking features, in addition to the disorder/order transition of the inhibitor site in the linker region which now becomes embedded at the active site in the cleft between the two lobes of the C-subunit. First is that the C-subunit serves as a stable scaffold for binding of the R-subunit using a large surface area that derives mostly from its large lobe. Second is that the R-subunit undergoes major conformational reorganization as it releases cAMP and wraps around the C-subunit. This change involves a reorganization of the two CBD domains as well as major changes within the α subdomain of each CBD. The details of this interaction are summarized below.

The RI α subunit, like PKI, requires Mg₂ATP to bind tightly to the C-subunit ($K_d = 0.1$ nM). As in the case of PKI [15], the C-subunit in the holoenzyme complex with RI α (91-244) assumes a fully closed conformation where the C-tail is folded over onto the core and the inhibitor site is wedged tightly in the cleft between the small and large lobes of the kinase core. Docking of the inhibitor peptide (Arg-Arg-Gly-Ala-Ile) to the active site cleft is thus a critical event that not only occludes the active site cleft thereby preventing binding of other substrates but also nucleates the large binding interface between the R and C subunits. For RI α , which is a pseudosubstrate with an Ala at the P Site, docking of this peptide also forces the molecule into a fully closed conformation. Essentially, the Ala pulls the entire N-lobe with its bound ATP into a closed conformation because there is no place to transfer the γ -phosphate of ATP. The C-tail, anchored to ATP through Phe327 and Tyr330, is dragged along with the N-lobe. The central portion of this interface is hydrophobic and ordering of the P+1 Ile is critical for bringing together two important hydrophobic motifs, one from the RI α -subunit and one from the C-subunit (Figure 5). From the C-subunit, it is the α G Helix that provides a critical tyrosine,

Tyr247. This α G-helix is exposed to solvent in the free C-subunit but protected from H/D exchange in the holoenzyme complex [27]. The H/D studies thus provided the first clue that this motif was part of the R/C interface. The PBC in RI α also contributes to this interface. Tyr205 at the tip of the PBC provides the other essential contact. The convergence of Tyr205 in RI α , Tyr247 in the C-subunit, and Ile99 in the Inhibitor Site of RI α defines an essential triad that distorts the PBC in Domain A. cAMP, a small ligand, and the C-subunit, a large protein, are thus both competing for the PBC in Domain A of RI α .

The surface of the C-subunit, especially the large lobe, serves as an extended stable scaffold for binding of the R-subunit. With the exception of closing the active site cleft, the conformation of the C-subunit remains mostly unchanged. The surface that is masked by the binding of RI α extends from the Activation Loop (residues 191-197) through the α G Helix (residues 244 – 252). The Activation Loop thus serves many roles. Not only does it stabilize the active conformation of the kinase so that it is optimal for catalysis [29,30], it also provides a docking surface for the RI α subunit. This is likely to be a general feature for most kinases – one side of the Activation Loop reaches out to stabilize the active site through phosphorylation of a residue that is equivalent to Thr197 while the other side faces outward towards the solvent where it serves as a docking site for binding to other proteins, either substrates or inhibitors. We believe that the α G Helix will also be a docking motif for most protein kinases [31]. Unlike the α E, α F, and α H Helices, which are buried in the hydrophobic core of the large lobe and not readily accessible to deuterium exchange, the α G Helix is solvent exposed in the free C-subunit but shielded from solvent in the holoenzyme.

4. Conformational Switching of the Regulatory Subunit and Cyclic Nucleotide Binding Domains

In contrast to the C-subunit, the RI α subunit undergoes complete reorganization of its subdomains as a consequence of binding to the C-subunit (Figure 6). The β sandwich, with the exception of the tip of the PBC, actually remains quite stable while the helical subdomain completely rearranges. The most striking feature of this reorganization is the extension of the α B/ α C Helix in Domain A. In the cAMP-bound state this segment forms a kinked helix; the α B and α C Helices fold over onto the cAMP that is bound to Domain A and become an integral part of the cyclic nucleotide binding motif. The α C Helix is anchored by Glu200, another conserved residue in the PBC, which hydrogen bonds to the ribose 2'-OH; in the cAMP-bound conformation Glu200 also interacts through an electrostatic bond with Arg241 in the α C Helix. The hydrophobic capping residue, Trp260 for Domain A in RI α , lies at the beginning of the α A Helix in Domain B.

In the holoenzyme the α B and α C Helices become fused as a single long helix that is buttressed up against the Activation Loop of the catalytic subunit. The α B/ α C Helix also interacts with the linker region that follows the inhibitor site. Many of the residues that form the interaction surface were exposed to solvent in the cAMP-bound conformation. The α N- α A helix also reorganizes and moves as a rigid body into the space that was opened up due to the movement of the α B/ α C helix. The global motions of the RI α^{AB} :C:Mg₂ATP complex are summarized in Figure 6.

5. Catalytic Subunit is a Scaffold as well as a Catalyst

Although we tend to focus on the catalytic properties of protein kinases, these molecules are also scaffolds, and their scaffold function is at least as important as the phosphoryl transfer function. It is the scaffold function that mediates protein:protein interactions, and, based on the recent PKA catalytic subunit complexes, we can now better appreciate that almost every part of the surface is utilized. Figure 7 summarizes how the N- and C-tails wrap around the

surface of the kinase core. These tails are not neutral tethering moieties but rather are an integral part of the active enzyme. Furthermore, as discussed above, the C-Tail is a conserved feature of all AGC kinases. Although many protein kinase structures have now been solved, in many cases it is only the conserved core, and it is thus likely that essential functional parts of the enzyme are missing. Perhaps this is why so few protein kinase structures have functional active sites such as we see in PKA. By solving structures of the PKA catalytic subunit bound to its different inhibitors, we can appreciate for the first time how the rest of the C-subunit is used to mediated diverse protein:protein interactions. Figure 7 at the bottom also shows how the various PKA inhibitors interact with the surface of the large lobe. Although PKI and RI α both have pseudo-substrate inhibitor sites, require ATP and two Mg⁺⁺ ions to form a tight complex, and bind with equally high affinity (0.1 nM), they each use different surfaces to achieve their high affinity binding. PKI docks into a hydrophobic groove formed by the α F- α G linker while the RI α subunit uses the large surface extending from the Activation Loop to the α G Helix for docking of the Domain A and a small region corresponding the α H- α I insert for docking of Domain B. RII α uses a similar surface but because it is a substrate as well as an inhibitor it does not recruit the small lobe or the N-tail nor does it require ATP [9]. Unlike RI α , it has an absolute requirement for docking to the α H- α I insert. The importance of the α H- α I Loop was only appreciated when we were able to crystallize complexes that contained both the A and B domains. As discussed below, this structure of the larger complex was also essential for us to be able to understand the mechanism whereby cAMP activates the holoenzyme.

6. Mechanism for the ordered activation of the RI α Holoenzyme is revealed by the complex of RI α (91-379), C-subunit, and Mg₂ATP

To understand how cAMP activates the holoenzyme required more information than the structures of the free R and C-subunits; the mechanism could not be deduced from the structures of the isolated subunits. For RI α the activation by cAMP is both cooperative and ordered (Figure 8). cAMP Binding Domain B serves as a “gate-keeper”. It prevents cAMP from binding to Domain A, which is the essential event that is required for release of the C-subunit [32]. To understand the mechanism of activation, we thus had to crystallize a complex that contained both cAMP binding domains. In an effort to overcome the dynamic properties of the complex, we engineered two mutant forms of RI α (91-379), where each of the essential arginines in the PBC, Arg209 in Domain A and Arg333 in Domain B, were replaced with Lys. This results in a significantly reduced affinity for cAMP. Both mutants and the wild type protein were set up for crystallization, but only RI α (91-379:Arg333Lys) gave well diffracting crystals. We refer to this mutant as δ RI α^{AB} and the complex as δ RI α^{AB} :C:Mg₂ATP. The model of this structure is shown in the right panel of Figure 4. Mutagenesis was used to confirm the ordered mechanism of activation that was revealed by this structure [8].

The location of the capping residues appears to be a crucial feature in the activation process, and neither of the capping residue was present in the initial structure which contained only the Domain A. The two capping residues in RI α (Trp260 for Domain A and Tyr2371 for Domain B) are both located in the domain B, and in the holoenzyme complex both capping residues are far removed from their respective PBCs. In addition, they are linked by a salt bridge between Glu261 and Arg365 (Figure 5). Both of these conserved residues were solvent exposed in the cAMP-bound conformation, and their function was not revealed until the holoenzyme structure was solved. Replacement of either residue with Ala made activation by cAMP much easier whereas mutation of the capping residues made it more difficult to activate the holoenzyme. Based on our mutagenesis and on our structure, we predict that cAMP binds first to the B Site because the A Site is partially occluded in the holoenzyme. Binding of cAMP to the B site will presumably recruit the capping residue, Tyr371, thereby breaking the salt bridge. This frees up Trp260 which can now be recruited to A Site upon binding of cAMP. Binding of cAMP to the A Site then recruits the tip of the PBC thereby breaking the interface between the RI α

subunit and the C-subunit. This is a highly coordinated process that is initiated by the small second messenger, cAMP. Stopped flow fluorescence showed that binding of cAMP leads to a 3 order of magnitude increase in the off rate for the R subunit while there is no change in the on rate [33].

7. Altered cAMP Binding Sites in the Holoenzyme Complex Provide Opportunities for the discovery of novel isoform-specific inhibitors and activators of PKA

The cAMP binding site A in the cAMP bound conformation and in the holoenzyme conformation is shown in Figure 8. One can appreciate here not only the spatial differences but also the electrostatic differences. Based on this finding, we developed a fluorescence anisotropy assay that allows us to screen for activators (agonists) and inhibitors (antagonists) of cAMP activation of PKA. In this assay, summarized at the top of Figure 9, the inhibitor peptide, IP20 (residues 5-24 from the heat stable Protein Kinase Inhibitor), is labeled with Texas Red. This labeled peptide then competes with the regulatory subunit for binding to the active site of the catalytic subunit. Measurement of the change in fluorescence anisotropy of the labeled peptide when it binds to free C-subunit gives a readout that reflects the inhibition state of the kinase. Upon addition of cAMP or another analog of cAMP, the regulatory subunit is released from the catalytic subunit and the inhibitor peptide binds to the free catalytic subunit. This assay principal is named ligand-regulated competition (LiReC).

This fluorescent competition assay has been adopted into three main assay modes; a high throughput screen (HTS) for antagonists and agonists, a dose response agonist screen, and a dose response antagonist screen. By using 384-well plates, each assay can be performed in a high throughput mode, with 384 compounds per plate in the full screening mode or 32 compounds in the dose response mode. Z factors of >0.7 are measured in the full high-throughput screening mode, well above the 0.5 minimum required for a HTS assay.

Preliminary screenings of commercially available cAMP analogues have yielded a successful proof of principal of the dose response mode. Both the antagonist and agonists modes have been tested using readily available compounds, and results show up to 5-fold selectivity for RI isoforms and 5-fold selectivity for the RII isoforms depending on the analogs that were used. Preliminary trends of activation data indicate a preference of the RII isoforms for N6 substitutions on the adenine ring while the RI complex shows a preference for C8 substitutions. This assay also does not identify ATP analogs as inhibitors and is thus quite distinct from conventional kinase inhibition assays. By using holoenzyme formed with RI α or RII β we hope to identify isoform-specific activators and inhibitors.

8. A Kinase Anchoring Proteins

Because PKA is a broad specificity kinase that can phosphorylate many substrates, it requires some mechanisms to achieve specificity. Over the past decade or more, we have come to appreciate that the isolated R and C subunits are not randomly scattered throughout the cell. Instead they are specifically localized and exist as part of larger signaling complexes that are assembled near their substrates such as an ion channel or a co-transporter or near organelles such as the mitochondria or the Golgi. Typically this targeting is mediated by a family of proteins called A Kinase Anchoring Proteins (AKAPs). We still do not know much about the full length AKAPs, but we do know a great deal about the mechanism for PKA targeting to AKAPs and about the PKA targeting motif. Early biochemical data predicted that the PKA binding domain in the AKAPs was a short amphipathetic helix that interacts with the dimerization/docking (D/D) domain of the R-subunits. Subsequent NMR studies showed that the D/D domains of RI α and RII α formed an antiparallel four helix bundle [34,35]. The AKAP

peptide typically binds with high affinity (1-2 nM) to this D/D domain, and the NMR structure of an AKAP peptide bound to RII α showed not only how the peptide was bound but also revealed the backbone dynamics involved in this unique protein:protein interaction [36].

This mechanism for targeting of PKA through AKAPs introduces another opportunity for interfering with kinase function since we can now design peptides that compete for the AKAP binding site and thus disrupt the PKA signaling. To understand the requirements of specific amino acids in this targeting motif, we used a peptide array where 25mers were attached to a paper filter (Figure 10). Each residue in the 25mer was then changed to every other residue. By overlaying with RI α and RII α , respectively, we were able to engineer AKAP peptides that were specific for RI α or RII α [37].

To obtain high resolution data on the AKAP binding mechanism we solved the crystal structure of a complex between the D/D domain of RII α and the helix from a dual specific AKAP, D-AKAP2, that binds to both RI and RII subunits (Figure 10) [10]. A similar structure of a PKA/AKAP complex was concurrently solved with an engineered AKAP that preferentially binds to the type II isoform [11]. Both structures show how the AKAP peptide docks to a shallow preformed groove on the surface of the D/D domain formed by one face of the helical bundle. In addition to the preformed surface, one of the N-termini becomes ordered and docks to the AKAP peptide. Over 400 waters were modeled in the structure, but none were found near the complex interface nicely illustrating this unique and exclusively hydrophobic protein:protein interaction.

9. Conclusions and perspectives

Since the protein kinases are known to be associated with many diseases, especially cancers, considerable effort has gone into the discovery of protein kinase inhibitors. However, the approach to inhibitor design is now aimed almost exclusively at ATP mimetics such as Gleevec [38]. Although in most cases we have structures of only protein kinase cores, we show in the case of PKA that the C- and N-tails are an integral part of the functional active enzyme. In addition, by solving crystal structures of holoenzyme complexes of PKA, we can for the first time understand the molecular features required for inhibition and for cAMP-induced activation. From these structures we can also better appreciate how the C-subunit functions not just as a catalyst but also as a scaffold. The large lobe in particular is a remarkably stable scaffold for mediating protein:protein interactions. The regulatory subunits, on the other hand, undergo major conformational changes as they release cAMP and wrap around the catalytic subunit. In the process of binding to the catalytic subunit, the cAMP binding sites are completely restructured. The PBC where the ribose phosphate docks, for example, is far removed from the residues that cap the adenine ring in the holoenzyme complex. This provides a new paradigm for designing novel agonists or antagonists for PKA. The AKAPs introduce another level of complexity into PKA signaling by localizing PKA in close proximity to its physiological substrates. The docking motifs are also valid targets for designing inhibitors that disrupt targeting. Thus with PKA, as we begin to gain an understanding of the higher level complexes, we are beginning to appreciate that there are a wide variety of strategies that can be employed to design therapeutic reagents that will disrupt PKA signaling in cells. These same strategies will likely be quite applicable to other protein kinases as well.

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Abbreviations

cAMP, Cyclic-3',5'-adenosine monophosphate
 PKA, cAMP-dependent protein kinase
 PKI, heat stable protein kinase A inhibitor
 PDK1, phosphoinositide-dependent kinase-1
 ATP, adenosine triphosphate
 D/D domain, dimerization/docking domain
 AKIP1, A Kinase Interacting Protein 1
 H/DMS, Hydrogen/deuterium exchange coupled with mass spectrometry
 PBC, phosphate binding cassette
 CNB domain, cyclic nucleotide binding domain
 C subunit, catalytic subunit
 R subunit, regulatory subunit
 N-lobe, amino-terminal lobe
 C-lobe, carboxy-terminal
 N-tail, N-terminal tail
 C-tail, C-terminal tail
 CLT, C-lobe Tether
 NLT, N-lobe Tether
 AST, Active Site Tether
 CLA, C-lobe Anchor
 CBD, cyclic nucleotide binding
 AKAP, A Kinase Anchoring Protein
 D-AKAP2, dual specific AKAP2
 PIP3, Phospho-inositide triphosphate
 Akt/Pkb, Protein Kinase B
 H/D exchange, hydrogen/deuterium exchange
 CAP, catabolite gene activator protein
 AGS kinase, PKA PKG PKC and related kinases
 PKC, Protein Kinase C
 LiReC, ligand-regulated competition
 HTS, high throughput screen

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Figure 1. Conformational states of PKA and candidate sites for engineering inhibitors of PKA signaling

The free catalytic (C) subunit can be inactivated with inhibitors to the active site and by inhibitors that disrupt tethering of protein substrates and inhibitors. Therapeutic intervention can also disrupt the activation of the PKA holoenzyme complex and the targeting of PKA through the AKAPs. C-subunit is yellow, R-subunit is teal and AKAP is red. Potential sites for therapeutic intervention are shown by black arrows.

Figure 2. N- and C-terminal tails of AGC kinases are cis-regulatory elements

The upper panels show the N- and C-terminal tails of the PKA catalytic subunit. On the left is the N-tail showing its different sites of covalent modification. On the right is the C-tail showing the Active Site Tether (AST) in red and the N- and C-lobe tethers in white. In the middle panel one can see how the α C Helix and the α C- β 4 Loop are anchored by the NLT and CLT. AGC conserved residues are also highlighted. The lower panels show how the features of the C-tail are conserved in the AGC subfamily of protein kinases. On the lower right are the segments of the C-tail: N-lobe Tether (NLT), Active Site Tether (AST), C-lobe Tether (CLT). The C-lobe Anchor (CLA) contains the AGC Insert between the α H and α I Helices. The panels on the left show the C-terminal tail conformation in three AGC kinase structures (PKA – PDB code:1ATP, AKT – PDB code:1GZN, and PKC – PDBcode:1XJD). The disordered regions are shown in dotted representation.

Figure 3. Domain organization of the R-subunit isoforms and the structure of the cAMP binding domains of RI α

The domain organization of RI α , RII α , and RII β is shown on the left. On the right is the structure of RI α (91-379) where the cAMP Binding Domain A is shown in dark teal and Domain B is in turquoise.

Figure 4. Models of the catalytic subunit bound to different inhibitors

On the left is shown the complex with the PKI(5-24) Inhibitor Peptide. In the middle is the RI α (91-244):C complex (PDB code:1U7E). On the right is the RI α (91-379):C complex (PDB code:2QCS). All three structures have bound Mg₂ATP and are in a closed conformation. The catalytic subunits are rendered as space filling models while the inhibitors are shown as ribbons. CBD A is in dark teal while CBD B is in turquoise. The α B/ α C Helix in CBD A is in red.

Site2

Figure 5. The active site cleft of the catalytic subunit is filled by the pseudosubstrate inhibitor segment of RI α

The left panel shows how the P-5 to P+1 peptide occupies the active site cleft. On the right is a close up of the site that is nucleated by the P+1 Val. Key residues that nucleate this site are Tyr205 from the PBC of Domain A and Tyr247 from the α G Helix of the C-subunit.

Figure 6. Dynamic malleability of the RI α subunit

On the top is shown the conformation of RI α (91-244) bound to cAMP (left) and bound to Mn₂ATP and the C-subunit. The α B/ α C Helix, shown in red, undergoes dramatic conformational rearrangement. On the bottom are the conformational changes associated with the RI α (91-379) construct that contains both the Domain A and B. The conformational changes in domain A are comparable to what was seen in the smaller construct, but the B-domain which immediately follows the Domain A is dramatically changed by the snapping open of the α B/ α C Helix, again shown in red.

Figure 7. The catalytic subunit is a scaffold as well as a catalyst

Panel A shows the conserved kinase core of the catalytic subunit (residues 40-300) while Panel B shows how the N- and C-tails wrap around the surface of the kinase core. The large lobe is in tan and the small lobe in cream. Panel C shows the surfaces that are masked by the inhibitor peptide (red), the PKI amphipathic helix (blue), the A Domain of RI α (green), and the P+5 Arg of the RI α Inhibitor Site.

Figure 8. Ordered pathway for activation of the RI holoenzyme

A cartoon of the ordered activation mechanism is shown at the top. Close up of the cAMP binding site in Domain A in the presence of cAMP is on the bottom left while the site in the absence of cAMP is shown on the bottom right. The PBC is shown as a ribbon through the transparent space filling model.

Figure 9. Fluorescence anisotropy assay for inhibitors and activators of PKA

The strategy for the assay is indicated at the top while the results of two different assays are indicated in the middle.

Figure 10. The AKAP binding motif docked to the dimerization/docking domain of the RII α subunit
 On the left is shown the peptide arrays where isoform specific properties of the AKAP peptide were identified. By overlaying the array with the RII α or RI α D/D domain, it was possible to identify isoform differences [37]. The hydrophobic surface of the AKAP peptides is shown between the two arrays. On the right is a model of the D-AKAP2 peptide bound to the RII α D/D domain, based on the recently solved crystal structure[10]. The peptide is shown in red while the two protomers of the D/D domain are shown in tan and grey. The density of the side chains is shown in green. There also are no water molecules in the interface.