Sensing Domain Dynamics in Protein Kinase A-Iα Complexes by Solution X-ray Scattering*

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The catalytic (C) and regulatory (R) subunits of protein kinase A are exceptionally dynamic proteins. Interactions between the R- and C-subunits are regulated by cAMP binding to the two cyclic nucleotide-binding domains in the R-subunit. Mammalian cells express four different isoforms of the R-subunit (RIα, RIβ, RIα, and RIβ), and all share the same domain organization. All of the R-subunits contain an N-terminal dimerization/docking domain that interacts with anchoring proteins, an inhibitor sequence that binds to the C-subunit active site, and two tandem cAMP-binding domains (designated Domains A and B). Despite these commonalities, each isoform differs in its cellular and tissue-specific distribution, abundance, sequence, structure, and biochemical properties. Moreover, each isoform is functionally nonredundant. Only RIα subunits are embryonically lethal (4, 5), and mutations in RIα are associated with Carney complex (6–8) and systemic lupus erythematosus (9, 10). Given the biological differences between the PKA isoforms and their relevance to disease, it is important to characterize their molecular properties more rigorously to better understand their functional differences.

The PKA-Iα holoenzyme crystal structure described by Kim et al. (11) illuminated the detailed interactions between the C- and RIα-subunits and elucidated a molecular mechanism for the ordered and cooperative activation of PKA by cAMP. Both the structure and mutational data highlighted the role of Domain B as the gatekeeper domain for Domain A. cAMP first binds to Domain B, triggering a conformational change that enables cAMP to bind to Domain A. This second cAMP binding step releases the active C-subunits (12). The first conformational change in RIα involves reorientation of the two cAMP-binding domains, induced by structural changes in the αβ and αC helices connecting Domains A and B (Fig. 1). In the C-subunit-bound conformation, the αβ and αC helices combine to form a single contiguous helix (αβ/C), producing an overall dumbbell-shaped structure where the two domains are separated (Fig. 1A, left panel). In the cAMP-bound conformation, the αβ/C helix is divided into three sections, producing an overall compact globular structure where the two domains share a common interface (Fig. 1A, right panel). Fig. 1B compares the changes in the αβ and αC helices when RIα shuttles between the cAMP-bound and C-subunit-bound conformations. In the cAMP-bound conformation, the αβ and αC helices form distinct segments with residues Gly235 and Tyr244 serving as two hinge points. In contrast, these same two residues are engaged to form the continuous helix in the C-subunit-bound conformation. These structural comparisons highlight the dynamic nature of the two domains within the RIα-subunit and show that the conformational transition between the active (cAMP-

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The large scale domain organization of Rln α altered within the R- C complex when mutations are introduced into either Domain A or B? 3) Can mutations at particular sites in the C-subunit be utilized to disrupt a specific interface in the R- C complex, and can SAXS detect these changes? 4) Finally, can we interpret and account for the SAXS differences between RI- and RII-subunits as a result of variations in the overall organization of the cAMP-binding domains and/or differences in the cross-talk between the R- and C-subunits? Answers to these questions should provide insights into how the diversity between R-subunit isoforms give rise to the unique and sophisticated mechanisms of isofrom-specific PKA regulation in cells.

To test our hypothesis that Domain B in PKA-α is highly flexible and the effects of the R333K mutation on this flexibility, we utilized a combination of site-directed mutagenesis, biochemical analysis, and small angle x-ray scattering to better understand the role of Domain B dynamics in PKA-α complexes. The SAXS results reported here show that heteroaldimers formed with the R333K mutant display a different SAXS profile from wild-type Rln α and that these differences stems from Domain B. Our data also indicate that in Rln α, Domain B is dynamic even when complexed with the C-subunit. In contrast to RIIβ where Domain B is essential for C-subunit interactions, the interaction of Domain B from Rln α with the C-subunit is dispensable for holoenzyme formation.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—The catalytic subunit was expressed and purified in *Escherichia coli* BL21 (DE3) cells (Novagen) as described previously (19). Expression and purification of the C-subunit mutant K285P followed the same protocol. All of the Rln α proteins (Rln α, Rln α, Rln αR209K, and Rln αR333K, where Rln α refers to constructs corresponding to residues 91–244 and 91–379, respectively) were expressed and purified as described previously (20).

Holoenzymes of Rln α mutants (Rln α, Rln α, Rln αR209K, and Rln αR333K) were formed by mixing each R-subunit with wild-type C-subunit in a 1:1.2 molar ratio and then dialyzing overnight at 4 °C in 10 mM MOPS (pH 7.0), 50 mM NaCl, 2 mM MgCl₂, 2 mM dithiothreitol, and 0.5 mM ATP. Each holoenzyme complex was separated from excess C-subunit by gel filtration chromatography. Care was taken to concentrate samples immediately prior to SAXS data collection because these complexes were extremely prone to aggregation at concentrations higher than 5 mg/ml.

**FIGURE 1.** A. Conformational change of Rln α upon binding to the C-subunit (left) and cAMP (right). The connecting αb and αc helices are shown in red, and the two hinge points are highlighted with yellow spheres. Domain B is rendered in surface representation for clarity. B, left, overlay of αb and αc helices in the C-subunit and cAMP-bound conformations. Right, the phosphate-binding cassette highlighting the role of a critical arginine (Arg333 for Domain A and Arg209 for Domain B) binding to the phosphate of cAMP. The phosphate-binding cassette is colored in yellow in A and B.
**Domain Dynamics of PKA-\(\alpha\)**

**SAXS Data Collection**—Small angle x-ray scattering measurements were collected at the University of Utah with an Anton Paar SAXSess instrument with line collimation and an image plate detector. The protein samples were concentrated to 2–5 mg/ml and filtered (0.22 \(\mu\)m) immediately prior to data collection. Scattering data for protein samples and their respective solvent blanks were collected in a 1-mm diameter quartz capillary with a 10-mm beam slit at 12 °C.

**SAXS Data Analysis**—Normalized buffer subtraction and data reduction to \(I(q)\) versus \(q\) (where \(q = (4 \pi \sin \theta)/\lambda, \theta\) is the scattering angle, and \(\lambda\) is the wavelength of radiation, 1.54 Å) were performed with the program SAXSquid1D (Anton-Paar, Austria). Radius of gyration (\(R_g\)) and zero angle scattering (\(I(0)\)) parameters were calculated using both GNOM (21) and by Guinier analysis with the program PRIMUS (22). Inverse Fourier transform calculations of \(I(q)\) to yield \(P(r)\) functions, \(I(0), R_g\), and the maximum dimension (\(D_{max}\)) were carried out using a \(q\) range of 0.013–0.17 /Å. CRYSSOL was used to calculate theoretical scattering intensity from Protein Data Bank coordinates of R-C crystal structures (23). The figures were made in PyMOL (DeLano Scientific LLC, San Carlos, CA).

**Amino Acid Analysis**—Amino acid composition of each R-C complex was determined after SAXS data collection to accurately quantify protein concentrations. Amino acid analysis was performed by Dr. Dennis Winge at the University of Utah. The molecular weight (MW) of each complex was determined experimentally with the relation \(I(0) = \pi^2 MW^2 c, \) where \(I(0)\) is the intensity at zero angle, \(\pi\) is a proportionality constant, and \(c\) is the concentration as determined by amino acid analysis. \(I(0)\) was determined by GNOM, and the proportionality constant, \(\pi\), was determined using lysozyme as a protein standard.

**Structural Models**—Ab initio shape restoration with the program DAMMIF (24) was used to generate three-dimensional structures from the one-dimensional scattering data of all seven PKA complexes. No symmetry constraints were applied, and default parameters were used in each calculation. Ten independent models for each heterodimer complex were generated, then aligned, and averaged using the program DAMAVER (25). DAMMIF models for \(\text{R\alpha}_{\text{A}}, \text{C}\) and \(\text{R\alpha}_{\text{AB}}, \text{R333K-C}\) complexes were aligned with their respective crystal structure coordinates (Protein Data Bank codes 3FHI and 2QCS, respectively) using the program SUPCOMB (26).

**Determination of Binding Kinetics by Surface Plasmon Resonance**—Binding experiments were performed on a Biacore 3000 instrument (GE Healthcare). Purified wild-type and K285P mutant C-subunits were diluted to 10–50 \(\mu\)g/ml in 10 mM potassium phosphate buffer (pH 6.0), 20 mM NaCl, 1 mM ATP, and 10 mM MgCl\(_2\). These proteins were immobilized onto CM5 Sensor Chips (GE Healthcare) via amine coupling to a ligand density response of ~300 response units (RU). Dilutions of \(\text{R\alpha}_{\text{AB}}\) ranging from 0.5 to 250 nM were prepared in 10 mM HEPES (pH 7.4), 150 mM NaCl, 0.0005% (v/v) surfactant P-20 (HBS-P buffer; GE Healthcare), 1 mM dithiothreitol, 1 mM ATP, and 10 mM MgCl\(_2\). Each \(\text{R\alpha}\) concentration was injected into the channels at a flow rate of 50 \(\mu\)l/min. The surface was regenerated with 200 \(\mu\)M CAMP and 2 mM EDTA in HBS-P buffer. Sensograms were analyzed using Biaevaluation software version 4.1 (GE Healthcare) to obtain kinetic parameters. Sensogram curves were fit to a 1.1 binding model.

**Inhibition of C-subunit by R\(\alpha\)**—The inhibition assay was carried out as described earlier (27). Briefly, C-subunit wild-type or K285P mutant proteins were incubated with varying concentrations of \(\text{R\alpha}_{\text{AB}}\) for 30 min at 30 °C. Each reaction mix contained 0.5–1 mM of the substrate peptide Kemptide (LRRASLG), 1–2 nM C-subunit, and 0.5 mM ATP. R-subunit was serially diluted to final concentrations ranging from 0.5 to 500 nM. The reactions were initiated with a mixture of Kemptide, ATP, and \([\gamma^32\text{P}]\)ATP, allowed to proceed for 20 min at 30 °C, and then quenched with 30% acetic acid. Free \([\gamma^32\text{P}]\)ATP was separated from protein-bound radioactivity by ascending chromatography on phosphocellulose p81 Whatman paper as described previously (28). Phosphate incorporation into Kemptide was detected by Cerenkov counting on a Beckman LS 6000SC liquid scintillation system. Curve fitting and IC\(_{50}\) calculations were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA).

**RESULTS**

SAXS data were collected for seven PKA-\(\alpha\) holoenzyme complexes formed with a combination of four different R\(\alpha\) mutants (termed \(\text{R\alpha}_{\text{A}}, \text{R\alpha}_{\text{AB}}, \text{R\alpha}_{\text{AB}}, \text{R333K}, \) and \(\text{R\alpha}_{\text{AB}}, \text{R209K}\) ) and two C-subunit proteins (wild type and K285P mutant; the \(\text{R\alpha}_{\text{AB}}, \text{R209K-C}(\text{K285P})\) complex was not examined). To study the contribution of the two cAMP-binding domains on PKA-\(\alpha\) dynamics, we used R\(\alpha\) constructs \(\text{R\alpha}_{91–244}\) and \(\text{R\alpha}_{91–379}\), which lacked the N-terminal dimerization domain and the linker region. The minimal sequence required to form a high affinity complex with the C-subunit includes the inhibitor sequence region (residues 91–100) and Domain A (residues 120–244). The SAXS intensity plots of \(I(q)\) versus \(q\), Guinier plots, and associated distance distribution \(P(r)\) functions are shown in Figs. 2 and 3. Guinier plots for all samples were linear at the low \(q\) range, indicative of monodisperse solutions with no presence of nonspecific aggregation during data acquisition. Because each sample contained monodisperse particles, these data were suitable for structural analysis. The intensity curves were fit, and inverse Fourier transforms were performed using the program GNOM (21) to generate \(P(r)\) curves.

**R\(\alpha\) Heterodimers**—To assess the general shape characteristics of \(\text{R\alpha}_{\text{AB}}, \text{C}\) heterodimers containing both Domains A and B, we measured the SAXS profiles of \(\text{R\alpha}_{\text{AB}}\) and wild-type C-subunit complexes (Fig. 2A). The \(P(r)\) functions for \(\text{R\alpha}_{\text{AB}}, \text{C}\) are similar to previous data from our laboratory (16), where a single peak with a maxima at 35 Å is followed by a long extended tail at the high \(r\) range. In the present experiments, the maximum dimension, \(D_{max}\) (105 Å), and \(R_g\) values (29.4 ± 0.1 and 32.4 ± 0.8 Å, from Guinier and GNOM analysis, respectively) are somewhat smaller than previously reported (\(D_{max} = 120–130\) Å and \(R_g\) values = 36.4–38.6 Å) (16). These differences are most likely due to differences in sample handling or variations from one preparation to the next as was noted in our previous report (16). In the present study, we were careful to concentrate samples immediately prior to data collection to minimize possible aggregation.
Effect of Deleting Domain B—To assess the contribution of Domain B to the overall shape of the heterodimer, we measured the x-ray scattering of a complex formed with RI<sub>A</sub>B/H<sub>9251</sub> containing only one of the two cAMP-binding domains (RI<sub>A</sub> residues 91–244, RI<sub>A</sub>/H<sub>9251</sub>A). This protein is the smallest fragment of RI<sub>A</sub>B/H<sub>9251</sub> that still binds to both cAMP and the C-subunit with high affinity. The resulting P<sub>(r)</sub> function resembles a spherical, globular-shaped particle with a symmetrical Gaussian curve and no indication of the broad shoulder at the high r region detected in the RI<sub>A</sub>B/H<sub>9251</sub>C complexes (Fig. 2B). The D<sub>max</sub> measured by SAXS was 70 Å, consistent with the maximum dimensions determined from the crystal structure solved with the same constructs (29).

In addition to differences in the P<sub>(r)</sub> curve, both the D<sub>max</sub> and R<sub>g</sub> values for RI<sub>A</sub>A are smaller than for RI<sub>A</sub>B (Table 1). Thus, not only does Domain B contribute to the overall size of the complex, it also contributes to the broad shoulder observed in the RI<sub>A</sub>B/H<sub>9251</sub>C P<sub>(r)</sub> function.

Effect of R333K on RI<sub>A</sub>Heterodimers—In contrast to the extended P<sub>(r)</sub> curve observed with the wild-type RI<sub>A</sub>B/H<sub>9251</sub>C heterodimer, the P<sub>(r)</sub> function for the RI<sub>A</sub>A/H<sub>9251</sub>C heterodimer displays a Gaussian distribution typical of a globular, spherical particle (Fig. 3A). GNOM analysis reveals a P<sub>(r)</sub> function with a peak maximum at 35 Å that goes to zero at a D<sub>max</sub> of 83 Å and an R<sub>g</sub> value of 28.4 ± 0.4 Å (the R<sub>g</sub> was estimated to be 28.3 ± 0.9 Å by Guinier analysis). The extended tail at the high r region in the RI<sub>A</sub>B/H<sub>9251</sub>C heterodimer data is not detected in RI<sub>A</sub>A/H<sub>9251</sub>C. Both the D<sub>max</sub> and R<sub>g</sub> values are smaller for RI<sub>A</sub>A/H<sub>9251</sub>C complexes compared with RI<sub>A</sub>B heterodimers without the R333K mutation. Moreover, PKA heterodimers formed with RI<sub>A</sub>A/R333K...
resemble the more compact shapes observed with RII heterodimers (16).

The crystal structure of the PKA-ια complex was solved using the R333K mutant, RιαAβR333K-C. We hypothesized that the R333K mutation trapped the R-subunit into a compact conformation enabling favorable crystal packing necessary to achieve crystals that diffract to ~3 Å. To assess the validity of the crystallographically observed conformation in light of our solution scattering studies, we calculated the theoretical scattering intensities from atomic coordinates of the RιαAβR333K-C structure (Protein Data Bank code 2QCS) using the program CRYSOL (23) and generated a P(r) function based on this theoretical scattering data. The experimental and calculated P(r) curves are both very symmetrical without any significant tail at long r values (Fig. 4). Furthermore, both D\text{max} and R_g values are in excellent agreement between the two data sets. Clearly, the experimental and calculated SAXS data closely overlap, confirming that the overall shape of the RιαAβR333K-C complex measured in solution is very consistent with what is observed in the crystal structure.

Based on the SAXS data of wild-type and RιαAβR333K-C heterodimers, the overall shape of these complexes are undoubtedly distinct (Fig. 3A). Although the P(r) functions of wild-type RιαAβ-C heterodimers have a broad tail at long vector lengths, both experimentally and calculated P(r) functions of RιαAβR333K-C heterodimers do not. The shoulder originates from Domain B because removal of this entire domain in RιαAβ-C complexes yield symmetrical P(r) curves. Clearly, the overall shape of the wild-type heterodimer in solution as observed by SAXS is not consistent with the static image presented in the RιαAβR333K-C crystal structure. In solution, the wild-type RιαAβ-C complex likely exists as an ensemble of conformational states ranging from compact to extended. The conformation observed in the crystal structure represents only one of many possible states available for the wild-type RιαAβ-C heterodimer in solution. We propose that when bound to the C-subunit, the R333K mutation pushes the equilibrium state of Rια toward a dumbbell shape (Fig. 1A, left panel), resulting in a very compact R-C complex. The SAXS data suggest that in the absence of the R333K mutation, Rια is more dynamic and spends a significant fraction of time in an extended conformation.

**FIGURE 4.** Calculated scattering intensity from the RιαAβR333K-C heterodimer crystal structure. A, calculated scattering intensity data using Protein Data Bank code 2QCS coordinates with the program CRYSOL (gray). The experimental data are shown in black. B, P(r) functions of the experimental (solid squares) and calculated (open diamonds) data of RιαAβR333K-C heterodimers.

**Effect of R209K on Rια Heterodimers**—The R333K mutation impairs cAMP binding to Domain B, and the corresponding mutation for Domain A is R209K. To test whether this mutation also affects the dynamics of the R- and C-subunit interaction, we measured the x-ray scattering of Rιβ heterodimers formed with R209K. Unlike the R333K mutation, the P(r) function for RιβR209K-C shows little difference in the overall shape compared with the wild-type R-C heterodimer (Fig. 3B). The P(r) function has a maximum peak at 34.7 Å, a D\text{max} of 105 Å, and an R_g value of 34.6 ± 0.4 Å (33.5 ± 0.9 Å from Guinier analysis; Table 1). Clearly, the compaction of the R-C complex is specific only to the R333K mutation in Domain B.

### Table 1

| R-subunit | C-subunit | \(R_g\) \(Å\) | \(D_{\text{max}}\) Å | Volume \(\text{Å}^3\) |
|----------|-----------|-------------|----------------|----------------|
| RιαAβ    | C         | 34.1 ± 0.2  | 106            | 175 \(\times 10^3\) |
| RιαAβ    | K285P     | 34.1 ± 0.2  | 106            | 175 \(\times 10^3\) |
| RιαAβ    | C         | 28.4 ± 0.4  | 83             | 133 \(\times 10^3\) |
| RιαAβ    | K285P     | 28.4 ± 0.4  | 83             | 133 \(\times 10^3\) |
| RιαAβ    | R333K     | 31.44 ± 0.3 | 105            | 158 \(\times 10^3\) |
| RιαAβ    | R333K     | 31.44 ± 0.3 | 105            | 158 \(\times 10^3\) |
| RιαAβ    | R209K     | 31.44 ± 0.3 | 105            | 158 \(\times 10^3\) |

**a** Derived using Guinier approximation.  
**b** Calculated using the program GNOM.  
**c** V is the molecular volumes calculated from DAMMIF. The expected dry volumes are calculated based on molecular mass and a partial specific volume of 0.073 cm³/g. Hydration layer effects are expected to give rise to increases in the experimentally derived volumes.  
**d** NA, not applicable.
Effect of the K285P Mutation on RIα Heterodimers—The PKA-1α holoenzyme crystal structure defined a novel interaction site between Domain B of RIα and a short S-shaped loop (αH-αl loop) in the C-subunit (see Fig. 6, A and B) (11). The surface of each protein precisely complements the other at this site. Specifically, Domain B docks onto the C-subunit through side chain interactions between Asp<sup>276C</sup>/Arg<sup>352R</sup> and Thr<sup>278C</sup>/Arg<sup>355R</sup> in addition to backbone interactions between Lys<sup>285C</sup> and both Arg<sup>355R</sup> and Leu<sup>357R</sup>. Mutation of Lys<sup>285</sup> to proline reduced the ability of RIIβ to inhibit C-subunit activity (27), providing biochemical evidence that Domain B (in the R-subunit) and αH-αl loop (in the C-subunit) interaction site is important for at least the RIIβ isoform. Bioinformatics analysis has also pinpointed a short segment within the αH-αl loop (Gly<sup>282</sup>–Gly<sup>286</sup>) to be a conserved AGC kinase-specific insert (26).

We predicted that the x-ray scattering of RIα and RIIβ heterodimers using a combination of SAXS and biochemical analysis would provide important insights for understanding the intrinsic behavior of RIα and C-subunit. Surface plasmon resonance was used to measure the binding constants between the RIα and C-subunits. C-subunits were immobilized to CM5 chips by amine coupling, and RIα<sub>AB</sub> was flowed across the chip surface. Compared with wild-type C-subunit, the K285P mutant exhibited a modest 5-fold decrease in binding affinity between the RIα<sub>AB</sub> and C-subunits. The <i>K<sub>D</sub></i> was 4.9 nm for the K285P complex compared with 0.9 nm for the wild-type R-C complex (Table 2). In contrast, the K285P mutation caused a 38-fold decrease in binding affinity for the RIIβ-C heterodimer, where the <i>K<sub>D</sub></i> was 2.9 and 111 nm between RIIβ and wild-type C-subunit and C(K285P) mutant, respectively. The difference between the binding constants is solely attributed to differences in the dissociation rate.

For the RIα<sub>AB</sub>R333K-C heterodimer, we investigated whether the complex nature of the complex would be disrupted by interfering with the interaction site between Domain B and the large lobe of the C-subunit using the C(K285P) mutant. Indeed, the RIα<sub>AB</sub>R333K-C(K285P) double mutant complex no longer exhibits the compact symmetric P(r) curve observed with RIα<sub>AB</sub>R333K-C (Fig. 3A). Instead, the P(r) curve exhibits an extended tail at the high r region, similar to but not as pronounced as in the wild-type R-C heterodimer. Comparison of <i>D<sub>max</sub></i> and <i>R<sub>g</sub></i> parameters between wild-type and K285P heterodimers show remarkable overlap. Both the double mutant complex and wild-type heterodimer have a <i>D<sub>max</sub></i> of ~105 Å, and the <i>R<sub>g</sub></i> of the double mutant is 31.4 ± 0.3 Å compared with 32.4 ± 0.8 Å for RIα<sub>AB</sub>-C. SAXS scattering parameters for all wild-type and mutant R-C heterodimer complexes are summarized in Table 1. The inability of the K285P C-subunit mutant to fully recapitulate the solution structure of the wild-type R-C heterodimer probably indicates that the RIα<sub>AB</sub>R333K mutant still retains some effect on R-C interactions that prevents Domain B from exploring the full conformational space accessible to the wild-type RIα<sub>AB</sub>-C complexes.

### DISCUSSION

Proteins are in continuous motion, constantly sampling different conformations. Although crystallography provides high resolution information, this technique does not guarantee that the conformational state of the crystallized protein is representative of the predominant conformational state under physiological conditions and can be occasionally plagued by artificial conformations induced by crystal packing. To fully understand the intrinsic behavior of RIα-C complexes, solution techniques must also be utilized. In this study, we analyzed PKA-1α heterodimers using a combination of SAXS and biochemical analysis to investigate domain dynamics within the protein complexes. SAXS is highly advantageous in that it provides low resolution structural information under a variety of solution-based physiological conditions and can be used to obtain size and shape information of large, highly dynamic proteins and protein complexes. Here, we compare our results to previous high resolution crystallographic data to obtain a more complete understanding of the large scale conformational dynamics of PKA. To explore the dynamics of Domain B in PKA-1α holoenzyme complexes, we measured the equilibrium

### TABLE 2

| R-subunit | C-subunit | <i>K<sub>D</sub></i><sup>10</sup> | <i>K<sub>D</sub></i><sup>100</sup> |
|-----------|-----------|-----------------|-----------------|
| RI<sub>AB</sub> | C         | 91.9 × 10<sup>-10</sup> | 8.44 × 10<sup>-4</sup> |
| RI<sub>AB</sub> | C(K285P)  | 90.6 × 10<sup>-10</sup> | 4.43 × 10<sup>-4</sup> |
| RIIβ<sub>AB</sub> | C         | 11 × 10<sup>-10</sup>  | 3.16 × 10<sup>-4</sup> |
| RIIβ<sub>AB</sub> | C(K285P)  | 11 × 10<sup>-10</sup>  | 123 × 10<sup>-4</sup> |

Biochemical Analysis of RIα and RIIβ Heterodimers—The previous data show that the K285P mutant played a pivotal role in RIIβ inhibition of the C-subunit; however, the R-C binding constants were not measured (27). Given the differences in SAXS profiles between RIα<sub>AB</sub>C and RIIβ<sub>AB</sub>C heterodimers, we postulated that the K285P mutation would not affect either the inhibition or binding affinities between RIα and the C-subunit.
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R-C dissociation constants, the inhibition of the C-subunit by the R-subunit, and the overall shape of various mutant RIα complexes by small angle x-ray scattering. The most pronounced result of our study is that a single point mutation, the K285P mutation, partially disrupted the interaction between Domain B and the C-subunit, it did not completely restore the full dynamic range of the wild-type RIα complex, suggesting that the average conformational state of Domain B in the RIαABR333K-C(K285P) complex is intermediate between the fully compact form observed in the RIαABR333K-C crystal and solution structures and the more extended RIαAB heterodimer solution structure. The RIαABR333K-C crystal structure highlights two additional ion pairs at the Domain B site: Asp276C:Arg352R and Thr278C:Arg355R (Fig. 6B, right panel). These two interactions may partially compensate for the release of the Lys285C/Arg355R interface. K285P was first identified in a yeast genetic screen (32) and was shown to reduce the ability of RIββ-subunits to inhibit C-subunit catalytic activity (27). If the interaction between Domain B (in RIα) and αH-αI loop (in the C-subunit) interface is stable as observed in the crystal structure, the K285P mutation would be expected to affect the overall shape characteristics in the RIαABR333K-C complex. In contrast, if the Domain B interaction with the C-subunit in wild-type RIαAB-C complexes is not stable, the K285P should not affect the binding affinities or inhibition. Indeed for RIαAB-C(K285P) complexes, we found that K285P did not affect the general size or shape characteristics (Fig. 2) or inhibition by RIα (Fig. 5) and had only modest effects on the binding affinity (Table 2).

The only differences between K285P and wild-type C-subunit were seen in complexes formed with the RIαABR333K mutant. The P(r) curve for the RIαABR333K-C shows that it assumes the most compact shape of all the RIα complexes, which is presumably why crystallization of this complex (and not the wild type) was successful. SAXS analysis of RIαABR333K-C(K285P) reveals a P(r) curve with a slightly extended tail at the high r region, but not to the large extent observed in RIαAB-C complexes. In other words, although the K285P mutation partially disrupted the interaction between Domain B and the C-subunit, it did not completely restore the full dynamic range of the wild-type heterodimer, suggesting that the average conformational state of Domain B in the RIαABR333K-C(K285P) complex is intermediate between the fully compact form observed in the RIαABR333K-C crystal and solution structures and the more extended RIαAB heterodimer solution structure.

**FIGURE 5. Inhibition of wild-type and K285P C-subunits by RIαAB.** Purified wild-type and K285P C-subunits were incubated with various concentrations of RIα-subunits. Catalytic activity was determined by measuring Kemptide phosphorylation via radioisotope ([γ-32P]ATP labeling). The data for wild-type C-subunit are shown as solid squares, and K285P mutant C-subunits are shown as open squares. The experiments were done in triplicate. The data for RIββ are taken from Ref. 27 (asterisks).

| R-subunit | C-subunit | IC₅₀(nM) |
|-----------|-----------|---------|
| RIαAB     | C         | 2 ± 1   |
| RIαAB     | C (K285P)| 3 ± 1   |
| RIββAB    | C         | 4 ± 1   |
| RIββAB    | C (K285P)| 16 ± 1  |

interface. K285P was first identified in a yeast genetic screen (32) and was shown to reduce the ability of RIββ-subunits to inhibit C-subunit catalytic activity (27). If the interaction between Domain B (in RIα) and αH-αI loop (in the C-subunit) interface is stable as observed in the crystal structure, the K285P mutation would be expected to affect the overall shape characteristics in the RIαABR333K-C complex. In contrast, if the Domain B interaction with the C-subunit in wild-type RIαAB-C complexes is not stable, the K285P should not affect the binding affinities or inhibition. Indeed for RIαAB-C(K285P) complexes, we found that K285P did not affect the general size or shape characteristics (Fig. 2) or inhibition by RIα (Fig. 5) and had only modest effects on the binding affinity (Table 2).

The only differences between K285P and wild-type C-subunit were seen in complexes formed with the RIαABR333K mutant. The P(r) curve for the RIαABR333K-C shows that it assumes the most compact shape of all the RIα complexes, which is presumably why crystallization of this complex (and not the wild type) was successful. SAXS analysis of RIαABR333K-C(K285P) reveals a P(r) curve with a slightly extended tail at the high r region, but not to the large extent observed in RIαAB-C complexes. In other words, although the K285P mutation partially disrupted the interaction between Domain B and the C-subunit, it did not completely restore the full dynamic range of the wild-type heterodimer, suggesting that the average conformational state of Domain B in the RIαABR333K-C(K285P) complex is intermediate between the fully compact form observed in the RIαABR333K-C crystal and solution structures and the more extended RIαAB heterodimer solution structure.

The RIαABR333K-C crystal structure highlights two additional ion pairs at the Domain B site: Asp276C:Arg352R and Thr278C:Arg355R (Fig. 6B, right panel). These two interactions may partially compensate for the release of the Lys285C/Arg355R interface. K285P was first identified in a yeast genetic screen (32) and was shown to reduce the ability of RIββ-subunits to inhibit C-subunit catalytic activity (27). If the interaction between Domain B (in RIα) and αH-αI loop (in the C-subunit) interface is stable as observed in the crystal structure, the K285P mutation would be expected to affect the overall shape characteristics in the RIαABR333K-C complex. In contrast, if the Domain B interaction with the C-subunit in wild-type RIαAB-C complexes is not stable, the K285P should not affect the binding affinities or inhibition. Indeed for RIαAB-C(K285P) complexes, we found that K285P did not affect the general size or shape characteristics (Fig. 2) or inhibition by RIα (Fig. 5) and had only modest effects on the binding affinity (Table 2).

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The RIαABR333K-C crystal structure reveals a previously unidentified single contact point between the αH helix in Domain B of RIα and the αH-αI loop in the C-subunit (Fig. 6A). We examined whether the interaction between the C-subunit αH-αI loop and the RIα Domain B observed crystallographically remains stable in solution or whether Domain B might have a more dynamic behavior in the PKA-Iα complex. Lys285 in the C-subunit αH-αI loop operates as a helix cap for the R-subunit αB helix (Fig. 6B, right panel), so we utilized the C-subunit mutation K285P as a strategy for disrupting the R-C interaction at the Domain B
the C-subunit, the αB and αC helices are extended into one contiguous helix. When Rlα is bound to cAMP, the same αB/αC helix is divided into three distinct helices separated by two bends at Gly235R and Tyr244R. There is a 22 Å difference in the maximum dimension between the most compact (RlαR333K-C) and the most extended (RlαAB-C) heterodimer, most likely because of hinge motions at the Gly235R position. Gly235R forms a hinge point at the C terminus of the αB helix (residues 226–235). This αB helix forms a hydrophobic interface with the C-subunit through Leu233R and Met234R in the R-subunit, and mutation of either residue permits activation of holoenzymes at lower cAMP concentra-
Domain Dynamics of PKA-Iα

Contrary to expectations, Domain B in the wild-type RI complex (33, 37) is highly mobile, and its interaction with the C-subunit is not critical for RI- or RII-β complex formation. Disruption of the interaction interface by the K285P mutation in the C-subunit resulted in a 38-fold decrease in $K_D$ for RI-β, suggesting that K285P is not critical for RI-α-C complex formation

To test our hypothesis that hinge motions are responsible for the extended structures seen in the wild-type RIAβC heterodimer, we took a computational approach and artificially introduced kinks at the Gly239R and Tyr344R hinge points of the RIAβR333K-C crystal structure and computed their scattering intensities and corresponding $P(r)$ functions (Fig. 6C). Movement of Domain B away from the C-subunit resulted in larger $R_g$ values, larger particle dimensions, and the presence of a shoulder at higher $r$ values. Although the extended tail in these modeled $P(r)$ functions is present in each curve, they are not as pronounced as the experimental data for the wild-type RIAβC complex. Previous modeling of scattering data with the programs DAMMIN (34) and CONTRAST (35) do show that Domain B is positioned far from the C-subunit (16). Our efforts in computing ab initio three-dimensional shapes for each of the seven PKA-Iα mutant complexes resulted in an array of molecular structures (Fig. 6D). The DAMMIF results for the RIAβ-C complex resulted in the most globular shape and superimposed very well with the crystal structure model (Protein Data Bank code 3FHI). The RIAβR333K-C complex with the additional Domain B resulted in a compact structure as well and also superimposed very well with the crystal structure (Protein Data Bank code 2QCS). In contrast, the DAMMIF envelope predictions for the remaining complexes (RIAβ-C, RIAβC(K285P), RIAβR333K-C(K285P), and RIAβR209K-C) all exhibited elongated shapes. These results corroborate our hypothesis that in the wild-type R-C complex, Domain B in RI-α is mobile in solution and samples an assortment of conformational states.

In light of data from other studies, it is apparent that the function of Domain B in RI-α is not to provide high affinity binding to the C-subunit but to facilitate cAMP-dependent activation of the holoenzyme. Deletion of Domain B from RI-α results in 500-fold higher concentrations of cAMP required to activate the holoenzyme and does not impair the ability of the remaining Rα structure to bind the C-subunit with high affinity (36). Similarly, preventing cAMP access to Domain B with the R333K mutation increases the activation constant for cAMP from 166 to $>1500 \text{nM}$ (12). Collectively, these data are in agreement with the notion that Domain B is dispensable for stable RIAβ-C complex formation. Instead, the role of Domain B in RI-α is to enhance activation of the RIAβ-C complex in response to cAMP.

From our data and others, it is unmistakable that the role of Domain B differs between RI- and RII-subunits. First, the activation of RIAβ-C is a stepwise process where cAMP must bind to Domain B before Domain A (12). For RII-β, sequential binding of cAMP is not required for activation, where cAMP binding to either domain is sufficient to release catalytically active C-subunits (37). Second, Domain B contributes to high affinity binding between RI-βC and the C-subunit, but not between RIAβ and the C-subunit. Disruption of the interaction interface by the K285P mutation in the C-subunit resulted in a 38-fold decrease in $K_D$ for RI-β, versus a modest 5-fold decrease in $K_D$ for RI-α, suggesting that K285P is not critical for RI-α-C complex formation but is significant for RII-β-C formation. Third, previous studies demonstrated that the K285P mutation reduced the ability of RII-β to inhibit the C-subunit and was sufficient to completely abolish BCY1 (the yeast homolog of mammalian RII subunits) inhibition of the C-subunit (27). Fourth, based on data from hydrogen/deuterium exchange-mass spectroscopy experiments, the αH-α loop is well protected in the RII-β-C complex (33, 37), whereas minimal protection is seen in the RIA-C complex (30). Lastly, SAXS analysis of the RII-β-C complex shows a $P(r)$ curve that reflects a compact particle (16), corroborating the view that Domain B forms a tight interaction with the C-subunit.

In this study, SAXS, in conjunction with mutagenesis and solution-based biochemical techniques, provides insights into domain dynamics within PKA complexes that are not feasible with x-ray crystallography alone. Taken together, our data suggest different roles of Domain B in RI-α and RII-β. For RII-β, not only does Domain B interact tightly with the C-subunit, it is also necessary for inhibition of the C-subunit. In contrast, for RI-α, Domain B is highly mobile, and its interaction with the C-subunit αH-α loop is not necessary for inhibition, but Domain B is essential for the highly cooperative process of cAMP-dependent activation. Given the sequence and overall structural similarity between the four isolated R-subunit PKA isoforms, it is surprising that their dynamic behaviors differ in the context of holoenzyme complexes, giving rise to unique and sophisticated modes of cAMP-mediated activation. RI holoenzymes are finely tunable multimeric complexes that require a sequential series of events, unlike RII holoenzymes. Our investigation of protein dynamics in RI-C and RII-C heterodimers provides yet another layer of complexity that distinguishes the molecular features between PKA isoforms. From a global perspective, these structural distinctions give rise to the functionally nonredundant roles each isoform performs within the cell.

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