Related Protein–Protein Interaction Modules Present Drastically Different Surface Topographies Despite A Conserved Helical Platform

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The subcellular localization of cAMP-dependent protein kinase (PKA) occurs through interaction with A-Kinase Anchoring Proteins (AKAPs). AKAPs bind to the PKA regulatory subunit dimer of both type Iα and type IIα (RIα and RIIα). RIα and RIIα display characteristic localization within different cell types, which is maintained by interaction of AKAPs with the N-terminal dimerization and docking domain (D/D) of the respective regulatory subunit. Previously, we reported the solution structure of RIIα D/D module, both free and bound to AKAPs. We have now solved the solution structure of the dimerization and docking domain of the type Iα regulatory dimer subunit (RIα D/D). RIα D/D is a compact docking module, with unusual interchain disulfide bonds that help maintain the AKAP interaction surface. In contrast to the shallow hydrophobic groove for AKAP binding across the surface of the RIIα D/D dimeric interface, the RIα D/D module presents a deep cleft for proposed AKAP binding. RIα and RIIα D/D interaction modules present drastically differing dimeric topographies, despite a conserved X-type four-helix bundle structure.

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Introduction

A prototypic example of a kinase which is involved in the supramolecular assemblies that regulate functions is cAMP-dependent protein kinase (PKA). PKA is anchored to specific targeted scaffold proteins in the cell (reviewed by Colledge & Scott¹). Compartmentalization ensures the fidelity and specificity of this enzyme. The PKA holoenzyme is composed of two catalytic (C) subunits held in an inactive conformation by association with a regulatory (R) subunit dimer.² The subcellular localization of the holoenzyme is mediated by protein–protein interactions between the R subunit dimer and A-Kinase Anchoring proteins (AKAPs).³ The multiplicity of R isoforms, each encoded by unique genes, suggests isoform-specific functional differences.³–⁸ These isoforms are not functionally redundant. For example, when the gene encoding RIIβ is deleted in mice, RIIα compensates.⁹,¹⁰ However, these mutant mice display a lean phenotype and the tetrameric RIα holoenzyme (RIα₂C₂) demonstrates greater cAMP sensitivity and increased kinase activity.¹⁰ Even though RIα is capable of rescuing the RIIβ knockouts, the converse is not true. RIα null mutants display early embryonic lethality with severe developmental

Abbreviations used: PKA, cAMP-dependent protein kinase; AKAP, A-Kinase anchoring protein; D-AKAP1, dual specificity AKAP1; R, cAMP-dependent protein kinase regulatory subunit; C, cAMP-dependent protein kinase catalytic subunit; RIα, Type Iα regulatory subunit of cAMP-dependent protein kinase; RIIα, Type IIα regulatory subunit of cAMP-dependent protein kinase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; GST, glutathione-S-transferase; NMR, nuclear magnetic resonance; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; CSI, chemical shift index; HSQC, heteronuclear single quantum coherence; RMSD, average root-mean-square deviation.

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abnormalities\textsuperscript{9}, indicating that Rlα stabilization may safeguard the cell. Mutations in the gene encoding Rlα cause both familial cardiac myxomas and Carney complex.\textsuperscript{11,12} These tumors demonstrate a reversal in ratios of the Rlα to RIIβ proteins. Rlα was also identified as within the tissue-specific extinguisher 1 (TSE-1), which down-regulates seven liver genes in cAMP-mediated gene expression.\textsuperscript{13,14}

Subcellular localization of PKA is also isoform-specific. While the RII subunits are mostly localized in particulate fractions, Rlα isoforms are predominantly in the cytosol.\textsuperscript{15–17} However, Rlα can also be localized \textit{in vivo}, frequently in a highly transient manner. For example, Rlα is associated with the plasma membrane of human erythrocytes\textsuperscript{18,19}, the “cap” site of activated T and B lymphocytes\textsuperscript{20,21}, the cardiac myocyte sarcolema\textsuperscript{19}, the activated EGF-receptor\textsuperscript{22}, and the fibrous sheath of mammalian spermatozoa.\textsuperscript{23} Rlα accumulates at the neuromuscular junction (NMJ) of skeletal muscle\textsuperscript{24} and is associated with microtubules during the entire cell cycle.\textsuperscript{25}

Furthermore, while initially AKAPs were isolated using RII, the identification of dual-specific AKAPs that can bind with high affinity to both RII and Rlα provides a mechanism for both isoforms contributing to an integrated signaling complex.\textsuperscript{26,27} Angelo & Rubin have identified AKAP CE, the first eukaryotic RI-specific binding protein.\textsuperscript{28,29} Another apparently Rlα-specific AKAP was recently identified\textsuperscript{30}; PAP7 binds to the peripheral-type benzodiazapiene receptors, is associated with cholesterol metabolism, and localizes to mitochondria. Mutation of Rlα amino acids involved in binding AKAPs abolished Rlα localization to the NMJ, stressing further the physical importance of the targeting of Rlα \textit{via} AKAP interactions.\textsuperscript{31}

To gain a molecular understanding of the isoform-specific functions of the type I and type II regulatory dimer subunits, we chose to study the N-terminal domains, 12–61 and 1–44, respectively. These domains are necessary and sufficient for dimerization and docking to AKAPs\textsuperscript{3,27,32–36}, and hence we refer to these modules as the Dimerization/Docking (D/D) domains. Previously, we have reported and analyzed the solution structure and the backbone dynamics of RIIa D/D, both free and bound to AKAPs.\textsuperscript{33,37–40} The first structural characterization of the Rlα D/D domain was based on solution NMR assignments and secondary structure analysis\textsuperscript{41}, which indicated a shared helical scaffold with Rlα, but distinct differences in secondary structure in the N-terminal amino acid residues.

We have now solved the solution structure of the dimerization and docking domain of the type lα regulatory dimer subunit (Rlα D/D). The structure of Rlα D/D, like that of RIIa D/D, is an X-type four-helix bundle. However, with its highly unusual interchain disulfide bonds, Rlα D/D forms a more compact docking module and presents a surface for AKAP interactions different from that previously seen. In contrast to the shallow hydrophobic groove across the surface of the RIIa D/D dimeric interface, the Rlα D/D structure forms a deep cleft. Isoform-specific dimeric topographies between type lα and lβ provide differing protein–protein interaction surfaces with distinct biological consequences.

**Results**

Some structural information on Rlα D/D is already available. Disulfide bonds were found to exist between Cys\textsuperscript{16} and Cys\textsuperscript{37} to create an antiparallel linkage between protomers.\textsuperscript{42} A high helical content had been assessed, based on CD.\textsuperscript{34} Solution NMR assignments and secondary structure analysis revealed isoform-specific differences at the N terminus.\textsuperscript{41} However, in order to provide a molecular basis for the biological effects of PKA targeting, high-resolution structural data are necessary. In particular, the packing and orientation may have profound consequences for the recognition surface presented to the AKAPs.

**Structure determination**

As the D/D domain (residues 12–61) and full-length Rlα bind with equal affinity to RPP8, the kinase binding domain of DAKAP2, we pursued structural studies on this fragment. We employed
three-dimensional, triple-resonance NMR methods in order to determine the solution structure of RIA \((12–61)\) D/D. A total of 435 distance, including 37 backbone dihedral and 13 hydrogen bond restraints (per protomer) were used to generate an ensemble of 21 structures with program X-PLOR 3.85143,44 (Figure 1). Structural statistics (Table 1) and the best-fit superposition of the 21 structures (Figure 1(A)) demonstrate that the structures are well defined and in excellent agreement with the NMR data, with no NOE violations.

### Table 1. Structural statistics and atomic RMSD differences

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<th>Structural restraints</th>
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### RMSD’s

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<td>(25–37, 25'–37') and (46–55, 46'–55')</td>
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<td>(25–37)</td>
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### Energy statistics

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<td>NOE</td>
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* Indicates that only one protomer was superimposed.

Table 1. Structural statistics and atomic RMSD differences

**Structure overview**

The D/D domain of RIA is a highly compact X-type four-helix bundle. The structure of each protomer begins with a short helix (helix N-1; residues 14–22) followed by a turn into a helix-turn-helix (Helix I, residues 25–39 and Helix II, residues 45–58) motif. Helix I and II serve as the platform for dimerization and formation of the four-helix bundle (Figure 1). Helices I and II of protomer 1 interact with or stack against Helices I' and II' of protomer 2 (Figure 2(B) and (C)). The protomers are oriented antiparallel to each other, with helix–helix interactions alternating between antiparallel and orthogonal for the four helices around the bundle. There is also extensive ordering of hydrophobic residues in the core (Figure 2(D) and (E)). Two inter-molecular disulfide bonds lock in the antiparallel orientation by linking Cys 16 in the N-1 helix of one protomer to Cys 37 in Helix I of the other protomer. This tethers helix N-1 to the four-helix bundle, making for a more compact module. It also helps to create an isoform-specific deep cleft, with surface residues of a different character from the RII D/D (see Discussion).

**Helix N-1 is isoform-specific**

Limited long-range restraints were detected for helix N-1 (residues 14–22). As seen in Figure 1(A), its absolute position is also the least clearly defined region in the ensemble of structures. However, overlays of helix N-1 (Figure 1(B)) indicate that the helix is well formed, even though its orientation with respect to the core is less well defined. Intermolecular NOEs between helix N-1 and Helix I and the disulfide linkage define the packing of helix N-1 in the quaternary structure. Lack of solvent protection for helix N-1 suggests a mobile region. His and Asn, which are the transition residues from helix N-I to Helix I, potentially serve as a hinge.
Helix N-1 at the N terminus is isoform-specific to the RI subunit and is necessary for function. Deletion of residues 1–17, including Cys16, does not abolish oligomerization or AKAP binding of R1α but deletion of residues 1–22 yields a monomer/dimer in equilibrium.35 Mutation of Tyr19 to Ala also abolishes dimerization in C. elegans R subunits.28 Additionally, mutation of Val20 to Ala, Ser21 and Leu24 (backbones shown in tan). Leu24(white), Phe25(green), and Phe27 (green) make key hydrophobic interactions which stabilize the dimer interface. (E) Interactions between Helices I and I′ and between Helices II(I′) and II(I′). Phe25, Phe25′ (green) and Tyr26′ from Helices II(I′) present a hydrophobic scaffold for Helix I(I′)–II(I′) interactions. Helix I–I′ interactions are composed mostly of long chain aliphatics (Val10, Ile25, Leu28 and Leu36).

Figure 2. Quaternary structure and packing interactions in R1α (12–61). (A) The D/D domain is comprised of two subdomains. Subdomain 1 (tan ovals) is critical for anchoring interactions. Subdomain 2 (red ovals) is composed of residues from Helices II (I′) which mediate key protomer–protomer contacts. Turn regions are shown in white and the isoform-specific region in R1α is shown in yellow. Residues that affect anchoring interactions are marked with a star. Dimerization is disrupted upon substitution of Phe47 and Phe52 to Ala (red arrows), within the context of a disulfide minus variant. (B) Ribbon diagram of R1α (12–61) shows the near antiparallel packing of the helices (orientation as in Figure 1(A)). Helix N-1 from each protomer is in yellow, Helices I and I′ are in grey, and Helices II and II′ in red. (C) 90° rotation about the x-axis of the view presented in (A). The alternating nearly antiparallel and nearly orthogonal packing of helices (I, I′, II and II′), characteristic of the X-type four-helix bundle, are apparent in this view. (D) Helical packing interactions between Helices II and II′. Key residues mediating interactions at the dimer interface are highlighted. Tyr51 (yellow) is within hydrogen bonding distance to the backbone of

Figure 3. Disulfide bonds in R1α (12–61). Ribbon diagram of R1α (12–61) D/D in a surface rendering shell. One protomer is in white and the other is in grey. Disulfide bonds are in yellow and Tyr19′, which stabilizes the disulfide bond, is shown in red. Conserved aromatic residues and important in mediating hydrophobic interactions rather than disulfide bond stabilization are shown in yellow.
in helix N-1, is sufficient to disrupt anchoring. Residues within helix N-1 contribute to the stability of the dimer formed by Helix I and II and are necessary for docking as well as dimerization.

Helix I and II form a conserved hydrophobic core

Helix I and II interact through hydrophobic interfaces to form a core that is conserved across all R subunits. The structure of Rla D/D, as with the structure of RIIa D/D, shows a hydrophobic core within a compact four-helix bundle (Figure 2(D) and (E)). Helix I (residues 25–39), which contributes the portion of the AKAP binding surface conserved along isoforms, makes important inter- and intra-molecular contacts in the core as does Helix II (residues 45–57), which is involved in dimerization. These strong, hydrophobic and non-covalent intermolecular contacts stabilize the antiparallel orientation of the monomers. With the exception of the disulfide bonds (see below) and a single hydrogen bond between Tyr41 in Helix II and the backbone atoms of Leu28 and Ser32 in Helix I, all helix packing interactions shown are hydrophobic (Figure 2(D) and (E)).

Disulfide bonds are isoform-specific

The inter-monomer disulfide bonds between Cys16 in helix N-1 and Cys37 in Helix I (Figure 3) reinforce, but do not ordain, monomer–monomer interaction along the dimer interface. An antiparallel alignment of the protomers had been predicted earlier by analysis of the disulfide linkages. However, although the disulfide bonds orient the protomers into an antiparallel alignment, they do not contribute to the packing of the two protomers.

Discussion

The mechanisms by which extracellular signals are relayed from the plasma membrane to specific intracellular targets form the basis of cellular regulation. The specificity of signal transduction is achieved by a coordinated assembly of multiprotein complexes. Scaffold, adapter, and anchoring proteins orchestrate the pleiotropic effects of kinases and phosphatases, which routinely possess broad substrate specificities. These proteins provide specific protein–protein and protein–phospholipid interactions, mediated by conserved protein modules.

![Figure 4](image-url)

**Figure 4.** Structural analysis of conserved residues in the Type I D/D domains. (A) Sequence alignment of the Type I D/D domains. From top to bottom, pig Rla, bovine Rla, human Rla, mouse Rla, rat Rla, mouse Rlb, rat Rlb, human Rlb, apllysia, Drosophila, and C. elegans R subunits. Conserved hydrophobic residues are highlighted in yellow. Val20 and Ile25 disrupt AKAP binding upon Ala substitution and are marked with a red circle. The residues involved in disulfide bond formation (Cys16 and Cys37) are marked with a black circle; Cys37 is also marked with a red asterisk, as mutation of this residue disrupts AKAP interaction. Y19, F47 and F52 are marked with a green circle as upon Ala substitution, dimerization is disrupted. (B) Conserved hydrophobic residues in the RI family are shown in yellow on the RIa ribbon structure, except for Val20 and Ile25 in red, and Tyr41, Phe47 and Phe52 in green. (C) 90° rotation about the x-axis of the view presented in (B). Residues are colored as described in (B). (D) Ribbon diagram of Rla D/D with the side-chains of key conserved residues. The view and coloring presented are the same as described in (B).
protein modules, with features both typical and atypical.

**Covalent linkages**

RLα D/D has unusual interchain disulfide bonds that help maintain the AKAP interaction surface. The disulfide bonds are extremely stable (up to 8 M urea), but once reduced, the protein does not dissociate into monomers nor does mutation of the cysteines prevent dimerization. This is not surprising, given the contribution of hydrophobic and aromatic amino acids at the dimer interface (see below). The unusual stability of the disulfide bonds in RLα can be explained by the packing of the aromatic ring of Tyr against the disulfide bond (see Figure 3). It is the only aromatic residue within hydrogen bonding distance to the backbone carbonyl of Cys in a subset of the calculated structures. Aromatic–disulfide interactions are very localized, and a sulfur atom can interact favorably with an aromatic ring via S–π interactions. Tyr in RLα is conserved in all species (Figure 4A). When Tyr is replaced in RLε (which is homologous to bovine RLα) with either tryptophan or phenylalanine, the protein is still capable of disulfide bond formation. Mutation of the tyrosine to any non-aromatic amino acid, however, yields a protein that is no longer disulfide bonded or even dimeric (Rubin, 2000, personal communication).

**Non-covalent dimerization determinants**

Sequence alignment of the RL family across several species (Figure 4A) shows conserved hydrophobic residues located in the Helix I and Helix II. Well-ordered hydrophobic interactions along the dimer interface seen in the RLα D/D structure (Figure 4B–D) maintain dimerization and are reinforced by the inter-monomer disulfide bonds. In the absence of disulfide bond formation, an increase in ionic strength stabilizes the dimeric bundle, which is consistent with the importance of hydrophobic interactions in the stability of the associated state. The contributions of specific residues to the stability of the dimer were analyzed in the background of the CysAla sequence variant, which disrupts the disulfide link but maintains a stable dimer capable of interacting with AKAPs. Residues forming an integral network of hydrophobic interactions at the dimer interface include Leu, Cys, Val, and Ile in Helix N-1, Ile, Leu, and Cys in Helix I, and Ala, Leu, Tyr, Phe, and Val in Helix II (Figures 4B–D and 2D).

A single turn and a half of Helix II, from 47–53, appears to pin the core together through its multiple contacts with Helix I and Helix I. Phe on Helix II is centrally located in the dimer interface and has numerous intermolecular NOEs with Helix II, including Phe (Figures 2D, 4B–D)). An alanine substitution at this site severely compromises dimerization of RLα in the absence of a disulfide bond. Phe on Helix II faces Helix I to promote intramolecular hydrophobic interactions. Replacement of Phe with Ala also hinders dimerization. Additionally, an important intermolecular interaction between Arg and Glu in Helix II and II' is observed. Finally, the hydroxyl proton of Tyr on Helix II forms an intramolecular hydrogen bond to the carbonyl backbone of Leu and the amide backbone of Ser in Helix I (Figures 2D, 4B–D)), which is the only tertiary H-bond not detected as part of a secondary structure pattern.

**An isoform-specific AKAP docking surface**

Helix N-1 is orthogonal to the normal of Helix I and I'. Note in Figure 5 (Helix N-1 is colored in yellow) how this creates a deep cleft that is part of the surface for AKAP binding. This is noticeably different from the orientation of the N-terminal extended strand to Helix I and I' in the RLα D/D domain (see below). Sequence alignment indicates the conservation of hydrophobic residues along Helix I among the various RLα D/D domains (Figure 4A). These residues, Ile, Leu, Leu, Ile, Val, Leu, and Cys, probably combine to provide a recognition surface as sequence...
alignments indicate complementary conservation of hydrophobic residues in the R binding regions of representative AKAPs (Figure 6). This is seen for both dual and RI-specific AKAPs. Replacement of Ile\textsuperscript{25} with Ala in Helix I is sufficient to abolish anchoring.\textsuperscript{56} Mutation of Val\textsuperscript{29} in Helix N-1 and Ile\textsuperscript{26} and Cys\textsuperscript{27} in Helix I to Ala either abolishes or reduces AKAP binding.\textsuperscript{35} Placement and orientation of these residues is shown in Figure 2(E). The contrast in the topography of a deep cleft in R\textsubscript{a} and a shallow hydrophobic groove in R\textsubscript{II} may explain differences in AKAP binding and therefore anchoring of PKA anchoring at the structural and biological level.

### The RI-D/D recognition elements

Information is available regarding R\textsubscript{a} binding proteins.\textsuperscript{26,27} To date, R\textsubscript{a}-specific binding proteins are AKAP\textsubscript{CE} in \textit{C. elegans},\textsuperscript{26,28} an RI-specific binding domain in the fibrous sheath protein in spermatozoaion,\textsuperscript{23} and PAP7 in mouse, humans, and rats.\textsuperscript{30} Other AKAPs capable of interacting with both R-subunits have been cloned.\textsuperscript{23,26,27,33} The binding affinities of R\textsubscript{a} for dual-specific DAKAP-1 and -2 are in the nanomolar range.\textsuperscript{34} The sequences of the R binding regions of RI-binding AKAPs vary (see examples in Figure 6), but all are predicted to form an amphiphatic \(\alpha\)-helix. Two RI-specific AKAPs, Ht31 and AKAP79, had different sequences but formed amphiphatic \(\alpha\)-helices upon binding.\textsuperscript{35,36}

### A molecular model for DAKAP–R\textsubscript{a} interaction

The cleft in R\textsubscript{a} (Figure 5) presents a distinct surface that should complement the proposed bound helical conformation of AKAP\textsubscript{CE}. Helix N-1 comprises part of the AKAP binding surface of R\textsubscript{a} and most likely repositions itself once the AKAP is bound, given its inherent flexibility relative to the core. Within AKAP\textsubscript{CE}, a central dipeptide, Phe\textsuperscript{243}-Ser\textsuperscript{244} is critical for interaction with R\textsubscript{a}.\textsuperscript{28} Mutation of the Phe to another aromatic residue maintains high affinity binding, whereas mutation to a non-aromatic residue suppresses binding. Cavities within the binding surface of R\textsubscript{a} could pack an aromatic residue and stabilize binding. Additionally, mutation of Phe\textsuperscript{243}-Ser\textsuperscript{244} in AKAP\textsubscript{CE} to Leu-Val switches its specificity from R\textsubscript{a} to R\textsubscript{II}. Long aliphatic chains in different AKAPs produce most of the NOEs to the R\textsubscript{II} D/D.\textsuperscript{38} For R\textsubscript{a} D/D, it appears that the recognition surface cannot accommodate aliphatic chains, but can accommodate the different steric requirements of an aromatic ring.

### Comparison of R\textsubscript{a} and R\textsubscript{II} D/D domains

The solution structure of R\textsubscript{II} (1–44) D/D, both free\textsuperscript{25} and bound\textsuperscript{39}, has been previously determined. Sequence alignment between R\textsubscript{a} and R\textsubscript{II} shows that a large percentage of residues are either conserved or are conservatively substituted (Figure 7). R\textsubscript{a} (12–61) D/D and R\textsubscript{II} (1–44) D/D have a shared helical scaffold and the overall domain organization is a helix-turn-helix motif (Figure 8(A) and (B)). Both have an overall global fold of a four-helix bundle. However, structural differences between the isoforms in the N-terminal domain lead to differences in surface topography (Figure 8(C)) and surface potential (Figure 8(D)).

Certain features are critical for dimer formation in both R\textsubscript{a} and R\textsubscript{II}. Based on the R\textsubscript{II} (1–44) D/D structure, residues forming important dimer contacts include Ile\textsuperscript{25}, Pro\textsuperscript{6}, Leu\textsuperscript{9}, Leu\textsuperscript{12}, and Tyr\textsuperscript{16}, Val\textsuperscript{29}, Leu\textsuperscript{21} in Helix I and Leu\textsuperscript{28}, Val\textsuperscript{29}, Ala\textsuperscript{32}, Val\textsuperscript{33}, Phe\textsuperscript{36}, Thr\textsuperscript{37} and Leu\textsuperscript{39} in Helix II. There is a striking similarity among these residues between R\textsubscript{a} and R\textsubscript{II}. In particular, Leu\textsuperscript{28}, Leu\textsuperscript{29}, Leu\textsuperscript{36}, Cys\textsuperscript{37} in Helix I of R\textsubscript{a} (Leu\textsuperscript{12}, Val\textsuperscript{13}, Val\textsuperscript{29}, Leu\textsuperscript{21} in R\textsubscript{II}), and Leu\textsuperscript{36}, Phe\textsuperscript{39}, and Leu\textsuperscript{39} in Helix II (Ala\textsuperscript{32}, Phe\textsuperscript{36}, Leu\textsuperscript{39} in R\textsubscript{II}), guide the antiparallel packing of the helices. These residues are either conserved or are conservatively substituted (Figure 7). The placement and orientation of these residues are shown in Figure 8(B), illustrating the similarity in core packing for both isoforms. One can appreciate the significance of the extensive hydrophobic interactions in maintaining the overall similar tertiary and quaternary fold. For both R\textsubscript{a} and R\textsubscript{II}, Helix I and II provide the hydrophobic core of the dimer interface.

The molecular surface of the putative AKAP binding surface in R\textsubscript{a} (12–61) is quite different relative to the surface presented to a typical anchoring peptide in R\textsubscript{II} (1–44) (Figure 8(C) and (D)). There is only one apparent groove where the peptide could fit in R\textsubscript{a}, based on AKAP binding studies and in analogy with R\textsubscript{II} (1–44)-AKAP interaction. However, the angle at which an AKAP would lie on the surface of R\textsubscript{a} (12–61) would depend on the specific molecular contacts. The tethering of Helix N-1 to Helix I in R\textsubscript{a} (12–61) has reoriented the surface topography and changed the overall protein shape from the extended structure seen in R\textsubscript{II} (1–44) to a more
Figure 7. Sequence alignments of R₁α and R₂α D/D domains. From top to bottom, bovine: bR₁α (P00514), human: hR₁α (P10644), Rattus norvegicus: rR₁α (P09456), Sus scrofa: pR₁α (P07802), Drosophila melanogaster: Dros R₁α (P16905), C. elegans: C.E. (P30625), human: hR₂β (P31321), rat: rR₂β (P81377), M. musculus: mR₁β (P12849), R. norvegicus: rR₂α (AAM97689), M. musculus: mR₂α (P12367), D. melanogaster: Dro R₁α (AA86976), strongylocentrotus purpuratus: S.U.RII (Q26619), and bovine: bR₂β (P31322). Sequence computation was performed using the BLAST network service. Hydrophobic residues are colored yellow, polar residues colored green, positively charged residues in blue and acidic residues in red.
**Figure 8.** Comparison of the RÎ± and the RÎ± D/D domains. (A) Ribbon representation of bovine (P00514): bRÎ± (12–61) (left) and *R. norvegicus* (AAM97689): rRÎ± (1–44) (right). The isoform specific region is shown in yellow, Helices I and I’ in tan, and Helices II and II’ in red, respectively. (B) Comparison of key residues in the hydrophobic core important for dimer contacts in RÎ± (12–61) (left) and RÎ± (1–44) (right). (C) GRASP surface rendition of RÎ± (12–61) (left) and RÎ± (1–44) (right) are shown. The isoform-specific N-terminal region is shown in yellow. (D) Electrostatic surface potential representation of RÎ± (12–61) (left) and RÎ± (1–44) (right). Acidic residues are red; basic residues are blue.
globular one (Figure 8(C) and (D)). Residues in the putative AKAP binding surface of RIα are much less accessible to intermolecular contacts. Analysis of the particular residues at the recognition sites of RIα (12–61) and RIIα (1–44) shows that they also differ in hydrophobic/hydrophilic character (Figure 8(D)). Hydrophobic residues, lle8, Thr10, Leu13, Lys16, Thr17, Val18, Leu21 in RIIα (1–44) have direct interactions based on NOE signals with the Ht31 peptide.38 In RIα (12–61), there are residues surrounding the putative AKAP binding surface that are charged and/or polar (Figure 8(D)). Residues such as Arg14, Glu15, Lys22, and Gln21 in the complexation.33,38 RIIα undergoes little structural reorientation upon restraints were obtained as described.41 In addition, a restraint was assigned44,58,59 (Table 1).

Two different AKAPs (Ht31 and AKAP79) in a peptide complex with RIIα recognize conserved amphipathic helices in a hydrophobic groove.39 The grooved channel of RIα becomes a deep cleft in RIα (12–61) because of the two N-1 helices that sit on top of a four-helix bundle. However, the dynamic disorder of the N-1 helices with respect to the helical core (see Results and Figure 1) suggests that they likely reorder once the AKAP is bound in RIα (1–44), the anti-parallel packing of helices I and I’ on top of II and II’ provide an extended docking surface for the AKAP. The hydrophobic groove in RIIα (1–44) preexists and undergoes little structural reorientation upon complexation.33,38 RIIα (1–44) accommodates a number of amphipathic helices with varying sequences and is tolerant to conserved hydrophobic substitutions. It is this geometric complementarity that allows intercalation of aliphatic peptide side-chains into the cavities of the protein surface and affords a mechanism for the binding of a wide array of peptides.38 The surface presented in RIα (12–61) appears to be more selective. The interface is less hydrophobic and less tolerant to variations in packing geometry and residue variation. Electrostatic properties play a role in the structure and stability of RIα D/D, influencing, for instance, quaternary packing.39 Given the increased electrostatic character of the AKAP-binding surface of RIα perhaps the charged residues at the interface play a role in peptide interaction, introducing a higher degree of selectivity.

Materials and Methods

NMR experiments

All NMR experiments were performed on either a Bruker DMAX500 or DRX600 spectrometer using a triple-resonance gradient probe. All experiments were processed using Felix 95.0 software (Molecular Simulations Inc.). RIIα (12–62) NMR sample conditions, resonance assignments, hydrogen bond and dihedral restraints were obtained as described.41 In addition, a 2D homonuclear NOESY (τ = 150 ms)55, and a 3D 13C-edited HMBC-NOESY experiment (τ = 150 ms) were acquired.60 Discrimination of intra-from intermolecular NOEs was aided by the preparation of an asymmetrically 13C–15N/13C–14N-labeled sample and collection of a 3D 13C-edited NOESY data set56 and a 3D 13C-edited (o2)13C-filtered (o1)15C-filtered (o3) NOESY (τ = 150 ms).67 22 inter-molecular NOE-derived distance restraints were assigned44,59,59 (Table 1).

Structure calculations

Initial monomer structures were calculated in X-PLOR 3.85.45,59,63 Structure calculations were carried out without any assumption of the potential for structural similarity with RIIα (1–44)(ref). Disulfide patches45 were introduced in the dimer calculations and the NOE data were interpreted as described.44 Additional NOE-derived distance restraints were classified into intra-molecular, inter-molecular or ambiguous if they had possibilities of both inter- and intramolecular contacts.

A total of 435 NOE-derived distance, 139 backbone dihedral and 13 hydrogen bond restraints, per monomer, were used to generate the final ensemble of 21 structures. Accepted structures had no NOE violations greater than 0.4 Å, dihedral angle violations greater than 5°, or improper angle violations (Table 1). The best fit superposition of these structures is presented in Figure 1. Structures were visually inspected in Insight II (MSI) and validated with PROCHECK-NMR62; 76% of the ordered residues fell into the most favored regions of Φ/Ψ space with 20% in the additionally allowed and 3.9% in the generously allowed regions.

Structural data and derived atomic coordinates will be deposited with the Protein Data Bank†.

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References


† Note that in the PDB, numbering goes by construct, i.e. +2 will be added.


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