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Defining A-Kinase Anchoring Protein (AKAP) Specificity for Protein Kinase A Subunit RI (PKA-RI)

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Abstract

A-Kinase Anchoring Proteins (AKAPs) act as spatial and temporal regulators of Protein Kinase A (PKA) by localizing PKA along with multiple proteins into discrete signaling complexes. AKAPs interact with the PKA holoenzyme through an α -helix that docks into a groove formed on the dimerization/docking domain of PKA-R in an isoform-dependent fashion. In an effort to understand isoform selectivity at the molecular level, a library of protein-protein interaction (PPI) disruptors was designed to systematically probe the significance of an aromatic residue on the AKAP docking sequence for RI selectivity. The stapled peptide library was designed based on a high affinity, RI-selective disruptor of AKAP binding, RI-STAD-2. Phe, Trp and Leu were all found to maintain RI selectivity, while multiple intermediate-sized hydrophobic substitutions at this position either resulted in loss of isoform selectivity (Ile) or a reversal of selectivity (Val). Since a limited number of RI-selective sequences are currently known, this study aids in our understanding of isoform selectivity and establishing parameters for discovering additional RI-selective AKAPs.

Keywords

Protein Kinase A (PKA); A-kinase Anchoring Protein (AKAP); cAMP signaling; PKA RI; stapled peptide

A primary effector of cAMP signaling is Protein Kinase A (PKA), which regulates a myriad of diverse cellular processes. In an inactive state, PKA is sequestered into an inactive tetrameric holoenzyme complex composed of a regulatory subunit dimer and two catalytic subunits (R_2C_2).^[1] There are four human isoforms of the regulatory subunits (RI α , RI β , RII α and RII β) that act as sensors for intracellular cAMP levels by binding two cAMP molecules each in response to increasing cAMP concentrations.^[2] In a cAMP-bound state, the R subunits release the C subunits into a catalytically active state to phosphorylate nearby

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substrates. In addition to regulating the catalytic activity of PKA, the R subunits also regulate PKA in a spatial and temporal manner through binding interactions with a class of proteins called A-kinase anchoring proteins (AKAPs).^[3]

AKAPs form small signaling complexes in cells that, in addition to PKA, may include various proteins including substrates, phosphatases, adenylyl cyclases and phosphodiesterases.^[4] Upon cAMP stimulation, PKA is activated and phosphorylates nearby substrates before being sequestered back into an AKAP complex, thereby forming microsignaling complexes in cells. A hallmark feature of all AKAPs is that they bind the PKA holoenzyme through interactions with an amphipathic α -helix that docks into a groove formed in the dimerization/docking (D/D) domain of the R subunits.^[5] Remarkably, although over 40 AKAPs have been identified,^[6] the majority demonstrate specificity towards the PKA-RII isoform. This is explained, in part, by structural differences between PKA-RI and PKA-RII. The RI D/D domain creates a deeper cleft that engages four helical turns of the AKAP docking helix^[5a] while the RII D/D domain creates a more superficial hydrophobic patch and only requires two helical turns of the AKAP helix for binding (Figure 1a,b).^[5b, 5c]

In efforts to delineate and define isoform-specific binding by the helical AKAP docking motif to the R-subunits, multiple peptide-based disruptors were previously developed.^[4] While the majority of disruptors demonstrate RII selectivity,^[7] a limited number were generated with specificity towards the RI isoform.^[8] Since determinants for RI specificity are still poorly understood, a bioinformatics approach was recently used to analyze known AKAP docking sequences as a means to establish docking consensus sequences.^[9] From this analysis, the RII-selective sequences were found to have a more defined consensus, likely due to the larger number of available RII-selective sequences.^[9] While the conserved residues in the RII binding consensus sequence were shown to be predominantly composed of relatively small hydrophobic residues (Ala, Val, Ile), the predicted RI binding motif demonstrated considerable variability at nearly every position. Interestingly, the RI consensus sequence highlighted the placement of an aromatic residue (Tyr or Phe) in the first hydrophobic register of the AKAP docking sequence which complements a pocket in the RI D/D binding groove (Figure 1a).^[5a] However, of the limited number of known RI-interacting sequences, an aromatic amino acid is not consistently found in this position (Figure 1c). Further, an intermediary class of AKAPs exists termed dual-specific AKAPs (D-AKAPs) and while D-AKAPs such as D-AKAP1 and D-AKAP2 can bind both isoforms, they preferentially bind the RII isoform.^[10] These D-AKAPs contain an aromatic residue near the first hydrophobic register of the PKA-AKAP binding interface, yet the aromatic residue is positioned on the hydrophilic face rather than the hydrophobic face of the amphipathic helix and therefore is not included in the protein-protein interface (PPI) (Figure 1a,b).^[5a] Thus, understanding how AKAPs maintain isoform specificity is poorly understood, particularly in the case of the RI isoform where an extremely limited number of sequences are known. Since there is considerable sequence variability amongst known RI-binding AKAP sequences, we sought to establish the significance of the aromatic residue on the hydrophobic binding interface and whether other amino acids would be tolerated at this position for maintaining RI selectivity in AKAP docking interactions. Further, we wanted to

investigate the effects the effects of introduced modifications at this position on RII engagement.

In order to evaluate the significance of an aromatic residue (position 4) on an RI-selective AKAP docking sequence, we synthesized a peptide library based on a high-affinity stapled peptide, RI-STAD-2.^[8c] In RI-STAD-2, this aromatic residue is predicted to engage the hydrophobic pocket (Figure 1a) and was previously deemed to be important for RI specificity.^[9] This cell-permeable, constrained peptide scaffold was designed based on a previously identified sequence, RIAD, that binds the D/D domain of RI and inhibits AKAP interactions.^[8b] Using RI-STAD-2 as a template, peptides were designed to contain various amino acids at position 4 while leaving the remainder of the sequence and N-terminal modifications unperturbed (Figure 2). Different substitutions were introduced in this position including aromatic hydrophobic residues (Phe and Trp), hydrophobic residues of varying sizes (Leu, Ile, Val and Ala), polar residues (Thr and Ser) and acidic residues (Asp and Glu).

Binding affinities were measured for each variant peptide by fluorescence polarization (FP). K_D values were determined for each peptide using full-length constructs of the human RI α and RII α subunits (Table 1, Figure 3 and Table S1). The R subunit isoforms were purified as previously described^[11] and tested over a concentration range of 2 pM to 10 μ M along with 0.5-1 nM of fluorescently labeled peptide. RI-STAD-2 was used as a RI-selective control since it demonstrates a greater than 20-fold preference for the RI isoform (Figure 3a).^[8c] Ratios of the K_D values determined for each variant are shown relative to the determined K_D values for RI-STAD-2 for each isoform (Table 1 and Figure 3). Overall, the RII isoform was found to be quite tolerant of nearly all amino acids introduced at this position with the exception of the acidic amino acids. The RI isoform, on the other hand, was considerably more selective for which amino acids could be tolerated. Indeed, aromatic substitutions of Tyr to either Phe or Trp caused a relatively minor change in binding affinity as expected by slightly decreasing affinity for RI, yet also caused a minor increase in affinity for RII. Surprisingly, while some hydrophobic amino acids did not significantly perturb binding, others caused significant decreases in affinity for the RI isoform.

Introduction of hydrophobic residues at position 4 resulted in unexpected alterations of isoform specificity. As a starting point, FP binding curves of RI-STAD-2 demonstrate preferential selectivity for the RI isoform (Figure 4a). While the Leu variant maintained RI selectivity, the Ile variant surprisingly bound to both isoforms with nearly comparable affinities (Figure 4b). Beta-branching of the amino acid side chain appears to be unfavorable in this position for RI binding but is tolerated for docking onto the surface of RII. Further, the Val and Ala variants demonstrated reversed selectivity with preference for the RII isoform (Figure 4c and Supplementary Figure S5). While Val is also beta-branched and therefore may not be readily accommodated in the binding pocket on the surface of RI, Ala is not under the same steric constraint. In this case, it is possible that Ala may not provide a sufficient hydrophobic surface for RI-selective binding. The polar variants containing Ser or Thr were found to have a pronounced loss of affinity for RI by greater than 150-fold. These variants were also found to have a slight preference for RII selectivity, albeit with K_D values in the mid nanomolar range (>100 nM). As expected due to introduction of a negatively

charged side chain at the PPI interface, the Asp and Glu variants had a significant loss in affinity for both isoforms with K_D s in the low μ M range.

In summary, position 4 of RI-STAD-2 can tolerate a limited number of substitutions and does not require an aromatic residue in order to maintain RI selectivity. However, the pocket on the surface of the RI isoform that complements this position appears to serve a critical role in increasing stringency for high affinity interactions. Beta-branched amino acids at this position are unfavorable for RI binding and appear to reverse isoform specificity. RII, on the other hand, appears to tolerate a variety of hydrophobic modifications at this position without causing any notable changes in K_D values. Surprisingly, the Ala variant was not well tolerated by either isoform, causing a loss in binding affinity by approximately 2-fold for RII and by over 100-fold for RI. Both biochemically and biologically, AKAP interactions with the RI isoforms are significantly less understood as compared to the RII isoforms. Since AKAPs are important regulators of cAMP-mediated signaling by regulating PKA both spatially and temporally, misregulation of AKAPs are implicated in a variety of diseases including cardiac-related diseases and cancer.^[12] A deeper understanding of PKA-R engagement at AKAP complexes will be critical for understanding the roles of the individual roles of each PKA-R isoform in signaling and disease. This study helps to further define parameters for isoform selectivity and understanding constraints for defining how selectivity may be reversed.

Experimental Section

Materials

(S)-N-Fmoc-2-(4'-pentenyl)alanine was purchased from Okeanos Tech. All other N- α -Fmoc amino acids and rink amid MBHA resin were purchased from Novabiochem. All other reagents and solvents were purchased from Fisher Scientific. HPLC-grade methanol, acetonitrile and trifluoroacetic acid were used for solutions involving peptide purification or analysis.

Peptide Synthesis

All peptides were synthesized on rink amide MBHA resin using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis as previously described.^[8c] Deprotection was performed using 25% (v/v) piperidine in 1-methyl-2-pyrrolidinone (NMP) for 20-30 min. For coupling steps, 10 equivalents were added for standard N- α -Fmoc amino acids. For couplings involving (S)-N-Fmoc-2-(4'-pentenyl)alanine or 11-amino-3,6,9-trioxaundecanoic acid (NH-PEG₃-CH₂COOH, ChemPep), four equivalents were used.

Olefin metathesis was performed on solid support before addition of the N-terminal PEG linker. The ring-closing metathesis reaction was done using 0.4 equivalents bis(tricyclohexylphosphine) benzylidene ruthenium(IV) dichloride (Grubbs Catalyst, 1st generation, Sigma). The reaction was performed at room temperature twice for one hour each with agitation.

N-terminal fluorescein labeling was performed using 2 equivalents of 5(6)-carboxyfluorescein (Acros) in DMF along with 0.046 M HCTU and 2% (v/v) DIEA

overnight at room temperature. Resin cleavage was performed using a mixture containing 95% trifluoroacetic acid, 2.5% water and 2.5% triisopropylsilane (Sigma) for 4-5 hours at room temperature. Cleaved peptides were precipitated in methyl-tert-butyl ether at 4 °C followed by lyophilization. All peptides were purified by high-performance liquid chromatography (HPLC) and verified by ESI mass spectrometry (ESI-MS). Peptides were quantified by measuring the absorbance of 5(6)-carboxyfluorescein at 495 nm using a Synergy 2 microplate reader (Bio-Tek).

The molecular weights of the purified peptides are as follows: RI-STAD-2 = 2758.5 (expected mass = 2759.1); F variant = 2742.2 (expected mass = 2743.1); W variant = 2781.3 (expected mass = 2782.1); L variant = 2708.4 (expected mass = 2709.1); I variant = 2708.4 (expected mass = 2709.1); V variant = 2694.4 (expected mass = 2695.0); A variant = 2666.2 (expected mass = 2667.0); T variant = 2696.4 (expected mass = 2697.0); S variant = 2682.2 (expected mass = 2683.0); D variant = 2710.2 (expected mass = 2711.0); and E variant = 2724.4 (expected mass = 2725.0).

Expression and Purification of Recombinant PKA-R Subunits Ia and IIa

The recombinant human PKA regulatory subunits hRI α and hRII α were expressed and purified using Sp-8-AEA-cAMP agarose as described previously.^[11] SDS-PAGE was used to monitor protein expression and purity. The recombinant proteins were purified to 95% homogeneity.

Fluorescence Polarization (FP)

The binding affinity of the RI-STAD-2 variant peptides were measured against the full-length human PKA regulatory subunits I α and II α using FP in a direct assay format.^[13] Increasing concentrations (2 pM to 10 μ M) of both PKA R-subunit isoforms were equally mixed with 0.5-1 nM of fluorescently labeled RI-STAD-2 or RI-STAD-2 variants in buffer containing 20 mM MOPS pH 7, 150 mM NaCl and 0.005% (v/v) CHAPS. Data were obtained in duplicates using a CLARIOstar (BMG LABTECH) plate reader at room temperature and a data acquisition of 0.1 s at Ex 482 nm/Em 520 nm in a 384 well microtiter plate (Perkin-Elmer Optiplat, black). Equilibrium dissociation constants (K_D) were calculated with a nonlinear regression dose-response curve using GraphPad Prism 6. The K_D values were normalized relative to RI-STAD-2 peptide for each protein preparation. At least two independent protein preparations were measured and the K_D -ratios are presented as a mean of the ratio to RI-STAD-2 $\pm$ SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

RI- versus RII-selective AKAPs. a) The RI D/D domain (blue) has a more extensive interface and engages four helical turns of the amphipathic AKAP docking helix (the dual-specific D-AKAP2 peptide is shown in red). The hydrophobic registers on the binding interface are shown in yellow. The location where the aromatic residue is positioned in an RI sequence is highlighted in orange with an arrow. b) RII D/D domain interactions with the D-AKAP2 peptide are notably different with a smaller binding interface (residues at the binding interface are shown in yellow). The position of the aromatic residue of an RI sequence is highlighted in orange with an arrow. c) AKAP docking sequences from various AKAPs demonstrates that some RI-selective sequences contain an aromatic residue in the first helical turn, but not all (i.e. PV-38). Dual-specific AKAPs such as D-AKAP1 and D-AKAP2 also lack an aromatic residue in this position but contain one on the hydrophilic face of the helix (shown in green). Unlike the RI isoform, this position (orange) does not complement a pocket on the surface of RII. Asterisks represent the positions of the non-natural olefinic amino acids in RI-STAD-2. Structures were rendered in PyMol using PDB files 3IM4 and 2HWN.

Figure 2. Modified RI-STAD-2 library. Using RI-STAD-2 as a template, peptide variants were generated by introducing substitutions at position 4 (orange). Aromatic, hydrophobic, polar and acidic substitutions were introduced at this site.

Figure 3.

Log scale of K_D ratios for the variants relative to RI-STAD-2. Relative K_D ratios for RI α are shown in black and ratios for RII α are shown in grey. While substitution changes were well tolerated by the RII isoform overall, the RI isoform demonstrated selectivity towards particular hydrophobic amino acids at this position.

Figure 4.

Normalized fluorescence polarization (FP) signals for RI-STAD-2 and select variants. a) RI-STAD-2 binding curves are shown for human PKA-RI α (black squares) and PKA-RII α (grey circles) using fluorescently labeled RI-STAD-2 b) Affinity comparison for the Leu variant (circles) and the Ile variant (squares) for the RI α and RII α isoforms. The Ile variant binds both isoforms nearly equally, while the Leu variant displays selectivity for RI α . Further the affinity of the Leu variant to RII α is comparable to the affinity of the Ile variant for both isoforms. c) In contrast to the binding preference for RI-STAD-2, the V variant depicts reversed selectivity with preferential binding to RII α . d) The Asp variant had a considerable loss of affinity for both isoforms.

Table 1

K_D ratio relative to RI-STAD-2 (mean $\pm$ SEM). K_D -values were normalized relative to the K_D values for RI-STAD-2 for each protein preparation. Each assay was performed using at least two independent protein preparations.

Peptide	RI α	RII α
RI-STAD-2	1	1
F variant	1.4 $\pm$ 0.02	0.6 $\pm$ 0.1
W variant	4.1 $\pm$ 0.5	0.5 $\pm$ 0.1
L variant	7.7 $\pm$ 1	0.8 $\pm$ 0.01
I variant	40 $\pm$ 6	0.8 $\pm$ 0.03
V variant	55 $\pm$ 1	0.9 $\pm$ 0.2
A variant	163 $\pm$ 35	2 $\pm$ 0.1
T variant	152 $\pm$ 44	2 $\pm$ 0.6
S variant	247 $\pm$ 33	4.2 $\pm$ 2
D variant ^[a]	>590	>30
E variant ^[a]	>390	>20

^[a]The minimum K_D ratio is shown for the D and E variants since they could not be determined accurately due to low binding affinities.