A Dynamic Mechanism for AKAP Binding to RII Isoforms of cAMP-Dependent Protein Kinase

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Summary

A kinase-anchoring proteins (AKAPs) target PKA to specific microdomains by using an amphipathic helix that docks to N-terminal dimerization and docking (D/D) domains of PKA regulatory (R) subunits. To understand specificity, we solved the crystal structure of the helical motif from D-AKAP2, a dual-specific AKAP, bound to the RIIz D/D domain. The 1.6 Å structure reveals how this dynamic, hydrophobic docking site is assembled. A stable, hydrophobic docking groove is formed by the helical interface of two RIIz protomers. The flexible N terminus of one protomer is then recruited to the site, anchored to the peptide through two essential isoleucines. The other N terminus is disordered. This asymmetry provides greater possibilities for AKAP docking. Although there is strong discrimination against RIIz in the N terminus of the AKAP helix, the hydrophobic groove discriminates against RIIz. RIIz, with a cavity in the groove, can accept a bulky tryptophan, whereas RIIz requires valine.

Introduction

Signal transduction is mediated by protein-protein interactions frequently regulated dynamically by peptides docking to relatively small binding motifs. SH2, SH3, WW, and PDZ domains are examples of signaling modules that interact with short linear peptides where specificity is typically achieved by mostly hydrogen bonds and electrostatic interactions (Cohen et al., 1995). In contrast, the targeting of cAMP-dependent protein kinase (PKA) is mediated primarily by docking of an amphipathic helix where specificity is achieved not by backbone/side chain contacts but rather by the interactions of hydrophobic side chains. One of the most important mechanisms for targeting PKA is through AKAPs where the signature motif of the AKAP is an amphipathic helix that docks to the PKA R subunits (Carr et al., 1991). AKAPs thus serve as scaffolds to target PKA and other signaling molecules to microdomains in the cell in close proximity to their substrates (Wong and Scott, 2004). This targeting is an essential feature of PKA signaling and is an important mechanism for achieving specificity.

The PKA holoenzyme is comprised of a homodimer of R subunits bound to two catalytic (C) subunits. Each protomer of R contains an N-terminal D/D domain followed by a PKA inhibitor site and two tandem cAMP binding domains. In most cases, the D/D domain is thought to be functionally well segregated from the cAMP binding domains where the C subunit binds (Heller et al., 2004). The D/D domain is an antiparallel four-helix bundle that provides the docking surface for the AKAP helix (Banky et al., 2003; Newlon et al., 1999, 2001). Although the fold of the D/D domain is conserved in all R subunits, sequence differences in RI and RII provide one mechanism for introducing specificity into the PKA signaling pathway (Hausken et al., 1994). Typically, AKAPs bind with high affinity (KD = 1–5 nM) to RII subunits (Carr et al., 1992; Herberg et al., 2000), whereas some AKAPs, termed dual-specificity AKAPs or D-AKAPs, bind to both RI and RII (Huang et al., 1997a, 1997b). RII subunits are typically localized to discrete sites in the cell, such as the plasma membrane, mitochondria, cytoskeleton, and centrosomes (Wong and Scott, 2004), whereas RII subunits tend to be more diffuse. Whereas docking of RII is more static, the RI subunits can be dynamically recruited to a specific site as seen in T cells, where normally diffuse cytoplasmic RI is recruited to the cap site (Skalhegg et al., 1994).

D-AKAP2 is considered a dual-specific AKAP because it binds with high affinity to both RI and RII (KD = 2 and 48 nM, respectively) (Burns et al., 2003). The biological importance of RI versus RII specificity was suggested by SNP analysis where a mutation in D-AKAP2 that reduces affinity only for RI has been associated with a shorter life span and possibly cardiac dysfunction (Kammerer et al., 2003). As summarized in Figure 1A, D-AKAP2 is a modular protein that contains two putative RGS domains followed by an A kinase binding (AKB) domain and a C-terminal PDZ binding motif (Gisler et al., 2003; Huang et al., 1997a). The biophysical properties of the AKB of D-AKAP2 have been characterized extensively by using a variety of solution methods. Protection of both the peptide and the D/D domains of RI and RII was mapped by hydrogen/deuterium exchange coupled with mass spectrometry (H/DMS) (Burns-Hamuro et al., 2005), whereas the specificity requirements for binding RI versus RII were mapped by using a peptide array (Burns-Hamuro et al., 2003). Although we can design AKAP peptides specific for RI and RII and can use them to disrupt cell function (Alto et al., 2003; Burns-Hamuro et al., 2003), we still cannot predict specificity a priori using an amphipathic helix as a template. How do we distinguish, for example, an amphipathic helix that has specificity for an AKAP from one that serves as a nuclear export signal (Wen et al., 1995)? Elucidating the stringent rules for AKAP specificity for PKA will require structures of complexes.

Here, we report the crystal structure of the D/D domain of rat RIIz bound to a 22 residue peptide from the AKB...
domain of D-AKAP2 (Figure 1A). Our structure reveals a unique docking mechanism and allows us to appreciate how hydrophobic side chains contribute to docking. In addition to a solvent-excluded hydrophobic interaction surface between the helical peptide and the groove formed by the antiparallel helices of the RIIα D/D domain described previously (Newlon et al., 2001), we see the critical contributions of the dynamic N terminus of RIIα, specifically Ile3R and Ile5R. The N-terminal tail of the second protomer within the dimer is disordered. Binding of the D-AKAP2 peptide thus introduces asymmetry into the D/D domain, and this asymmetry provides greater docking possibilities for the AKAPs. The crystal structure, correlated with extensive biochemical and biophysical data, defines the atomic determinants for RI and RII specificity of AKAP docking. Although many mutations in the N terminus of the AKAP peptide discriminate against RIIα, the N terminus of the D/D domains for RIβ and RIIα are flexible and also different. Thus, we cannot establish definitive isoform specificity for this region until we have a structure of the RIβ docked to the AKAP peptide. However, the hydrophobic groove formed by helix I and helix I’ is more rigid, and here, there is one isoform-specific site that discriminates strongly against RIIα. In the corresponding groove for RIIα, there is a cavity that can accept bulky side chains, whereas RIIα has a flat hydrophobic surface. RIIα has a strong preference for Val at position 13 of the peptide and cannot accept larger residues, whereas the AKAP peptide with Trp at the same position binds to RIIα. This site allows AKAP peptides to discriminate between RI and RII isoforms, thus contributing to isoform-specificity as demonstrated in vitro and in cells.

Results and Discussion
Summary of the RIIα D/D-D-AKAP2 AKB Complex Structure

The fully refined structure of the D/D domain bound to a 22 residue AKAP peptide from DAKAP2 showed two dimers-AKAP complexes in the asymmetric unit (dimer I and dimer II) (Figure 2). Although each dimer-AKAP complex has a unique set of crystal contacts, they align with an rmsd of 0.32 Å for 64 equivalent Cα atoms. Crystallographic data and refinement statistics are included in Table 1. The backbone trace of the D/D domain of our model forms a four-helix bundle and agrees well with the previous NMR solution structures (Newlon et al., 2001), as discussed later. Each protomer contains two helices that pair to form an X-type, four-helix bundle. As previously reported, helices I and II from one protomer interact in an antiparallel manner with their counterparts on the other protomer, helix I’ and II’, respectively (Figure 2A). The two molecules in the asymmetric unit are joined through a stacking interaction (Figure 2D).
between RIIγ residues Pro25R and Pro26R of one molecule (dimer I) and His2R of the other molecule (dimer II). Gln24R of dimer I also forms a hydrogen bond with Gln4R of dimer II. Symmetrically related molecules cluster together by the N termini of the D/D domains as well as by contacts between several residues of the D-AKAP2 peptide with D/D domains of neighboring molecules. For example, Trp6R of the D-AKAP2 peptide forms a crystal contact with Pro25R and Pro26R of RIIγ in a symmetrically related molecule.

The dimer interface of each D/D domain is comprised of hydrophobic side chains from each helix (residues Leu12R, Leu13R, Tyr16R, Thr17R, Val20R, Leu21R, Leu28R, Val29R, Phe31R, Ala32R, Val33R, Tyr35R, Phe36R, Thr37R, and Leu39R from each protomer) forming a tight, solvent-excluded core. The structure includes over 200 water molecules, none of which are found within the dimer interface. The center of the dimer is especially enriched with phenylalanines and tyrosines, which stack together to form the core (Figure 2E). Additionally, two motifs, characterized by two conserved adjacent prolines (Pro6R and Pro7R), create an additional hydrophobic groove that nicely complements the surface displayed by RIIγ. As mentioned, no water was found at the hydrophobic interface between the RIIγ D/D domain and the D-AKAP2 peptide. The interface occludes an area of 360 Å² (Lee and Richards, 1971).

Figure 3C summarizes the multiple contacts between the D-AKAP2 peptide and the RIIγ D/D domain. A cluster of hydrophobic residues from each protomer of RIIγ D/D (Leu9R, Thr10R, Leu13R, Val16R, Thr17R, and Leu21R) creates a preformed hydrophobic ridge that nicely complements the surface displayed by RIIγ. As mentioned, no water was found at the hydrophobic interface between the RIIγ D/D domain and the D-AKAP2 peptide. The interface occludes an area of 360 Å² (Lee and Richards, 1971). Figure 3C summarizes the multiple contacts between the D-AKAP2 peptide and the RIIγ D/D domain.

Assembly of an Asymmetric, Hydrophobic Binding Pocket Is Revealed by the Crystal Structure

Two elements in the D/D domain, one stable and one flexible, create an asymmetric surface for the AKAP helix (Figure 3A). A preformed, stable hydrophobic groove is formed by the antiparallel packing of helix I and helix I₀, whereas a flexible component derives from the N-terminal tail. Because only one tail is recruited to the site, binding of the AKAP peptide creates an inherent asymmetry. Side chains from the D-AKAP2 peptide form a hydrophobic ridge that nicely complements the surface displayed by RIIγ. As mentioned, no water was found at the hydrophobic interface between the RIIγ D/D domain and the D-AKAP2 peptide. The interface occludes an area of 360 Å² (Lee and Richards, 1971). Figure 3C summarizes the multiple contacts between the D-AKAP2 peptide and the RIIγ D/D domain.

A cluster of hydrophobic residues from each protomer of RIIγ D/D (Leu9R, Thr10R, Leu13R, Val16R, Thr17R, and Leu21R) creates the preformed surface where the AKAP helix docks (Figure 3B). Many of these hydrophobic side chains are also in close proximity to each other on the exposed surface of the D/D domain. As indicated above, Pro25R and Pro26R also contribute to crystal packing, suggesting that this surface may have a propensity to bind other proteins.
chains also stabilize the dimer. Leu21R in this stable core is the only hydrophobic residue known to disrupt AKAP binding, but not dimerization (Li and Rubin, 1995). As indicated in Figure 1B, mutations of other residues abolish dimerization and, as a secondary consequence, prevent AKAP binding (Li and Rubin, 1995). As discussed later, the position of the side chain of Leu13R differs in RIIz and RIIx and is a specificity determinant for this hydrophobic groove.

Our structure reveals that the solvent-exposed and flexible N-terminal segment (residues 1–5) of RIIz is also an essential feature of the AKAP binding site. The side chains of Ile3S and Ile5S of one protomer extend in toward the peptide, making multiple hydrophobic contacts with D-AKAP2 (Figure 4A); the N terminus of the other protomer is disordered (residues 1–3) and has higher temperature factors, suggesting that the N terminus is flexible, especially the disordered end. Although Ile5 of the disordered end is seen, it is displaced 1.6 Å from its position compared to the ordered end and shows a high-temperature factor (Figure 5). Ile3S contacts Leu4S, Ala5S, and Ile8S of the D-AKAP2 peptide, whereas Ile5S contacts Ile8 and Ile12S. Leu21R from the other protomer also extends upward into the docking surface to contact the D-AKAP2 peptide where it is juxtaposed against Ile3S and Ile5S. As seen in Figure 4, Ile3S, Ile5S, and Leu21R, previously identified by mutagenesis to be required for AKAP binding (Hausken et al., 1994; Li and Rubin, 1995), create a well-defined hydrophobic pocket. Our structure confirms that these residues are critical AKAP contact points. This binding module was not revealed by the previous NMR structures, as the N termini were not well ordered. This essential hydrophobic recognition site requires the clustering of at least three residues. Leu21R is part of the stable core, whereas Ile3S is recruited from the flexible tail. Ile5S is reoriented as well for binding. The side chain of Leu9R, which initiates helix I, is also a stable part of this pocket; however, no mutants of Leu9R have been studied. The symmetry-related site in the same molecule is partially disordered, allowing one to appreciate the dynamic features of this binding element. Leu21R and Leu9R, both part of the stable core, are in place, Ile5R is close, but Ile3R is not seen at all. Residues 4–7 are seen, but backbone and side chain positions are different. Residues 1–3 are not seen at all. Gly8S appears to be the clear hinge point for the N-terminal element. It is also important to note that we have two dimers in each asymmetric unit and in all cases the disordered N terminus is not influenced or biased by crystal packing, thus serves as an internal control. This reinforces the argument that one N terminus is disordered.

Docking of the nonpalindromic D-AKAP2 peptide to the hydrophobic pocket of RIIz induces inherent asymmetry. Because the D/D domain is symmetrical, there are two possible binding modes for the AKAP, in principle. Thus, the chances of forming a productive complex are increased, because the AKAP can dock with multiple approaches and orientations. Leu21R (21R) and Leu9R (9R) are located at the C- and N termini of helix I (I) and are all held in place by the packing of the four-helix bundle. However, only one N terminus is ordered by AKAP binding; the other is disordered, suggesting that the stable docking site for the AKAP is only assembled as a tight fit when the AKAP is bound. This induced fit, stimulated by AKAP docking, most likely accounts for the slow off rate associated with the interaction (Burns et al., 2003) and may contribute to the high-affinity binding of the RII subunit. It is most likely why mutations of either Ile3S and Ile5S can no longer pull down AKAPs (Hausken et al., 1994; Li and Rubin, 1995).

The Crystal Structure Combined with Biochemical and Biophysical Studies Support the Binding Mechanism Suggested for D-AKAP2

Several biophysical methods have been used previously to characterize this same AKAP peptide, and these data are all consistent with the above model. Using peptide arrays, the AKB of D-AKAP2 was systematically mutated previously to identify the determinants for RI and RII specificity (Burns-Hamuro et al., 2003). The array data (Figure S1 in the Supplemental Data available with this article online) confirmed the helicity of the AKAP peptide, the specific importance of two turns of the helix (boxed in the figure), and the stringent requirement for Ile-specific sites. Based on the Ala scan, the most critical residues for RIIz binding on our peptide are Ile8, Ala9S, Ile12S, and Val13S; RIIx binding was abolished for nearly all mutations at these sites. These residues define the center of the hydrophobic ridge on the...
peptide that docks to the hydrophobic surface of the D/D domain. They represent only two turns of the D-AKAP2 helix where the first turn complements the portion of the docking surface created by the N-terminal tail and the second turn goes mostly to the hydrophobic groove created by helices I and I'. In the crystal structure, Ile8P of the peptide contacts Ile3R and Ile5R of the D/D domain, and only an isoleucine is uniquely able to make these multiple contacts. The branching of the side chain at Cβ is critical for bridging to both Ile3R and Ile5R. Ile12P also uses both branches to contact Ile5R and Leu13R of the D/D domain. Peptide residues Leu4P and Ala5P, which also participate in docking but can accept most hydrophobic substitutions, are not at all buried in the pocket. Moreover, the N terminus of RIIα is flexible enough to accommodate multiple substitutions at these positions. The fourth essential residue, Val13P, does not bind to the tail; it binds to the center of the hydrophobic groove created by helices I and I', and bulkier side chains are not accepted. We will discuss this residue later as a determinant for specificity. Val16P, also located in the hydrophobic groove, is clearly preferred for its position, but the peptide can bind with reduced affinity when Val16P is replaced with other hydrophobic residues. This is also consistent with our structure.

Backbone amide H/D exchange followed by mass spectrometry (H/DMS) was also used to characterize

Figure 3. Complementary Hydrophobic Surfaces on RIIα D/D Domain and D-AKAP2 Mediate a Tight Interaction between Helices
(A) The D-AKAP2 peptide is removed from the interface and rotated 180° in order to see the buried interacting surfaces displayed by the peptide and the RIIα D/D domain. The hydrophobic residues of the peptide form a ridge required for PKA binding. RIIα D/D domain displays a complementary hydrophobic surface, formed by helices I and I', that allows the peptide’s hydrophobic ridge to dock. Dotted lines outline the residues of both peptide and the D/D domain that pack together in the hydrophobic interface.
(B) Helix I and helix I' interact in an antiparallel manner to form the stable binding site for AKAP docking. Arrows represent the hydrophobic interactions between the two helices.
(C) Specific side-chain interactions between residues of the D/D domain and peptide were elucidated and listed.
the binding interfaces of D-AKAP2 and RI and RII D/D domains (Burns-Hamuro et al., 2005). The H/D protection data are consistent with our structure and also correlate nicely with the B factors (Figure 5). Although the D-AKAP2 fragment (residues 623–662) alone was highly deuterated, indicating that it was fully exposed to solvent, increased amide protection was detected upon binding to the RIIα D/D domain. Based on higher deuteration levels at both ends, the binding surface was restricted to 634–647. Residues 1–19 of our peptide coincide with 631–649 of D-AKAP2, whereas residues 4–17 correspond to the protected regions. This region was shown to be involved in binding to the D/D in the structure. Furthermore, there is a rise in B factors for residues 18–22 on the peptide consistent with the lack of protection. The H/D exchange profile was also characterized for the RIIα D/D domain in the presence and absence of the D-AKAP2 peptide. Protection of RIIα D/D in the absence of the AKAP was localized on helix I and helix II, and both of these helices showed increased protection upon D-AKAP2 binding. As reported previously, AKAP binding is sensed across the helix bundle, not just at the binding interface (Burns-Hamuro et al., 2005; Fayos et al., 2003; Newlon et al., 1999).

**Comparison of the Crystal Structure of RIIα D/D-AKAP2 and the NMR Solution Structures of RIIα D/D with Ht31 and AKAP79**

The NMR solution structures revealed that the hydrophobic faces of the AKAP amphipathic helices of Ht31 and AKAP79 docked onto a well-ordered solvent-accessible hydrophobic groove on the surface of the D/D domain (Newlon et al., 2001). Additional solution studies revealed that the residues in the solvent-accessible groove experience increase in dynamics upon complex formation with an AKAP peptide (Fayos et al., 2003). In addition, protomer-specific assignments revealed reduced dynamics for one protomer (including Ile3) and disorder in the other protomer upon binding to a different natural AKAP peptide (Ht31). We hypothesized that this increase in dynamics would facilitate binding to the diverse sequences within the AKAP family of proteins, as the hydrophobic groove could accommodate multiple sequences by altering the contour of the hydrophobic groove once bound to an AKAP. The current structure of the RIIα D/D-AKAP2 complex supports this prediction and allows us to expand upon this theme.

Despite the obvious similarities in global structure, the new crystallographic model reveals how the D/D domain...
surface can accommodate peptide binding. The importance of the N-terminal extended region of RIIα containing Ile3R and Ile5R was first revealed by mutagenesis (Hausken et al., 1994). Although mutagenesis first demonstrated the importance of Ile3R and Ile5R and interactions involving these side chains were suggested in the NMR analysis, the presence of two protonated histidines at pH 4.0, necessary for NMR analysis, likely deemphasized their hydrophobic interactions (Newlon et al., 2001). The crystal structure demonstrates clearly the importance of these two isoleucines and explains their stringent requirement for binding.

In addition, the conformational flexibility of the C terminus of the AKAP peptide apparent in the NMR analysis (Figure 6A) correlates with the B factors in the crystal structure and H/DMS (Figure 5). In Figure 6A, the NMR structures of Ht31 and AKAP79 bound to the RIIα D/D domain are aligned with the crystal structure with the D-AKAP2 peptide. The inherent flexibility of the N- and C termini of the D/D domain is most apparent from the NMR structures but also correlates with the temperature factors in the crystal (Figure 5). The peptides bind in nearly the same space; however, the crystal structure reveals a change in helical register between the AKAPs by as much as a quarter turn. Despite this, the Ht31 and AKAP79 peptides display a hydrophobic ridge similar to what we see with D-AKAP2 (Figure 6C). This highlights that helix-helix interactions do not determine the precise position of the helix backbone, because only the side chains are involved in the recognition process. In the case of D-AKAP2, Ht31, and AKAP79, all naturally occurring AKAP peptides, the ridge comprised of hydrophobic side chains represents the recognition motif. We expect, given the diversity of AKAPs and lack of an absolute consensus sequence, that a variety of peptide orientations can be accommodated.

**AKAP Binding Specificity for RI versus RII Isoforms of PKA**

Although some AKAPs, such as D-AKAP2, have specificity for RI and RII subunits, most AKAPs do not bind well to RI. With the peptide array data, in combination with this structure, we can now define isomeric specificity more precisely. As indicated in Figure S1, the D-AKAP2 peptide can be divided into two segments: (1) the helical segment where side chains recognize the RIIα N terminus and (2) the segment where the side chains dock to the hydrophobic groove. Clearly RII is much more tolerant of
substitutions in the N-terminal site, as many point mutations in this region will abolish high-affinity binding of RIIα. Without a structure of this peptide bound to the RIIα D/D domain, however, we cannot interpret those specificity differences more precisely. We can, however, address specificity differences in the hydrophobic groove, specifically the docking site for Val13P.

In contrast to the RIIα subunit, the RIIβ subunits are much more accommodating in terms of the AKAP sequences they can tolerate for binding. Many single point mutations will abolish RI binding; however, the peptide arrays revealed only one site in the D-AKAP2 peptide that strongly discriminates selectively against RIIα. This site is Val13P. RI subunits readily accept a Trp at

Figure 6. Crystal and NMR Structures Reveal Conserved Features for AKAP Docking to RIIα and Provide Insight into RI Binding

(A) Both dimer I and dimer II of the crystal structure (in red) are aligned with NMR structures of RIIα D/D bound to Ht31 (gray) and AKAP79 (olive) (Newton et al., 2001). The structures are aligned based purely on helix I and helix I'. The AKAP peptides differ in helical register.

(B) The sequences of D-AKAP2, Ht31, and AKAP79 involved in RIIα binding are aligned according to the structural alignment. The two turns of the helix that are critical for binding the D/D domain are shown in the box. The arrow points to Val13P, which is important for RI versus RII specificity.

(C) Despite the diversity of sequence, these AKAPs display a hydrophobic ridge that interacts with the D/D of RIIα. The two turns of the helices that are crucial for binding are boxed for each. Ht31 and AKAP79 peptides are each taken from one structure in an ensemble. The C terminus of AKAP79 is not illustrated as a ribbon because it does not form a well-ordered helix in the NMR structures. The axis of each peptide helix is shown to demonstrate that the D/D domain can accommodate different orientations of the AKAP helix.

(D) A cartoon representation of the D/D domain of RIIα subunits demonstrates the presence of a stable core and two flexible N-terminal tails. Upon binding to an AKAP, one of these tails is stabilized. RI subunits possess two conserved cysteines (Cys16R and Cys37R), which can stabilize the N-1 helix when disulfide bonded. Cys16R and Cys37R are located in analogous positions to Ile3R and Leu21R of RIIα (Banky et al., 2003), which are essential for binding. Crystal structures of RIα D/D may detail the specific involvement of these residues in AKAP docking.
this position, but replacing Val13P with Trp reduces RII affinity by 40-fold (Burns-Hamuro et al., 2003). Our crystal structure shows why Trp is too bulky to fit in the hydrophobic groove and would disrupt peptide docking by causing steric clashing with the surface created by Thr10R, Leu13R, Thr17R, and Leu13 R (Figures 7A and 7B). Because helices I and I align very well in RI and RII in the NMR structures, we aligned the NMR structure of the RI D/D domain with our crystal structure. The homologous surface on RI (created by Gln26R, Leu29R, Ile33R, and Leu29 R) forms the surface highlighted in yellow and can accommodate the bulky Trp side chain (Figure 7C). The sequence of the V13W mutation is shown above. This is located in the segment of the peptide that is critical for binding the D/D domains. The surfaces of the wild-type and mutant peptides are quite different because the Trp protrudes from the peptide surface. The specificity of this mutation is demonstrated in vivo. HeLa cells were transfected with wild-type, null, RI-, or RII-specific versions of the D-AKAP2 peptide fused to a mitochondrial targeting sequence. Localization of these constructs is shown in the column on the left. RII was stained with fluorescent antibodies, which is shown in the middle column. Each AKAP construct was targeted to the mitochondria; however, only the wild-type and RII-specific constructs successfully redirected the RII away from the golgi and to the mitochondria.

Figure 7. AKAP Specificity for RII Subunits of PKA Can Be Toggled by Val13P Mutations
(A) Val13P, highlighted in black, binds to the surface of the D/D domain at the center of the hydrophobic groove comprised of Thr10R, Leu13R, Thr17R, and Leu13R, which are highlighted in green.
(B) Mutating Val13P to Trp eliminates binding to the RII D/D domain, as shown previously (Burns-Hamuro et al., 2003). Modeling this mutation into the D-AKAP2 in our structure causes steric clashes with the D/D domain; therefore, we believe this mutation disrupts the hydrophobic packing within the interface.
(C) By modeling our structure with the NMR structure of the RI D/D domain (Banyk et al., 2003), we observe a pocket in the RI D/D domain that can accommodate the V13W mutation. This is consistent with peptide array data. Gln26R, Leu29R, Ile33R, and Leu29 R form the surface highlighted in yellow and can accommodate the Trp mutation in RII.
(D) The sequence of the V13W mutation is shown above. This is located in the segment of the peptide that is critical for binding the D/D domains.
(E) The specificity of this mutation is demonstrated in vivo. HeLa cells were transfected with wild-type, null, RI-, or RII-specific versions of the D-AKAP2 peptide fused to a mitochondrial targeting sequence. Localization of these constructs is shown in the column on the left. RII was stained with fluorescent antibodies, which is shown in the middle column. Each AKAP construct was targeted to the mitochondria; however, only the wild-type and RII-specific constructs successfully redirected the RII away from the golgi and to the mitochondria.

Plasticity of the D/D Domain and the AKAP Facilitates the Diversity of cAMP Signaling
More than 50 structurally diverse, but functionally similar, AKAP family members have been discovered to date (Michel and Scott, 2002). All AKAPs contain an amphipathic helix of 14–18 residues, which binds to the
N-terminal D/D domain of the R subunits of PKA (Carr et al., 1991; Newton et al., 1997, 2001). The crystal structure of the RIIz D/D domain and D-AKAP2 provides insight into how RIIz binds such a wide array of AKAPs with high affinity. In the structure, the N terminus of one protem of RIIz is tightly bound to the AKAP whereas the other is disordered (Figure 6D). The malleability of the N terminus and the presence of two potential docking modes in the dimer enable the D/D domain to accommodate various sequences of AKAP peptides. The variability in helical register for bound AKAPs in the crystal and NMR structures confirms the plasticity and versatility of the D/D domain. In addition, peptide array data showed that only four residues on D-AKAP2 have strict sequence requirements for binding RIIz. The structure demonstrates how the RIIz D/D binding pocket can accommodate various side chains at the other positions of the AKAP peptide, especially at the N terminus. This tight interaction can be summarized as a stable helix docking to a bipartite surface, where the surface created by helix I is rigid and the N-terminal tail is flexible (Figure 6D). The plasticity of the D/D domain, conveyed by the flexible N termini, is likely to be crucial for facilitating PKA interactions with numerous AKAPs, allowing for amplified cAMP signaling at many cellular microdomains.

D-AKAP2 also has enhanced versatility compared to most AKAPs, because it can bind to both RI and RI isoforms with high affinity. Although the hydrophobic cores of RIIz and RIIz are superimposable, the RI isoforms contain an abundance of charges on their AKAP binding surface (Banky et al., 2003). In addition, the N terminus of the RI subunit contains a well-ordered helix, called the N-terminal extended tail in RIIz. The flexible N terminus of the RIIz subunit is positioned by two conserved prolines (Pro6 and Pro7) that are linked to the hydrophobic core by an intervening glycine. In contrast, the N terminus of RIz is regulated by an intramolecular disulfide bond. The disulfide bond between Cys16R and Cys37R provides a stable anchor to the C terminus of helix I of the opposite protomer. Surprisingly, these two cysteines correlate spatially with Ile3 R and Leu21 R in the RIIz structure (Figure 6D). The N terminus of RI is clearly more flexible when the cysteines are reduced. Moreover, the linker between the N-1 helix and helix I is dynamic, based on NMR structures (Banky et al., 2003). Crystal structures of RIIz D/D-AKAP2 complexes will reveal how D-AKAP2 can adapt to such differences. We expect that a network of both hydrogen bonds and hydrophobic contacts the N terminus of the RIIz D/D-AKAP2 complex.

Concurrent with our studies, a crystal structure of the RIIz D/D domain complexed with AKAP-IS, an AKAP peptide engineered for enhanced affinity and specificity for binding RII subunits was solved (Gold et al., 2006 [this issue of Molecular Cell]). As seen in Figure S2, the D/D domain of this structure aligns closely with ours. Based on the alignment of the D/D domains, the AKAP peptides align in register such that the critical residues of D-AKAP2 for R binding (Ile8R, Ala9R, Ile12R, and Val13R) are located in the same space as Leu8P, Ala9P, Ile12P, and Val13P of AKAP-IS. This structure provides independent confirmation that these positions are structurally conserved, including Val13P, which is crucial for RII specificity. In addition, the AKAP-IS structure reveals ordering of Ile8 in only one protomer of the RIIz, which contacts the N terminus of the AKAP-IS, and thus independently confirms our model for binding. The AKAP-IS structure, however, is anchored by additional engineered hydrogen bonds and hydrophobic contacts that are not present in our structure.

Experimental Procedures

Protein Expression and Purification

Rattus norvegicus RIIz D/D (I–44) (Figure 1B) was cloned into a pET15b vector (Novagen) downstream of the His tag and thrombin cleavage sequence, using Ndel and BamHI restriction sites. The clones were expressed in BL21 (DE3)-competent E. coli (Stratagene) and grown in YT media containing 10 μg/ml ampicillin. Cells were grown to OD600 = 0.8 and then induced for 1 h with 1 mM IPTG for PII. Cells were resuspended in 20 mM Tris, 100 mM NaCl and ruptured in a French pressure cell. The His-tagged RIIz D/D was purified with ProBond resin (Invitrogen) and eluted with 10 mM EDTA. The His tag was then cleaved with thrombin for 3 h. The protein was purified further by using a Mono S cation exchange column in 20 mM potassium phosphate (pH 5.0) with a gradient of 10–500 mM KCl followed by gel filtration on an S75 column (Pharmacia). Gel filtration was performed in the final buffer consisting of 10 mM Na acetate (pH 5.0), 75 mM NaCl, 1 mM TCEP, and 10% glycerol. The protein was quantified based on the extinction coefficient 5960 M−1 cm−1 at 280 nm.

Crystallographic Data Collection and Refinement

Data was collected at cryogenic temperature (100 K) by using 20% glycerol as a cryoprotectant. Using the same single crystal, two complete data sets were collected, first on a rotating anode X-ray generator at UCSF, and on synchrotron beamline 5.0.3 of the Advanced Light Source, Lawrence Berkeley National Labs (Berkeley, California) (Table 1). Data were processed by using Denzo/HKL2000 (Otwinowski and Minor, 1997). Due to dense ice rings at 3.75, 2.24, and 1.92 Å resolution, data bins corresponding to these Bragg spacings showed 63.5%, 60.8%, and 56.4% completeness, respectively. The final data, however, showed a completeness of 87.9% for the resolution range of 35.0–1.60 Å. The initial model for the molecular replacement was constructed by using the least-varied region of the RIIz dimer from the NMR ensemble (residue 100–440) (PDB access code 2DRN) (Newlon et al., 2001). Molecular replacement using MOLREP (Vagin and Teplyakov, 1997) of the CCP4 package (CCP4, 1994) found two dimers in the asymmetric unit using all data between 25.0 and 2.5 Å resolution. The resulting electron density map showed clear density for each dimer, but not D-AKAP2 peptides. After a few rounds of manual model building followed by refinement with REFMAC5 (Murshudov et al., 1997), the improved phases clearly showed additional density for the peptides, at the periphery of each dimer that was used to model the peptides. After the addition of 214 waters using ARP/WARP (Lamzin and Wilson, 1993), the final refinement implementing TLS refinement (Winn et al., 2001) for each chain converged to R and Rfree values of 0.208 and 0.238, respectively, with excellent geometry.
(Table 1). The final model includes residues 1–43 of one protomer and residues 5–43 of the other protomer. Both molecules in the asymmetric unit are nearly identical with an rmsd of 0.17 Å for the 96 equivalent Cα atoms. The shorter protomer is denoted with an (’) for the sake of distinguishing between the two. Residues 2–22 and 2–20 were included for the AKAP peptides. Molecular representations in Figures 2, 3, 4, 6, and 7 were made with PyMOL (DeLano Scientific) (DeLano, 2002).

Amide H/D Exchange Coupled with Mass Spectrometry

The H/D exchange experiments performed on D-AKAP2 and RIIα/D/D domain were previously described (Burns-Hamuro et al., 2003).

Peptide Array Binding Studies

Peptide array experiments were performed with the C terminus of D-AKAP2 as previously described (Burns-Hamuro et al., 2003).

Immunofluorescence Studies

The targeting constructs containing the C-terminal 156 residues of D-AKAP2 with different mutations have a 30 residue mitochondrial targeting sequence from D-AKAP1α on the N terminus and a Flag tag on the C terminus as described previously (Burns-Hamuro et al., 2003). These constructs were used to transfect HeLa cells with Polyfect (Qiagen) according to the manufacturer’s instructions. The cells were fixed with 4% paraformaldehyde for 10 min, 15 hr after transfection. The cells were then treated with 0.2% Triton X-100 in PBS for 5 min before staining with antibodies sequentially in 1% BSA in PBS. A rabbit antibody against RIIα (Santa Cruz) and a fluorescent-conjugated secondary antibody both at a 1:100 dilution were used to stain endogenous RIIα. A cryo-conjugated anti-Flag antibody (Sigma) at a 1:4000 dilution was used to stain the transfected targeting constructs. The images were taken with a confocal microscope (BioRad MRC1000).

Supplemental Data

Supplemental Data include two figures and three movies and can be found with this article online at http://www.molecule.org/cgi/content/full/24/3/397/DC1/.

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Accession Numbers

The coordinates have been deposited in the Protein Data Bank under the accession code 2HWN.