Isoform-specific Differences between the Type Iα and IIα Cyclic AMP-dependent Protein Kinase Anchoring Domains Revealed by Solution NMR*

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Cyclic AMP dependent protein kinase (PKA) is controlled, in part, by the subcellular localization of the enzyme (1). Discovery of dual specificity anchoring proteins (β-AKAPs) indicates that not only is the type II, but also the type I, enzyme localized (2). It appears that the type I enzyme is localized in a novel, dynamic fashion as opposed to the apparent static localization of the type II enzyme. Recently, the structural dimerization/docking (D/D) domain from the type II enzyme was solved (3). This work revealed an X-type four-helix bundle motif with a hydrophobic patch that modulates AKAP interactions. To understand the dynamic versus static localization of PKA, multidimensional NMR techniques were used to investigate the structural features of the type I D/D domain. Our results indicate a conserved helix-turn-helix motif in the type I and type II D/D domains. However, important differences between the two domains are evident in the extreme NH₂ terminus: this region is extended in the type II domain, whereas it is helical in the type I protein. The NH₂-terminal residues in RIα contain determinants for anchoring, and the orientation and packing of this helical element in the RIα structure may have profound consequences in the recognition surface presented to the AKAPs.

Extracellular signals are relayed from the plasma membrane to specific intracellular targets with precision and speed. Many signaling pathways do so by altering the phosphorylation state of the target proteins. Kinases and phosphatases have broad substrate specificity, and mechanisms exist to organize and effectively concentrate the correct repertoires of enzymes into distinct signaling cascades. In the case of cAMP-dependent protein kinase (PKA), the importance of signal integration and

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†The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAP, A-kinase anchoring protein; D-AKAP1, dual specificity AKAP1; R, cAMP-dependent protein kinase regulatory subunit; D/D, dimerization and docking domain; C, cAMP-dependent protein kinase catalytic

coordinated assembly of signaling cascades has been established (1). The opposing actions of adenylate cyclase and phosphodiesterases generate localized gradients of cAMP (4), which will exert its greatest influence only when concentrated pools of PKA are colococalized in an inactive conformation (5, 6). To account for this, Scott and co-workers (7) have proposed the "targeting hypothesis." This hypothesis states that phosphorylation events are not only controlled by the balance of kinase and phosphatase activity, but also by their respective subcellular localization (7).

It is the regulatory subunits of PKA that mediate subcellular compartmentalization via their binding to A-kinase anchoring proteins (AKAPs) (6, 8). The anchoring of the type II PKA holoenzyme has been extensively investigated, and AKAPs have been found in centrosomes, mitochondria, Golgi, microtubules, filopodia, dendrites, and the plasma membrane (1, 9–15). More recently, the structural basis for this high affinity interaction was revealed by solution NMR (3). The X-type four helix bundle topology in RIα(1–44) provides a hydrophobic interaction surface for an amphipathic helical motif in the AKAPs (3, 16).

Two novel AKAPs, β-AKAP1 and β-AKAP2 (2, 17), were recently isolated and were designated dual specificity AKAPs, since they interacted with the type I as well as the type II regulatory subunits. RIα, however, binds this class of proteins with a 25–500-fold higher affinity than RI (2, 17). Since the cloning of β-AKAPs, other RI-binding proteins have emerged (18–21). Recently, a novel Caenorhabditis elegans AKAP that binds C. elegans R-subunit, R_C, has been cloned (21). AKAP_C binds R_C with nanomolar affinity, and neither RIα nor RIβ competitively inhibit formation of AKAP_C-R_C complexes. These studies suggest that RIα dimers, like RIβ, have a site available for interactions for anchoring proteins. Although RIα is mostly cytoplasmic in certain cell types, it is clearly anchored in vivo (22–25). The dynamic nature of RIα anchoring, in contrast to the static anchoring of RIα, is of critical importance in vivo and needs to be understood at the molecular as well as the structural level.

The recent solution NMR structure of the RIα dimerization/docking domain (D/D) revealed an X-type four-helix bundle topology (3). Although one would expect a conserved fold among these family members, the differential affinities to the various

subunit; RIα, type Iα regulatory subunit of cAMP-dependent protein kinase; RIα, type IIα regulatory subunit of cAMP-dependent protein kinase; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; CSI, chemical shift index; HSQC, heteronuclear single quantum coherence; IPTG, isopropyl-β-D-thiogalactopyranoside; HMQC, heteronuclear multiple quantum coherence.
AKAPs and other target proteins, and the absence of disulfide bonds in RI suggest that there are potentially some subtle, yet critical, structural differences dictating different mechanisms of homodimerization and AKAP interactions. We bacte-
rially expressed, 13C/15N isotopically enriched, and purified a 50-residue fragment, corresponding to the D/D domain of R1a. Residues 12–61 of R1a were previously identified as the tryptic core responsible for dimerization (26). This region is highly stable and is capable of binding the d-AKAPs (2, 27). We report here the 1H, 15N, and 13C backbone resonance assignments and secondary structure analysis of uniformly 15N- and 13C-enriched R1a (12–61), as determined by multidimensional NMR techniques. Like R1a (1–45), this region contains two regions of α-helical structure separated by a turn. A key difference, however, is observed when the secondary structures are com-
pared. The extreme NH2 terminus in R1a (1–44) is extended, however, in R1a (12–61), this region is helical. This altered structural element in R1a-(12–61) at the extreme NH2 terminus has implications for the unique quaternary contacts in R1a and affects homodimerization as well as AKAP binding.

**EXPERIMENTAL PROCEDURES**

*Expression of R1a-(1–61)—cDNA encoding recombinant R1a-(1–61) was inserted into the EcoRI site of pRSETc (Invitrogen, Inc.). This construct introduced a His6 tag at the amino terminus and allowed for rapid purification of the expressed protein by Ni2+ affinity chromatography. Residues 12–61, identified previously as the trypsin-resistant core, were then purified to homogeneity by gel filtration and high performance liquid chromatography (26). The gene encoding R1a (1–61) contains two rare arginine codons for expression in E. coli (15N, 15C backbone resonances and assignments and secondary structure analysis of uniformly 15N- and 13C-enriched R1a (12–61), as determined by multidimensional NMR techniques. Like R1a (1–45), this region contains two regions of α-helical structure separated by a turn. A key difference, however, is observed when the secondary structures are compared. The extreme NH2 terminus in R1a (1–44) is extended, however, in R1a (12–61), this region is helical. This altered structural element in R1a-(12–61) at the extreme NH2 terminus has implications for the unique quaternary contacts in R1a and affects homodimerization as well as AKAP binding.*
RESULTS

Optimizing NMR Conditions—Full-length Rlα is a dimeric protein of 98 kDa molecular mass (55). The NH₂-terminal 61 residues are responsible for maintaining the integrity of the dimer as well as v-AKAP interactions (2, 27). Initial two-dimensional homonuclear TOCSY experiments (41) of Rlα-(12–61) were acquired in 50 mM potassium phosphate at pH 6.5. The efficiency of TOCSY transfer, under these conditions, is extremely poor (data not shown). The protein had an apparent molecular mass of 18 kDa at pH 6.5 (actual molecular mass of the dimeric fragment = 10 kDa), as determined by gel filtration chromatography, suggesting a higher order association. Moreover, dynamic light scattering indicated a heterogeneously dispersed sample (data not shown). A variety of buffer conditions were screened to determine the optimal sample conditions. The ideal sample conditions were observed at a pH of 4.0 and 150 mM sodium chloride (Fig. 1A). Under these conditions, the extent of magnetization transfer in the two-dimensional TOCSY was dramatically improved. These conditions were thus chosen for future NMR studies.

Even though working at low pH conditions is preferred for NMR studies due to lower amide exchange rates, there is a potential risk of compromising the structural integrity of the protein under study. To address this issue, Rlα-(12–61) was analyzed by circular CD to monitor the overall secondary structure. At both pH 6.5 and pH 4.0, the spectra show the classical double minima at 210 and 222 nm expected for helical structures in solution. In addition, the spectra are similar under the two different buffer conditions, suggesting the absence of any gross structural changes due to the different solution conditions (Fig. 1B). Additionally, the functionality of this domain is preserved at these buffer conditions, as indicated by analysis of peptide binding by NMR (61).

Resonance Assignments—The two-dimensional 1H-15N HSQC of Rlα-(12–61) is shown in Fig. 2. All 47 expected nonproline backbone resonances, excluding the NH₂-terminal ser-
Fig. 3. A, representation of NOE contacts, $^{13}$Ca chemical shifts, NH exchange, and $3^J_{HN}$ coupling constants relevant for secondary structure analysis. The chemical shift differences for $^{13}$Ca are reported. They are based on the CSI method; a value of $+1$ is assigned to the residue if it falls downfield of the random coil value 0.7 ppm (helical), and a value of $-1$ is assigned to the residue if it falls upfield of the random coil value $+0.7$ ppm ($\beta$-sheet). Regions corresponding to each element of secondary structure are indicated. The symbols above the sequence indicate $3^J_{HN}$ coupling constants. Red circles denote coupling constants of less than 5 Hz; gray circles represent coupling constants of $>5$ Hz, and the open circles are for those with coupling constants $>8$ Hz. Symbols below the sequence represent NH exchange data. Filled circles, open circles, and filled diamonds represent those amide protons present after 2, 7, and 24 h, respectively. B, secondary structure comparison of RII and RIIa DD domain. $^{13}$Ca secondary shift analysis of RII and RIIa are compared.

ine were observed. The spectrum is well dispersed and well resolved. Even though RII-(12–61) is a disulfide-bonded covalent dimer, the number of resonances observed is equivalent to the number of backbone resonances expected for a monomeric RII-(12–61). Therefore, it is inferred that RII-(12–61) is indeed in a stable, symmetric homodimeric conformation.

In a dimeric system it can be difficult to distinguish inter- from intraresidue NOEs (56, 57). Analysis of sequential connectivities is simplified using triple resonance experiments. Comparison of the intraresidue $^1$HN, $^{15}$N to $^{13}$Ca and $^{13}$C$^\beta$ connectivities in the CT-HNCA and the HINCAB with sequential intraresidue $^1$HN, $^{15}$N to $^{13}$Ca and $^{13}$C$^\beta$ connectivities observed in the CBCA(CO)NH allowed complete identification of the sequential connectivities (Fig. 3). Side chain assignments were obtained using TOCSY-HSQC, HCCH-TOSCY, HC(NH)-TOCSY, and C(CO)NH-TOCSY. All $^1$HN, $^{13}$N, Ca, $C^\beta$, and over 80% of the other side chain assignments were completed.

Secondary Structure Analysis—Carbon chemical shift, short and medium range NOEs, amide proton exchange rates, as well as spin-spin coupling constants ($^3$J$_{HN}$) were used collectively to assess the secondary structure conformation in RII-(12–61). Initial evaluation of secondary structure was done by determining the $^{13}$Ca chemical shifts and subsequently comparing them to random coil chemical shift information. According to the chemical shift index method (CSI), the $\phi$, $\psi$ angles of the polypeptide backbone change in $\alpha$-helix and $\beta$-sheet configuration, which leads to characteristic shifts in the $^{13}$Ca chemical shifts (34, 36, 37). The secondary shifts as a function of residue number are plotted in Fig. 3. These data indicates that RII-(12–61), contains two helices separated by one turn. The first helix, however, does not appear "classical," since it is interrupted at His$^{23}$, Asn$^{24}$, and Ile$^{25}$. This discontinuity in secondary shifts may be explained by the intermolecular disulfide bond that could cause a contortion of the polypeptide backbone leading to unusual $\phi$, $\psi$ angles not normally expected in an $\alpha$-helix.

$\alpha$-Helices, $\beta$-strands, and $\beta$-turns display characteristic NOE cross-peaks that help distinguish among them fairly conclusively (58). Analysis of the three-dimensional HSQC-NSESY experiment generated a pattern of NOE cross-peaks for RII-(12–61) that confirmed the secondary structure analysis of the $^{13}$Ca secondary shift data presented in Fig. 3.

$\alpha$-Helical Structures—RII-(12–61) demonstrates a high proportion of $\alpha$-helix when analyzed by circular dichroism as well as NMR spectroscopy. The first $\alpha$-helical segment is subdivided into two segments, since according to many of the NMR parameters, it cannot be defined as a long uninterrupted helical segment. The first helical segment spans residues Arg$^{14}$ through Asn$^{24}$ as observed from strong $d_{NN}$, medium $d_{NN,(+1)}$ cross-peaks. In addition, weak intensity $d_{NN,(+2)}$ and $d_{NN,(+3)}$ and $^{13}$C secondary shift analysis further indicate this region to be helical. This helix is disrupted at Cys$^{16}$, according to $^{13}$Ca chemical shift analysis, which could be explained easily by the disulfide bonding that has resulted in unusual torsion angles. Additional indication for the helical nature of this region comes from analysis of coupling constants ($^3$J$_{HN}$). Residues Arg$^{14}$ through Glu$^{22}$ have $^3$J$_{HN}$ values less than 5 Hz, indicative of an $\alpha$-helix. Despite the presence of a medium intensity $d_{NN,(+3)}$ between Glu$^{21}$ and Asn$^{24}$ and $d_{NN,(+3)}$ between Asn$^{24}$ and Ala$^{27}$, His$^{28}$, and Asn$^{24}$ have $^3$J$_{HN}$ values of 9.4 and 6.6 Hz, respectively. This region could be kinked or bulged, resulting in aberrant torsion angles. Furthermore, the $^{13}$Ca secondary shifts for these residues are zero.

Helix I spans Ile$^{25}$ through Ala$^{39}$ and is defined by strong $d_{NN}$ cross-peaks, medium intensity $d_{NN,(+2)}$, as well as weak $d_{NN,(+3)}$ and $d_{NN,(+3)}$. Analysis of $^{13}$Ca secondary shifts also indicates this region to be helical (Fig. 3). Additionally, residues Ile$^{25}$ through Thr$^{38}$ have $^3$J$_{HN}$ values less than the 5.5 Hz characteristic of $\alpha$-helical structures. Residue Arg$^{40}$ is not included in the COOH-terminal end of Helix I because of the lack of $d_{NN,(+3)}$, as well as $d_{NN,(+3)}$. Above all, this residue has $^3$J$_{HN}$ > 5 Hz. Finally, Helix II spans residues Met$^{45}$ through Glu$^{58}$ by similar criteria. This helix is defined by characteristic $\alpha$-helical NOEs, coupling constants, and $^{13}$Ca secondary shift analysis.

Hydrogen exchange experiments were used to detect slowly exchanging amide protons in RII-(12–61). Amide protons that exchange slowly are either solvent inaccessible and thus buried within the protein or are involved in hydrogen bonding interactions, implying stable secondary structure elements. The slowly exchanging protons in RII-(12–61) lie in the regions defined as $\alpha$-helical from both the NOESY and CSI method, as well as coupling constant analysis (Fig. 3). The main exception is the amide exchange rates for the first helical moiety. These residues show fast exchange rates, despite their helical nature, suggesting that perhaps this helix does not participate in numerous quaternary contacts.

Turns—A turn defined by residues Pro$^{31}$-Glu$^{42}$-Arg$^{43}$-Pro$^{44}$ separates Helix I from Helix II. Side chain identification of the prolines was done using the HCCH-TOSCY as well as the three-dimensional $^{13}$C-edited HMBC-NOSY experiments. NOE cross-peaks, $^{13}$Ca secondary shifts, dihedral angles measurements derived from the HNHA experiment, and $^2$H exchange data suggest a type I conformation. The second and
third residues in the turn, Glu$^{12}$ and Arg$^{43}$, have $^{3}J_{\text{HNH}}$ values of 4.7 and 9.4 Hz, respectively. These values correspond to those expected for the second and third residues in a Type I turn (58). NOE cross-peaks involved in determining this turn include a medium $d_{\text{NH}}$-correlation between Glu$^{42}$ and Arg$^{43}$, a weak $d_{\text{NO}}$-correlation between Pro$^{41}$ and Glu$^{42}$, and finally a weak $d_{\text{NO}}$-correlation between Glu$^{42}$ and Arg$^{43}$.

Data obtained from the three-dimensional $^{13}$C-edited HMQC-NOESY experiment reveals that the two proline residues are, in fact, in trans-peptide bonds with their respective preceding residues. In addition, Pro$^{41}$ and Pro$^{44}$, show medium $d_{\text{NO}}$-cross-peaks, which are indicative of trans-peptide bonds. $d_{\text{NO}}$-NOEs, which would indicate a cis-peptide bond for the sequence Xaa'$^{\text{Pro}}$ showed no NOEs.

**DISCUSSION**

The identification of a family of anchoring proteins that bind RIIα has offered novel insights into the unique and dynamic nature of PKA anchoring via RIIα. By analogy with RIIα, it is the D/D domain of RIIα that is responsible for interaction with AKAPs. There are isoform-specific differences in the nature of these interactions that need to be addressed at the structural and molecular level. In addition, the type I R-subunits are maintained as a dimer by two interchain disulfide bonds, whereas the type II R-subunit is noncovalent dimer (55). We, therefore, pursued structural characterization of the RIIα D/D domain by NMR to address the potential differences in the molecular basis for homodimerization as well as anchoring. Newlon et al. (3) recently determined the solution structure of RIIα-(1–44) by multidimensional NMR. Given the overall conservation in function among the two R-subunits, one would expect a conserved fold for the NH$_2$ terminus. However, there is biochemical evidence to expect differences in quaternary structure. We expressed isotopically enriched RIIα-(12–61) with $^{15}$N and $^{13}$C nuclei and purified it to homogeneity. This region is necessary and sufficient for binding d-AKAPs (2, 27).

**Secondary Structure Comparison with RIIα**—Secondary structure analysis of RIIα and RIIβ shows that elements of secondary structure are conserved among the two domains. Both contain two conserved regions of helicity separated by a turn. As speculated earlier in our mutagenesis studies of RIIα and demonstrated in the NMR solution structure of the RIIα D/D domain, these two helices correspond to two functional subdomains each serving a distinct role (3, 27, 31). Helix I, or subdomain 1, contains determinants for anchoring, whereas Helix II, or subdomain 2, contains determinants for dimerization. In contrast to these conserved helices there are major differences in the secondary structure at the extreme NH$_2$ terminus in the small segment that precedes Helix I. $^{13}$C secondary shift analysis shows that instead of an extended strand for the first 9 residues in RIIα, RIIα has a helical segment (Fig. 3B). We propose that the region corresponding to the extended β-strand in RIIα, and a helical region in RIIβ, are isoform-specific, variant in sequence and secondary structure, and may provide additional requirements for docking. In fact, the two critical isoleucines (Ile$^{8}$ and Ile$^{10}$) that have been implicated in AKAP binding to RIIα reside in this region (3, 59). The importance of this region in mediating anchoring interactions will be addressed eventually in the solution structure of RIIα-(12–61) in complex with an anchoring protein.

**Structural Implications**—The RIIα-(1–44) dimer folds as an X-type four helix bundle that encompasses an extended hydrophobic dimerization interface as well as an AKAP binding surface (3). Extensive, well ordered hydrophobic interactions define the dimerization interface in RIIα. Residues important in dimer contacts reside in Helix I as well as Helix II as shown in Fig. 4A. Sequence alignment with RIIα shows that these residues are mostly conserved. The conserved residues among
the two isoforms are depicted on the RIα structure in green space filling models (Fig. 4B). Given the preponderance of the hydrophobic interactions in dictating the dimerization interface in RIαs and the conservation of these residues in RIβ, it is likely that the two proteins have a similar quaternary fold. The extreme NH₂ terminus that is extended in RIα is colored red in Fig. 4B. This is the region that corresponds to the first helical segment in RIα. Once the first 23 residues are deleted in the full-length RIα, the stability of the dimer is compromised due to the shift in the monomer/dimer equilibrium to the monomeric species, although the dimeric species is still present (27). This deletion mutation corresponds to deleting Helix N-1. This helix is most likely contributing to the stability of the dimerization interface, and its quaternary contacts could affect the dimer core in RIα.

RIα contains two interchain disulfide bonds: Cys¹⁶ and Cys³⁷, in the extreme NH₂-terminal region, is linked in an antiparallel orientation to Cys³⁷ in Helix I (55). Mapping these sites on the RIα-(1–44) structure would suggest that in RIα, the region that parallels the extended region in RIα-(1–44) must somehow contact Helix I to accommodate the interchain disulfide linkage with the correct geometry.

Anchoring Interactions—In addition to the hydrophobic core, RIα also presents an extended, solvent accessible hydrophobic surface (3). This surface is formed by the antiparallel arrangement of helices I and I’ (3). Residues that show direct contact with Hs31 are shown in Fig. 5. Only three of these residues are conserved between RIα and RIβ, which could explain the differences in the affinities of these domain for the various AKAPs. It is important to stress that two critical residues for AKAP binding in RIα, Ile³⁷ and Ile³⁸, lie within the β-strand extended region. These determinants are missing in RIβ and may account for the differences in d-AKAP binding affinity. Moreover, the position and orientation of the helical segment in RIαs and its quaternary contacts could have an important consequence in terms of the surface presented for AKAP binding. Due to the antiparallel linkage of Cys¹⁶ and Cys³⁷, the helical segment in RIαs must somehow fold back onto Helix I and potentially occlude the binding interface that is accessible in the case of RIα-(1–44). The three-dimensional solution structure of RIα is in progress to specifically address the nature of the peptide binding surface present in RIα.

Conclusions—The different isoforms of the R-subunits do not have redundant functions in vivo and, in fact, contribute distinct regulation of PKA activity. Targeted disruption of the RIα gene results in viable mice with no physiological defects, whereas RIα knockout mice are embryonically lethal (60). The potentiation of the L-type Ca²⁺ channel in skeletal muscle cells from RIα knockout mice is retained, and, in fact, immunocytochemical studies show that the C subunit of the type I holoenzyme is colocalized in transverse tubules with the L-type Ca²⁺ channels. In RIα and RIα knockout mice, there is an increase in the level of RIβ protein in tissues that normally express the β isoforms (60). Compensation by RIα represents a crucial biological mechanism for safeguarding the cells from unregulated PKA activity. Understanding the molecular and structural differences in isoform-specific PKA anchoring interactions is thus of utmost importance.

We report the first structural characterization of the RIα D/D domain. Comparison of this domain with the solution structure of the RIα D/D domain indicates that they both share a conserved helical scaffold contributing to two distinct functions, namely dimerization and anchoring. However, the extreme NH₂ terminus is distinct structurally. This region will contribute to the dimerization interface, since it contains one of the cysteines involved in the antiparallel disulfide linkage. More importantly this region in RIαs contains determinants for anchoring, and the orientation and packing of this helical element in RIα will have consequences in terms of the recognition surface presented to AKAPs. The solution structure of RIα alone and in complex with an anchoring peptide will help delineate the contributions of this unique element of secondary structure in RIs that sets it apart from RIαs structurally and functionally.

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