R-subunit Isoform Specificity in Protein Kinase A: Distinct Features of Protein Interfaces in PKA Types I and II by Amide H/2H Exchange Mass Spectrometry

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Introduction
The adenosine 3′, 5′ cyclic monophosphate (cAMP) signaling pathway plays a critical role in the cell, serving to transduce the action of a wide variety of hormonal stimuli across species ranging from bacteria to mammals.1 In most eukaryotic cells, cAMP exerts a broad influence inside the cell primarily through its activation of protein kinase A (PKA), a key enzyme with numerous intracellular substrates.2 PKA is a master switch that controls a wide range of cellular functions, which are in turn regulated by cAMP. In the absence of cAMP, PKA exists in an inactive state as a tetrameric holoenzyme composed of a homodimeric regulatory (R) subunit and two catalytic (C) subunits. Binding of cAMP leads to dissociation of the holoenzyme to unleash the active C subunit. The R-subunit is thus a primary locus for cAMP in the cell. By toggling between C-subunit bound and cAMP-bound states, the R-subunit functions as a cAMP-dependent regulator of PKA phosphotransferase activity.3

There are two principal isoforms of the R-subunit (type I and type II)† each further sub classified into α

Abbreviations used: H/2H exchange, hydrogen/deuterium exchange; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight; PKA, protein kinase A; Mops, 3-(N-morpholino)propane sulfonic acid; cGMP, guanosine 3′-5′-cyclic guanosine monophosphate; AMP-PNP, 5′-adenylimidodiphosphate; TFA, trifluoroacetic acid.

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and β subtypes. R-subunits are highly modular with an N-terminal dimerization/docking domain (D/D domain) joined by a variable linker to two cAMP binding domains (domains A and B) (Figure 1(a)). The linker region contains a PKA phosphorylation consensus motif, which serves as a docking site for the C-subunit and can be considered the primary C-subunit recognition site (RS1). Differences in primary sequence within this motif distinguish the two isoforms of the R-subunit. In the RI isoforms, the site of phosphorylation is substituted by an alanine or glycine making this segment a pseudo-substrate inhibitor while in the RII isoforms, this site is a serine that is phosphorylated by PKA and functions as a substrate inhibitor of PKA. Sequence alignments of mammalian RIα and RIIβ show high homology (>60% sequence identity) within the cAMP-binding domains but variability within the D/D domain and linker.5 Despite high sequence similarities and a similar domain organization, the R-subunit isoforms show major differences in their subcellular localization and physiological function.6–10 The molecular basis for the dramatic differences in physiology and function of the two R-subunit isoforms are not yet completely understood. One approach to understanding the molecular basis for isoform specificity has been to characterize the unique non-overlapping protein–protein interactions mediated by the R-subunits with two important protein partners: A-kinase anchoring proteins (AKAPs) and the PKA C-subunit. Here we have focused on interactions of the RIIβ isoform with the C-subunit.

Deletion mutagenesis of the R-subunit has been successfully used to map its interactions with the C-subunit. The D/D domain which is the binding site for AKAPs is not required for high affinity holoenzyme complex formation.11 Studies first carried out with the RIα isoform, revealed a multivalent mode for binding of the R-subunit with the C-subunit, requiring interactions at more than one site to achieve high-affinity binding (KD = 0.2 nM).11–13 These sites included the pseudo-substrate, primary interaction site14 and a second site within the cAMP-binding domains which together confer high affinity binding for the C-subunit.11 Complementary amide H/2H exchange mass spectrometry15 and X-ray crystallographic analyses of the RIα-C complex16 have provided detailed descriptions of the RIα–C interface. Amide H/2H exchange mass spectrometry (MS) revealed decreased exchange in RIα at both the pseudo-substrate site and a peripheral recognition site within domain A15 and at the active site cleft and C-lobe of the C-subunit in the RIα–C complex. These sites were subsequently confirmed in the crystal structure of the RIα–C complex.16

Previous amide exchange studies on the full length RIIβ isoform mapped differences in exchange between RIIβ, free in solution and in complex with the C-subunit.17 However, for a detailed mapping of
the interface it is necessary to also identify the interface contributed by the C-subunit. Here we have used deletion mutagenesis to localize interactions of different regions of RIIβ to C-subunit amide H/2H exchange MS to map interfaces of complexes of the C-subunit with different deletion fragments of RIIβ. We have also tested the effects of Mg2+-ATP on the RIIβ–C complex. Our results have revealed important differences in the intersubunit interface between the RIIα and RIIβ holoenzyme complexes and provide new insights into isoform-specificity in PKA.

Results

Deletion mutagenesis of RIIβ and contribution of cAMP binding domain–A to high affinity interactions with the C-subunit

Following earlier work on RIIα,11 we initially engineered a deletion mutant of RIIβ, spanning the primary C-subunit interaction site and domain A and tested its ability to bind the C-subunit. This mutant, RIIβ (108–268) (Figure 1(b)) is analogous to RIIα (91–244) which binds the C-subunit with high affinity in the presence of Mg2+ ATP.11 Since the γ-phosphate is transferred to the substrate site in the Mg2+ ATP-bound RIIβ–C holoenzyme complex,18 we replaced Mg2+-ATP with the non-hydrolyzable analog, adenylylimidodiphosphate in the presence of MnCl2 (Mn2+-adenylylimidodiphosphate (AMP-PNP)). This analog mimics ATP with a nitrogen substitution at the oxygen connecting the β and γ-phosphate groups and is an excellent analog to study active conformations of kinases because of its ability to trap kinases in their transition states and provide new insights into isoform-specificity in PKA.

Table 1. Kinetic association rate constants kα(M−1s−1), dissociation constants k−1 (s−1) and affinity constants (Kd) for PKA catalytic subunit binding to RIIβ in the presence and absence of Mn2+-AMP-PNP, an ATP analog, by surface plasmon resonance (Biacore)

<table>
<thead>
<tr>
<th>Complex</th>
<th>kα (M−1s−1)</th>
<th>k−1 (s−1)</th>
<th>Kd (μM)</th>
<th>χ2a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIIβ(108–268)–C</td>
<td>2.0 ± 0.3 × 106</td>
<td>2.4 ± 0.1 × 10−2</td>
<td>12.0 nM</td>
<td>0.5</td>
</tr>
<tr>
<td>Mn2+-AMP-PNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIIβ(108–402)–C</td>
<td>1.5 ± 0.2 × 106</td>
<td>2.4 ± 0.1 × 10−4</td>
<td>1.6 nM</td>
<td>0.3</td>
</tr>
<tr>
<td>RIIβ(108–402)–C</td>
<td>4.2 ± 0.6 × 105</td>
<td>8.4 ± 2.6 × 10−5</td>
<td>0.2 nM</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a The Kd value is calculated from kα and k−1 by Kd = kα/k−1.

b A χ2 value less than 2 can be considered a good fit. The standard errors shown have been calculated from at least two independent experiments.

Solvent accessibility changes in RIIβ and C-subunits by amide exchange mass spectrometry

We next set out to measure solvent accessibility by amide H/2H exchange MS in the free C-subunit and in complex with the different engineered deletion mutants of RIIβ. Our analysis was primarily targeted to the C-subunit and included only a limited number of peptides from the RIIβ subunit since changes in RIIβ upon complexation with the C-subunit by amide exchange mass spectrometry have been characterized.17 These earlier studies identified a single peptide spanning residues 253–268, from among 38 peptides of RIIβ analyzed, that showed decreased exchange in the RIIβ–C complex under similar experimental conditions (Figure 1(c)). We included seven peptides from RIIβ(108–402) covering 28% of the sequence and two peptides from RIIβ(108–268) covering 19% of the sequence in our analysis. Both sets included the critical C-interface peptide RIIβ(253–268). Amide H/2H exchange experiments were carried out over a five minute period at 23(±1) °C by preparing tenfold dilutions of protein samples in deuterated buffer (5 μl of protein solution with 45 μl of deuterated buffer (50 mM Mops, 50 mM NaCl, 1 mM DTT (pH read 7.0)), followed by “on-exchange” incubation for varying times (0–5 min)) prior to quenching in 0.2% (w/v) trifluoroacetic acid TFA (pH 2.5) at 0 °C followed by pepsin digestion. Mass spectrometry data were analyzed and the average numbers of deuterons were calculated as described in Materials and Methods. Fourteen peptides from the C-subunit could be analyzed from all of the samples, and these covered nearly 40% of the sequence of the C-subunit similar to coverage studied in previous studies on the RIIα–C complex.22 Centroids were calculated from the mass envelopes that had the natural abundance isotope profiles removed by deconvolution. This method was shown in previous studies to increase sensitivity and accuracy by removal of stochastic widening and by noise reduction due to local averaging.23 The software package DEX was used for both the deconvolution and centroid calculations. The average number of deuterons (Ds) exchanged (2 min) for each peptide is given in Table 2. For those segments where changes were observed, plots of the time-course of deuteration are also shown and either computing the average exchange at the 2 min time point or calculating the exchange rate from the plot results in the same conclusions.24 Extending the time-course of the experiment also did not result in observation of any further differences. In comparing the average numbers of deuterons exchanged between samples,

(KD = 0.2 nM in the presence of Mg2+-ATP20 or full-length RIIβ (KD = 0.6 nM).21 In the absence of Mn2+ and AMP-PNP, no binding of RIIβ(108–268) to the C-subunit was observed in SPR-Biacore as well as gel filtration chromatography (S.H.J.B. & S.S.T., unpublished observations).
only differences in exchange greater than 1 Da have been interpreted to be significant. Exchange after 2 min. of deuteration for one peptide each from RIIβ (m/z = 2281.31) and the C-subunit (m/z = 1793.97), residues 247–261 are shown in Figures 2 and 3, respectively.

Except for two overlapping peptides, C8, C(163–172) and C9, C(164–174), no solvent protection was seen in any of the other C-subunit peptides in the RIIβ(108–268)–C complex in the presence of Mn²⁺-AMP-PNP compared to the C-subunit bound to Mg²⁺ ATP (Table 2). There was also no solvent protection in any of the RIIβ peptides analyzed in the RIIβ(108–268)–C complex plus Mn²⁺-AMP-PNP (Table 2).

**Contribution of cAMP binding domain–B to high affinity interactions with the C-subunit**

The observed weak binding of RIIβ(108–268) to the C-subunit suggested that domain B might be important for high-affinity interactions with the C-subunit. To test this, we characterized the complex formed by the C-subunit and a larger fragment of RIIβ, spanning both cAMP binding domains. However, because the C-terminal residues of RIIβ(403–416) are susceptible to proteolytic cleavage during purification of the holoenzyme, it was necessary to use a slightly truncated construct RIIβ(108–402). The resulting truncated protein was very stable and retained all of the properties of the larger construct including C-subunit and cAMP binding (S.H.J.B. & S.T., unpublished observations). Biacore-SPR analysis revealed that the RIIβ(108–402) construct alone binds with higher affinity to the C-subunit unlike the shorter construct containing domain A alone. Mn²⁺-AMP-PNP further enhances interactions with the C-subunit (Table 1).

Amide H²/²H exchange analysis showed that within a single region of RIIβ(108–402) spanning residues 253–268 (m/z = 2281.31) (Figure 1(c)), nearly ten amides were protected from solvent in the RIIβ(108–402)–C holoenzyme complex (after 2 min deuterium (Figure 2 and Table 2). Interestingly, most of the other peptides analyzed, including peptide 354–371 that spans the cAMP binding site of domain B in RIIβ showed increased exchange in the holoenzyme complex. This is consistent with previous studies on the full-length RIIβ–C complex that showed this is the only region in full-length RIIβ that showed decreased exchange under similar short timescale deuteration “on exchange” conditions.  This is also consistent with earlier studies describing allosteric communication between the C-subunit and cAMP-binding sites in Rα.  

All peptides with the exception of the α–C helix spanning peptides C5, C(92–100), and C1, C(27–40) both within the N-lobe of the C-subunit showed protection in the RIIβ(108–402)–C (+Mn²⁺-AMP-PNP)
complex relative to the C-subunit bound to Mg\textsuperscript{2+} ATP (Figure 4; Table 2). The peptide, C(44–54) corresponding to the glycine-rich loop showed solvent protection (∼two amides) in the RIIβ(108–402) complex (Figure 4(a)). This protection seen was localized to ∼one amide protection each within residues 41–44 and 45–54 through subtractive analysis using exchange data from a larger, overlapping fragment, C3, C(41–54) (Table 2). These results suggest that the domain B somehow enhances binding of the substrate/product inhibitor region of RIIβ(108–112) to the active site cleft of the C-subunit. The C-subunit residues 163–172 corresponding to the catalytic loop were completely shielded from solvent in both RIIβ(108–268)–C-subunit and RIIβ(108–402)–C-subunit complexes (Figure 4(b)). The protection (one amide after 2 min) for both complexes presumably reflects locking-in of the Mn\textsuperscript{2+} AMP-PNP in the active site of the C-subunit, completely burying these residues from solvent. Results of amide exchange in the three C-lobe peptides of the C-subunit are shown in Figure 5. There were no significant differences in exchange between the free C-subunit and RIIβ(108–402) complexes within residues 212–221 corresponding to the APE-α-F loop (Figure 5(a)). Decreased exchange was observed in the α–G helix C(247–261) (Figure 5(b)) (two amides protected after 2 min exchange) in the RIIβ(108–402)–C complex. The C-subunit residues 278–289, corresponding to the α–H–α–1 loop, also showed a significant decrease in solvent accessibility when RIIβ(108–402) was bound (Figure 5(c)).

**Role of ATP in holoenzyme complex formation**

To examine the effects of Mg\textsuperscript{2+} ATP on RIIβ interactions with the C-subunit, we compared the RIIβ–C binding kinetics and amide exchange of the RIIβ(108–402)–C-subunit complex in the presence

![Figure 2](image-url)  
**Figure 2.** Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) spectra of one of the peptides spanning the α–C helix (residues 253–268) in RIIβ that showed decreased exchange in complexes of C-subunit with the deletion fragment, RIIβ(108–402). The spectra are expanded so as to show the isotopic distribution for the peptide of interest (m/z=2281.31). (i) Undeuterated sample. The higher mass peaks in the envelope are caused by naturally occurring isotopes. The isotopic envelope for the same peptide after 2 min of deuteration from: (ii) free RIIβ (residues 108–402), (iii) RIIβ (residues 108–402)–C (minus Mg\textsuperscript{2+} ATP/Mn\textsuperscript{2+} AMP-PNP), and (iv) RIIβ (residues 108–402)–C (plus Mn\textsuperscript{2+} AMP-PNP).

![Figure 3](image-url)  
**Figure 3.** MALDI-TOF spectra of one of the peptides spanning the α–G helix (residues 247–261) in the C-subunit that showed decreased exchange in complexes of C-subunit with the deletion fragment, RIIβ(108–402). The spectra are expanded so as to show the isotopic distribution for the peptide of interest (m/z=1793.97). (i) Undeuterated sample. The higher mass peaks in the envelope are caused by naturally occurring isotopes. The isotopic envelope for the same peptide after 2 min of deuteration from: (ii) C-subunit (minus Mg\textsuperscript{2+} ATP), (iii) C-subunit (plus Mg\textsuperscript{2+} ATP), (iv) RIIβ (108–268)–C complex+Mn\textsuperscript{2+} AMP-PNP, (v) RIIβ (108–402)–C complex +Mn\textsuperscript{2+} AMP-PNP, and (vi) RIIβ (108–402)–C complex (minus Mg\textsuperscript{2+} ATP/Mn\textsuperscript{2+} AMP-PNP).
and absence of Mn\textsuperscript{2+}AMP-PNP. A relatively small (∼eightfold) increase in RII\(\beta\)•C-subunit binding affinity was observed in the presence of Mn\textsuperscript{2+}AMP-PNP (Table 1).

Two of the N-lobe peptides (C4, C(66–83) and C5, C(92–100)) showed increased exchange while the glycine-rich loop peptide C3, C(41–54) showed decreased exchange in the RII\(\beta\)(108–402)–C complex-bound to Mn\textsuperscript{2+}AMP-PNP compared to the RII\(\beta\)(108–402)–C apo complex. There were no significant differences in amide H/2H exchange between the two complexes in three C-lobe peptides (C10, C11 and C12 in Table 2, Figure 6) of the C-subunit that are part of the RS2 interaction interface. This is consistent with Mg\textsuperscript{2+}ATP not being obligatory for high-affinity RII\(\beta\)–C interactions.\textsuperscript{21} Residues 163–172, corresponding to the catalytic loop, were completely protected whether Mn\textsuperscript{2+}AMP-PNP was present or not in the RII\(\beta\)(108–402)–C complex (Figure 6).

**Comparison of RII\(\alpha\) and RII\(\beta\) complexes with the C-subunit in the presence of Mn\textsuperscript{2+}AMP-PNP**

Comparison of amide exchange (2 min deuteration) in the C-subunit bound to Mg\textsuperscript{2+}ATP, the C-subunit in complex with RII\(\beta\)(108–402) plus Mn\textsuperscript{2+}AMP-PNP and the C-subunit in complex with full-length RII\(\alpha\) plus Mg\textsuperscript{2+}ATP revealed many differences between the two isoforms (Figure 7). While all peptides showed decreased solvent accessibility upon complex formation, the extent of protection observed varied widely in the two complexes. Two of the C-lobe peptides showed more protection for the RII\(\beta\)(108–402) complex than the full-length RII\(\alpha\)–C complex. After deuteration for 2 min, close to three amides were solvent protected in C-subunit residues 278–289 in the RII\(\beta\)(108–402) complex, while only one amide showed protection in the RII\(\alpha\)–C complex (Figure 7(a)). More than two amides were protected in C-subunit residues 247–261 in the RII\(\beta\)(108–402) complex, while roughly one amide showed protection in the RII\(\alpha\)–C complex (Figure 7(b)).

Within the N-lobe of the C-subunit, residues 163–172 and residues 133–145 showed no differences in exchange between the two complexes (Figure 7(d) and (e)). However, the glycine-rich loop represented by C-subunit residues 44–54 revealed significant differences between the two complexes. This region of the C-subunit showed no significant protection in the RII\(\beta\) complex in contrast to the RII\(\alpha\) complex (∼two amides protected) (Figure 7(f)). The results indicate an inverted pattern of solvent protection between the RII\(\alpha\) and RII\(\beta\) complexes. In the RII\(\beta\) complex, the C-lobe residues 212–221 showed almost no changes in the RII complex in contrast to the large protection in the RI complex,
Figure 5. Deuterium exchange in three C-lobe peptides of the C-subunit bound to Mg\(^{2+}\)ATP (x), bound to RII\(\beta\) (residues 108–268) and Mn\(^{2+}\)AMP-PNP (open squares) and bound to RII\(\beta\) (residues 108–402) with Mn\(^{2+}\)AMP-PNP (filled circles). (c) C-subunit residues 278–289 (\(\alpha\)-H-\(\alpha\)-I loop); (b) C-subunit residues 247–261 (\(\alpha\)-G); (a) C-subunit residues 212–221 (APE-\(\alpha\)-F loop). (d) Structure of the C-subunit (PDB access code 1ATP) with the N lobe colored white and the C lobe in wheat and above three fragment peptides (A: C(212–221), B: C(247–261), C: C(278–289)), in red.

Figure 6. (a)–(f) Deuterium exchange in six fragment peptides of the C-subunit bound to RII\(\beta\) (108–402) with (filled circles) and without Mn\(^{2+}\)AMP-PNP (open circles). (a) Residues 278–289; (b) residues 247–261; (c) residues 212–221; (d) residues 163–172; (e) residues 133–145; (f) residues 44–54.
while the glycine-rich loop showed the opposite with a greater protection in the R1α complex.

**Discussion**

**R-subunit isoform differences in holoenzyme complexes: unique importance of cAMP-B domain in RIIβ**

A critical aspect in PKA research is the existence of non-redundant R-subunit isoforms that show major differences in tissue specificity, sub-cellular localization and function and, consequently, have distinct physiological functions despite having similar domain organization and conserved cAMP-binding domains. Extensive deletion mutagenesis and structural studies on the R1α complex with the C-subunit have provided detailed information on the R–C inter-subunit interface. These reveal multivalent modes of binding of R1α to the C-subunit using the pseudo-substrate/inhibitor site or recognition site 1 (RS1), and the cAMP-binding domains contributing the peripheral interaction site or recognition site 2 (RS2) for binding C-subunit. These
Amide H⁻²H Exchange PKA RIIβ

studies also showed that domain A contributed the bulk of the Rα–C interface and showed nearly identical binding affinity as reported for the full-length complex. 13,20 In Rα, the α–A and α–C helices contributed to interface formation, 21 later confirmed by X-ray crystallography, 19 whereas in RIIβ, only one peptide (residues 253–268) spanning helix α–C (Figure 1(c)) showed decreased solvent accessibility in the full-length RIIβ–C complex 22 reconfirmed here with deletion mutants of RIIβ. Small angle X-ray scattering 27 and protein footprinting studies 28 have previously hinted at differences in overall shapes of the R subunit type I and type II PKA complexes.

Here we show that in contrast to Rα, the binding of RIIβ to C critically depends on the presence of domain B. This domain enhances the binding affinity by 40–60-fold and is not ATP-dependent. Furthermore, despite high sequence conservation between Rα(91–244) and RIIβ(108–268), the RIIβ(108–268)–C complex in the presence of AMP-PNP is very different from the Rα(91–244)–C complex. RIIβ(108–268) shows a rapid dissociation rate and lower affinity for the C-subunit compared to Rα(91–244) while amide exchange revealed several regions in the C-lobe of the C-subunit that are associated with decreased exchange in the Rα(91–244)–C complex but are unaffected when complexed to RIIβ(108–268) under identical experimental conditions with the same sequence coverage and peptides analyzed. Based on the H⁻²H exchange data alone, it is unclear how domain B contributes to RIIβ–C interactions. Absence of solvent protection in the C-terminal peptide in the RIIβ(108–268)–C(+)Mn²⁺ AMP-PNP, R2, RIIβ(253–268) relative to the RIIβ(108–402)–C(+)Mn²⁺ AMP-PNP suggests that the C terminus in RIIβ(108–268) and RIIβ(108–268)–C(+)Mn²⁺ AMP-PNP might be disordered and consequently shows a weaker affinity for binding the C-subunit. Domain B might facilitate direct interactions with the C-subunit either through direct interface contacts between domain B, RIIβ(269–402) and the C-subunit. Alternatively, RS2 might still be contributed entirely by domain A but unlike in Rα, domain B might be required for proper folding of domain A. X-ray crystallographic analyses of RIIβ(108–268)–C(+)Mn²⁺ AMP-PNP show that the α–C helix, RIIβ(253–268) is ordered and mediates direct interactions with the C-subunit (S.H.J.B. & S.S.T., unpublished results). However, the ordering might be a consequence of crystal packing as the α–C helix, RIIβ(253–268), in the absence of domain B is disordered in solution from our results. A direct role for domain B in mediating direct interactions with the C-subunit is evident from X-ray crystallography on a deletion mutant of RIIα containing both cAMP-binding domains 29. In this structure, domain B has been found to mediate direct interactions with the αH–αI loop of the C-subunit. It is not clear if there are similar direct interactions between domain B of RIIβ and C-subunit since amide H⁻²H exchange studies on the full-length RIIβ–C complex showed no regions of significant solvent protection in domain B. 17 It is therefore possible there are different roles for domain B in RIIα–C and RIIβ–C complexes.

Distinct, overlapping surfaces on the C-subunit mediate interactions with Rα and RIIβ

The results presented here reveal a clear difference between the binding of the C-subunit with the Rα and RII isoforms. Previous amide H⁻²H exchange studies on the Rα–C holoenzyme complex revealed large decreases in amide exchange in two surface segments of the C-subunit; residues 212–221 and 247–261. In addition, only a slight protection was observed in residues 278–289 when domain B was present (full-length Rα). 22 RIIβ binding also decreased solvent exchange in residues 247–261 but to a lesser extent than Rα (Figure 7). Greater protection was observed for residues 247–261 whereas no significant protection was observed for residues 212–221. In addition, when RIIβ(108–402) was bound to the C-subunit, three fewer amides exchanged within residues 278–289, whereas less than a single amide was protected for this region when in complex with Rα.

Allostery between residues 278–289 (C) and the active site of the C-subunit

Interestingly the peptide, 278–289 (Figure 5(c)) showed greater exchange in the RIIβ(108–268)–C complex than was observed for the C-subunit bound to Mg²⁺ ATP. This increase in exchange was observed concomitantly with decreased exchange in residues 163–172, an N-lobe fragment contributing to RS1 and spanning the catalytic loop of the kinase. Usually, we have interpreted such increases in exchange with long-range conformational effects as “allostery” although this term is not used in the traditional sense. In other words, binding of the RIIβ pseudo-substrate sequence and ATP at the active site subtly alters the conformation and/or dynamics of residues 278–289. The increase in exchange observed for RIIβ binding was not observed for Rα binding where the analogous truncated Rα(94–244) showed very weak protection that was only slightly greater in full-length Rα. 22 In fact, this very weak protection was misinterpreted as interface protection causing a slight skew of the initial models proposed from H⁻²H exchange and docking. 30 There is a greater protection in this region in the larger RIIβ(108–402)–C complex than observed for full-length Rα strongly suggesting that in the case of RIIβ, this region is a major contributor to the RS2 interface. These amide H⁻²H exchange results therefore suggest that binding at RS1 is allosterically linked to binding at RS2 for the RIIβ subunit, but much less so for the Rα subunit.

These results are very consistent with other parallel studies on kinases. Residues comprising the loop between αH and αI are unique to the AGC family of protein kinases (Figure 7(g) and (h)), and are
thought to play a regulatory role in other cases as well.\textsuperscript{31} Mutagenesis has also highlighted allosteric cross-talk between the active site and the αH–αL loop. Amide H/\textsuperscript{2}H exchange studies have shown that a mutant Tyr\textsuperscript{204}Ala that decreased catalytic rates for phosphotransfer showed increased exchange in three peptides in the C-terminal lobe including the αH–αL loop indicating long range allosteric networks coupling the active sites with this region.\textsuperscript{32} Conversely, a mutation in this loop (Lys\textsuperscript{285}Pro) was isolated in a screen that rescued growth of PKA, and this mutant protein blocked interactions with the RII subunit but not RI highlighting both the importance of this region in interactions with the RII subunit in addition to being important for allosteric networks in the kinase (J.Y. & S.S.T., manuscript submitted). The importance of this region in recognition of protein substrates also has been highlighted by a novel genetic approach for identifying PKA substrates in Saccharomyces cerevisiae.\textsuperscript{33} In this study the region on the kinase spanning the αH–αL loop was a secondary site for substrate recognition.

**ATP is not obligatory for formation of high-affinity RIIβ–C holoenzyme complexes**

ATP and two Mg\textsuperscript{2+} ions are required for formation of a high-affinity Rα–C complex. In its absence, the K\textsubscript{D} value for R–C interactions is approximately 100-fold weaker.\textsuperscript{34} A primary classification of the R-subunits into RI and RII isoforms is based on the ability to accept the γ-phosphate at its PKA at the pseudo-substrate/autoinhibitor site or RS1. Rα contains an inhibitor sequence that cannot be phosphorylated while RIIβ contains a pseudo-substrate sequence that is phosphorylated.\textsuperscript{35} Thus, it was a surprise that the RIIβ subunit appeared to bind with similar affinity whether or not the ATP analog was present, and tight binding depended mostly on the presence of the cAMP-binding B-domain. When only the cAMP-binding domain A was present, protection of RS1 residues 44–54, 133–145, and 163–172 is not seen (Table 2). This is most likely due to the rapid dissociation of the truncated RIIβ(108–268) mutant. When both cAMP-binding domains were present, the protection observed at RS1 was nearly identical in the presence and absence of ATP (Table 2). It is interesting to note, however, that even in the RIIβ(108–402)–C holoenzyme complex, the glycine-rich loop showed greater exchange than in the Rα–C holoenzyme complex (Figure 7). This reinforces the idea that the primary determinant for formation of a high affinity complex of Rα with the C-subunit is occupancy of the active site cleft by the pseudo-substrate site (RS1) whereas binding of RIIβ is dependent primarily on RS2 interactions that require both cAMP-binding domains. Furthermore this binding is insensitive to the presence or absence of ATP reported previously.\textsuperscript{36} It therefore appears that the ability to bind in the absence of ATP (by strong RS2 binding) allows RIIβ to regulate the C-subunit in low ATP environments such as adipose tissue.\textsuperscript{25}

In summary, mechanisms for PKA regulation mediated by RI and RII are very different. RIIβ requires both cAMP binding domains to interact with the C-subunit. This RS2 interface site in RIIβ complex might compensate for decreased interactions at RS1 to maintain a high-affinity complex even in the absence of ATP. This might explain the distinct differences in nature of C-subunit inhibition by the two R-subunit isoforms with the RI and RII functioning as competitive and non-competitive inhibitors, respectively.\textsuperscript{37} Our results clearly indicate that the C-subunit of the kinase shows multiplicity in molecular interactions with different R-subunit isoforms and provides a basis for isoform-specificity in the PKA R-subunit.

**Materials and methods**

**Materials**

ATP, cAMP, Mops and cAMP immobilized on 6%(w/v) agarose were obtained from Sigma. Deuterium oxide, \textsuperscript{2}H\textsubscript{2}O (99.9% deuterium) was obtained from Cambridge Isotopes.

**Expression and purification**

Proteins were expressed in Escherichia coli BL21 (DE3) cells (Novagen) and purified as described using cAMP-agarose resin.\textsuperscript{36} Following cell lysis, protein was precipitated from the soluble fraction by 60%(w/v) saturated ammonium sulfate (AS) at 4 °C. The AS pellets were resuspended, incubated overnight with the cAMP-resin and eluted at room temperature. For cAMP-bound RIIβ, the protein was eluted with buffer containing 25 mM cAMP. For cAMP-free RIIβ, the protein was eluted with buffer containing 25 mM cGMP. The protein eluates were then purified over a S75 gel filtration column to remove excess cGMP, in buffer A (50 mM Mes, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 mM DTT (pH 5.8)). Proteins were concentrated with 10 kDa molecular mass cutoff Millipore concentrators prior to deuterium exchange (RIIβ(108–268) (45 μM), RIIβ(108–402)(75 μM)). The C-subunit was prepared as described\textsuperscript{37} and concentrated to 75 μM prior to deuterium exchange experiments. The purified C-subunit (apo) was incubated with 50 mM Mops, 50 mM NaCl, 1 mM DTT, 2 mM MgCl\textsubscript{2} and 0.2 mM ATP to prepare samples of C bound to ATP, concentrated to 125 μM prior to deuterium exchange experiments.

**Holoenzyme formation**

To prepare holoenzyme in the absence of ATP, cAMP-free RIIβ was added to wild-type C-subunit in a 1:1.2 molar ratio and dialyzed at 4 °C against buffer B (10 mM Mops, 50 mM NaCl, 1 mM EDTA, 2 mM DTT (pH 6.5)). To prepare holoenzyme in the presence of ATP analog the same dialysis steps were followed with buffer C (buffer B modified to include 1 mM MnCl\textsubscript{2} and 0.2 mM AMP-PNP (ATP analog).\textsuperscript{16} To remove excess C-subunit, the complex was purified by elution through a S200 gel filtration column and then concentrated with 30 kDa molecular...
mass cutoff Millipore concentrators (RIIβ(108–402)–C complex (40 μM), RIIβ(108–402)–C complex (50 μM) (+Mn2+ AMP-PNP), RIIβ(108–268)–C complex (75 μM)) prior to deuterium exchange and stored in buffer B or buffer C, respectively, at 4 °C.

**Surface plasmon resonance**

Surface plasmon resonance was used to measure the interaction kinetics of the C-subunit and RIIβ subunits using a Biacore 3000 instrument (GE Healthcare Life Sciences). The C-subunit was immobilized to a CM Dextran surface sensor chip (Biosensor amine coupling kit). All binding interactions were performed at 25 °C in 20 mM Mops, 150 mM KCl, 1 mM DTT (pH 7.0), 0.005% (w/v) polysorbate 20 (pH 7.0) buffer. All AMP-PNP samples were done with 0.2 mM AMP-PNP and 1 mM MnCl2. After injection of the R-subunit, the C-subunit surface was regenerated by injection of 1 min (25–50 μl) of 30 μM cAMP in the running buffer. Kinetic constants were calculated using the Biacore pseudo-first-order rate equation and affinity constants (Kd) were calculated from the equation $K_d = k_d/K_a$.

**Deuterium exchange experiments**

Deuterated samples were prepared at 23(±1) °C by diluting 5 μl of protein solution with 45 μl of deuterated buffer A(50 mM Mops, 50 mM NaCl, 1 mM DTT (pH 7.0)), followed by “on-exchange” incubation for varying times (0–10 min) prior to quenching in 0.2% TFA (pH 2.5) at 4 °C. The exchange mixtures for the C-subunit in the presence of Mg2+ and ATP included 2 mM MgCl2 and 0.2 mM ATP with buffer A. The exchange mixture for the RIIβ(108–402)–C and RIIβ(108–268)–C complexes contained 2 mM MnCl2 and 0.2 mM AMP-PNP with buffer A. The quench buffer for free RIIβ(108–268), RIIβ(108–402) and RIIβ(108–402)–C complex included 1 mM EDTA with buffer A. Deuterium exchange at time t = 0, was determined by adding the protein solution in H2O (5 μl) to a mixture of 0.5 μl 0.1% TFA and deuterated buffer A (45 μl). A mock experiment was performed to determine the amount of 2% TFA required so that upon quenching, the pH would be pH2.5. A portion of the quenched reaction (0.1 ml) was mixed with 50 μl of pepsin bead slurry (previously washed two times in 1 ml of cold 0.1% TFA). The mixture was incubated with ice with occasional mixing for 5 min, centrifuged for 15 s at 12,000g at 4 °C, divided in aliquots, frozen in liquid N2, and stored at −80 °C until analyzed.

Frozen samples were quickly defrosted to 0 °C, mixed with matrix (5 mg/ml a-cyan-o-4-hydroxycinnamic acid in 1:1:1acetonitrile, ethanolo, 0.1% TFA, final pH 2.5 at 0 °C), and 1 μl was spotted on a chilled MALDI target. The target was quickly dried and analyzed on a Voyager DE STR Biospectrometry Workstation (Applied Biosystems Inc., Foster City, CA) as described.

**Data analysis**

Mass spectra were calibrated as described and then converted to ASCII text for further analysis. The mass spectrometry data were analyzed using DEX software to remove the natural isotopic abundances for all but one peptide (residues 278–289 of the C-subunit). Centroid determination was calculated automatically for each sample by DEX, and checked manually for verification.

To calculate the centroids, all measurable peaks, or populations, for each peptide sample were used, and the centroids were adjusted by average noise levels. The side-chain exchange was determined to be 4.5% of fast exchanging side chain hydrogen atoms based on dilution factors. Side-chain deuteration models were not used in the deconvolution, but their centroid values were subtracted from the centroids to show deuterium exchange of the backbone amides exclusively. The only exception was peptide m/z = 1347.75 (residues 278–289), which was not deconvoluted prior to centroid analysis, due to variability in signal–noise ratio at different time points analyzed. Back exchange was found to be ~50%, so all centroid values were multiplied by a back exchange factor or 2.0 to calculate the experimental deuterium exchange levels. The average number of deuterons exchanged and standard deviations reported were determined from at three independent experiments for most peptides. Averages and standard deviations were calculated from measurements from three independent experiments for most peptides. Due to high noise in data sets for certain peptides, fewer measurements were obtained and consequently no standard deviations were calculated. The kinetic plots of deuteration for most peptide fragments fit best to single exponential model ($D = B_{\text{max}}(1-e^{-kt})$), where $D$ represents the number of deuterons on the peptide at any time and $B_{\text{max}}$ is the value at which the number of deuterons plateau) accounting for deuterons exchanging at a rapid rate (predominantly solvent exchangeable amides). The fit was implemented in KALEIDOGRAPH 3.0 (Synergy Software, Inc., Reading, PA).

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**References**


