Dimerization/Docking Domain of the Type Iα Regulatory Subunit of cAMP-dependent Protein Kinase

REQUIREMENTS FOR DIMERIZATION AND DOCKING ARE DISTINCT BUT OVERLAPPING*

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Based on increasing evidence that the type I R subunits as well as the type II R subunits localize to specific subcellular sites, we have carried out an extensive characterization of the stable dimerization domain at the N terminus of Rα. Deletion mutants as well as alanine scanning mutagenesis were used to delineate critical regions as well as particular amino acids that are required for homodimerization. A set of nested deletion mutants defined a minimum core required for dimerization. Two single site mutations on the C37H template, Rα(F47A) and Rα(F52A), were sufficient to abolish dimerization. In addition to serving as a dimerization motif, this domain also serves as a docking surface for binding to dual specificity anchoring proteins (D-AKAPs) (Huang, L. J., Durick, K., Weiner, J. A., Chun, J., and Taylor, S. S. (1997) J. Biol. Chem. 272, 8057–8064; Huang, L. J., Durick, K., Weiner, J. A., Chun, J., and Taylor, S. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11184–11189). A similar strategy was used to map the sequence requirements for anchoring of Rα to D-AKAP1. Although dimerization appears to be essential for anchoring to D-AKAP1, anchoring can also be abolished by the following single site mutations: C37H, V20A, and I25A. These sites define “hot spots” for the anchoring surface since each of these dimeric proteins are deficient in binding to D-AKAP1. In contrast to earlier predictions, the alignment of the dimerization/docking domains of Rα and RII show striking similarities yet subtle differences not only in their secondary structure (Newlon, M. G., Roy, M., Hausken, Z. E., Scott, J. D., and Jennings. P. A. (1997) J. Biol. Chem. 272, 23637–23644) but also in the distribution of residues important for both docking and dimerization functions.

cAMP-dependent protein kinase (PKA),1 one of the first proteins to be purified and characterized (4), is maintained as an inactive tetrameric holoenzyme complex of regulatory (R) and catalytic (C) subunits, in the absence of cAMP (5). Upon elevation of intracellular cAMP and its subsequent binding to the R subunit, the holoenzyme complex dissociates into an R subunit dimer and two free and active C subunits (5). Although PKA is one of the best understood protein kinases in terms of the structure and function of the R and C subunits (5–8), a further level of control is introduced by interaction of the R subunits with A-Kinase Anchoring Proteins (AKAPs) that target the enzyme to specific subcellular sites (9–12).

Until recently the R subunits have been thought of simply as physiological inhibitors of PKA (6, 13–15). However, like many other proteins involved in signal transduction, these highly modular proteins are also multifunctional (16). The structural and functional independence of the domains within the R subunits were first recognized by limited proteolysis (17). Although the two tandem cAMP-binding sites at the C terminus as well as the inhibitor site are required for high affinity binding to C (18–21), it is the dimerization domain at the N terminus that is important for interacting with AKAPs (22–24).

Numerous AKAPs have now been identified, each having distinct subcellular locations (16, 25–28). Some AKAPs also appear to act as scaffolds for assembling multiprotein complexes. For example, AKAP79 localizes to the actin cytoskeleton and interacts with the calcium and calmodulin-dependent protein phosphatase 2B (calcineurin) and protein kinase C as well as PKA (29, 30). Anchoring is thought to be mediated by a small helical segment in the AKAPs that binds specifically to the N-terminal dimerization domain of RI (22–25).

Although most AKAPs were thought initially to interact exclusively with RII subunits, PKA can also be tethered via Rα. In T lymphocytes, for example, both Rα and RIIα are expressed. In resting cells, type I holoenzyme is evenly distributed in the cytoplasm, whereas the type II holoenzyme is particulate. Once the T cell is stimulated by antigen-specific activation of the T cell receptor, however, Rα is tightly associated with the capped T cell receptor-CD3 complex (31). Rα also colocalizes with the antigen receptor in stimulated B cells (32). In human erythrocytes Rα is tightly bound to the plasma membrane (33). A recent report also showed that Rα localizes at the neuromuscular junction (34). Moreover, Rα was shown to interact with the activated EGF receptor through Grb2 (35). The Grb2-binding site is speculated to lie C-terminally to the dimerization domain in a proline-rich sequence typical for recognition by SH2-binding proteins. Therefore, Rα potentially

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1 The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAP, A-kinase anchoring protein; D-AKAP1, dual specificity AKAP1; R, cAMP-dependent protein kinase regulatory subunit; C, cAMP-de
contains specific subdomains geared for protein-protein inter-
thactions that may fine-tune type I holoenzyme action in vivo.
Two new families of anchoring proteins, referred to as dual
specificity AKAPs (D-AKAPs), were identified recently from a
yeast two-hybrid screen using an RII fusion protein as bait (1, 2). These proteins not only bind RII but also RI subunits. The
R-binding region of D-AKAP1 localized to a 125-residue frag-
ment, RPP7. A human homolog of D-AKAP1, AKAP84, was
identified in sperm (36).
So far characterization of the various AKAPs and PKA has
focused on RII subunits. With the discovery of D-AKAP1, we
set out to determine sequence requirements not only for dimer-
ization of RIα but also for D-AKAP1 binding. A further goal was
to determine whether RI and RII mediate homodimerization
and anchoring by similar mechanisms. Deletion mutants, chi-
meric proteins, and site-specific mutants were used to deline-
ate critical regions as well as particular amino acids involved
in homodimerization and also to identify sequence require-
ments for RII and D-AKAP1 interaction. We have shown that
distinct but overlapping determinants are required for dimer-
ization and docking. Furthermore, and in contrast to earlier
predictions, we find strong similarities as well as subtle differ-
ces in the dimerization domains of RI and RII.

EXPERIMENTAL PROCEDURES

Materials—The following materials were purchased as indicated: ATP,
cAMP, protease inhibitors, Triton X-100, nickel-NTA resin (Qiagen),
cAMP-agarose and GST-agarose (Sigma), enzymes used for DNA manip-
ulations (Life Technologies, Inc.), and the DNA sequencing kit (U. S.
Biochemical Corp.). All oligonucleotides were synthesized at the Peptide
and Oligonucleotide Facility at the University of California, San Diego.

Construction of Mutants—All mutations in RIα were engineered by
site-directed mutagenesis using the Kunkel et al. (37) method as de-
scribed previously (38). N-terminal truncation mutants of RIα were
designed previously (38). N-terminal truncation mutants of RIα were
engineered by introducing an NheI site at codons encoding residues 17,
22, 37, and 45 using the vector pRSETb-RIα. Residues 1–17, 1–22, 1–37,
and 1–45 were then excised using NheI to make (Δ1–17)RIα, (Δ1–
22)RIα, (Δ1–37)RIα, and (Δ1–45)RIα, respectively.
Three classes of specific site mutations were also made. The cysteines
in the dimerization domain, Cys16 and Cys37, were mutated singly and
in pairs in order to extend our understanding for the role of the cys-
teines in dimerization and anchoring of D-AKAP1. Leon et al. (39) had
previously engineered RII(C37H) and shown that the disulfide bonds
are not required for dimerization. Two additional single site mutants
were made, RII(C16A) and RII(C37A). A double mutant was also
constructed, RII(C16A,C37A).

An additional set of mutants was used to assess requirements for
dimerization. These mutations were double mutants where Cys77 had
been mutated to either His or Ala. Mutating one of the cysteines was
sufficient to remove the two interchain disulfide bonds and allowed us
to examine the role of the additional residues for dimerization. Double
mutations with C37H were engineered by changing the following resi-
dues: V20A, I25A, L29A, L28A, Ile33/Val34, F52A and F47A.
A final set of mutations, V20A and I25A, was engineered that had only
single site mutations. These were used specifically to assess binding to
D-AKAP1. Arg44 and Arg57 also were both mutated to alanine to make the
double mutant, RII(R44A,R57A), as described previously (40, 41). A
summary of all the mutants used for analysis is presented in Fig. 1A.

Construction of Chimeric Proteins—Two chimeric proteins of RIα and
RIα were constructed and expressed as polyHis fusion proteins. First,
an Nhel site was engineered just before the initiation Met on pRSETb-
RIα and pRSETb-RIα, respectively. An MunI site was then engineered
at residue 62 of RIα and residue 46 of RIα on these two vectors
containing the new Nhel sites. One protein, designated (His6)RIα/RIα,
was constructed by excising the cDNA encoding the first 62 residues of
RIα with Nhel and MunI and then ligating this oligonucleotide frag-
ment to cDNA encoding residues 47–379 of RIα. This chimeric protein
contains the N-terminal dimerization of RIα fused to the C terminus of
RIα. The other protein, designated (His6)RIα/RIα, was constructed by excising
the cDNA encoding the first 45 residues of RIα with Nhel and
MunI and ligating this fragment to the cDNA encoding residues 63–379
of RIα. This chimeric protein contained the N-terminal dimerization
domain of RIα fused to the C terminus of RIα (Fig. 1B).

Expression and Purification of R Subunits—Two methods were used to
purify the wild type and mutant R subunits. In method 1, the deletion
mutants were expressed in BL21(DE3) cells. The BL21(DE3) cell strain
was a gift from Bill Studier (Brookhaven National Laboratories). The R
subunits were then purified on a DE52 ion exchange column as de-
scribed previously (42). Further purification was achieved using MonoQ
(Amersham Pharmacia Biotech) ion exchange chromatography. In
method 2, the R subunits were expressed in 222 cells (42) and then
affinity purified on cAMP-agarose resin (43). Further purification of these
mutants was achieved using gel filtration (Superdex 200). Cells were
lysed in 20 mM phosphate buffer containing 5 mM β-mercaptoethanol
and protease inhibitors at pH 6.5 (42). After centrifugation, the supernatant
was incubated with cAMP resin for 6 h at 4 °C. After extensive washing,
the R subunits were eluted in lysis buffer using 50 mM cAMP.

Analytical Gel Filtration—Analytical gel filtration was carried out
using a Superdex 200 HR 10/30 column with a flow rate of 0.4 ml/min
at 22 °C in MOPS buffer (20 mM, pH 7.0) containing 100 mM KCl on an
Amersham Pharmacia Biotech FPLC. Proteins were loaded onto the
Superdex 200HR 10/30 column at an initial concentration of 1 mg/ml.
Mutants were compared with wild type RIα dimer and the monomeric

FIG. 1. A, mutations in the dimerization/docking domain of RIα. Residues 12–61 that define the DD domain of RIα are shown. Residues that were
mutated are indicated with a filled circle. The deletion mutants are represented by lines below the sequence. B, schematic representation of RIα/RIα
and RIα/RIα chimeras. The chimeric constructs of RI and RII are schematically shown. The boundaries of the fusion proteins are indicated.
deletion mutant (Δ1–91)RIα. To determine the apparent molecular weight and Stokes radii of the various mutants, the column was calibrated using the Amersham Pharmacia Biotech Calibration Kit.

**Electrophoresis of Proteins**—Samples of wild type and mutant RIα subunits were denatured in gel loading buffer and subjected to electrophoresis in polyacrylamide gels as described previously (39). Non-denaturing gel electrophoresis was performed in a 7.5% polyacrylamide gel at 50 V for 4–6 h (44). All electrophoresis was performed using Mini-Protein II electrophoresis system (Bio-Rad). SDS-PAGE reagents were prepared according to Laemmli (45). Proteins were visualized by staining with Coomassie Blue.

**D-AKAP1 Binding Assay**—A deletion mutant of D-AKAP1, RPP7 (1) which contains the R-binding region, expressed as either a polyHis-tagged fusion protein or a GST fusion protein, was used in these binding assays. This fragment corresponds to residues 284–408 in the D-AKAP1 core. Bacterial cell lysates containing His6-RPP7 or GST-RPP7 were incubated with either nickel-NTA resin or glutathione resin, respectively, for 2 h at 4 °C in phosphate-buffered saline (10 mM potassium phosphate, 150 mM NaCl, pH 7.4) with 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 5 mM β-mercaptoethanol and then washed extensively with the same buffer. Full-length and RI mutants (100–200 mg) were then added to the resin and incubated for 2 h at 4 °C. After washing the resin extensively with phosphate-buffered saline, proteins associated with the resin were released by boiling in SDS gel-loading buffer and analyzed by SDS-PAGE.

**RESULTS**

**Minimum Core Requirements for Dimerization of RIα**—The following nested N-terminal deletion mutants were made, expressed, and purified to homogeneity as described under "Experimental Procedures": Δ(1–17)RIα, Δ(1–22)RIα, (Δ1–37)RIα, and Δ(1–45)RIα. Once the purity of each protein was assessed by SDS-PAGE (Fig. 2A), the oligomerization state was analyzed by both analytical gel filtration and electrophoresis under non-denaturing conditions (Fig. 2, B and C). (Δ1–17)RIα eluted as a single peak with a retention volume that was identical to the wild type dimer, RIα. (Δ1–37)RIα and (Δ1–45)RIα both eluted as a single peak but were monomeric based on their retention volume (Fig. 2B). The apparent molecular weight and Stokes radii of all the mutants were compared with the wild type RIα and presented in Fig. 2D.

In contrast to the above mutants, deletion of five additional residues beyond 17 yielded a protein, (Δ1–22)RIα, that displayed an intermediate behavior (Fig. 3A). It eluted as two distinct peaks, one migrating at the characteristic dimer position, and the other migrating with a retention volume corresponding to a monomer. Based on SDS-PAGE analysis, the protein was >95% pure, indicating that the two peaks were not due to heterogeneity (Fig. 3B). This behavior is characteristic of two species in equilibrium if they are in slow exchange. To test this possibility, the concentration dependence of the elution profile was tested. After loading (Δ1–22)RIα at three different chromatography elution profile for RIα deletion mutants. (Δ1–17)RIα (I), (Δ1–22)RIα (II), and (Δ1–45)RIα (III) were loaded at a concentration of 1 mg/ml. The Superdex 200 column was run in 150 mM potassium chloride, 20 mM MOPS, pH 7.0, 1 mM dithiotheitol at a flow rate of 0.4 ml/min. Column effluent was monitored at an absorbance of 280 nm. Arrows indicate the position of the dimeric and monomeric R subunits. (Δ1–17)RIα migrates as the wild type dimeric RIα. (Δ1–22)RIα migrates as two distinct species, one with the molecular weight corresponding to dimeric RIα and the other corresponding to monomeric RIα. (Δ1–37) and (Δ1–45)RIα migrate with retention volumes characteristic of monomeric RIα. Only one representative profile of these three is shown. C, native gel analysis of deletion mutants. A correlation between oligomerization state and mobility in a non-denaturing polyacrylamide gel was established for RIα deletion mutants. D, determination of Stokes radii for wild type and RIα mutants. Samples of wild type and mutant proteins were characterized by gel filtration as described under "Experimental Procedures." The data were plotted according to Siegel and Monty (52). Arrow 1 indicates the position of the (Δ1–37)RIα and (Δ1–45)RIα; arrow 2 indicates the position of the monomeric phenylalanine mutants; arrow 3 indicates the position of the dimeric RIα. V_M, retention volume of dimer; V_M, retention volume of monomer.
concentrations, the ratio of the dimeric peak to the monomeric peak was monitored. As shown in Fig. 3A, the slower migrating peak, corresponding to the monomer, was more prominent as the loading concentration of the protein decreased, whereas the peak corresponding to the dimer decreased. Moreover, when the first peak was collected, concentrated, and reloaded onto the analytical Superdex 200, two species were generated thus confirming that both species exist in a slowly exchanging equilibrium (Fig. 3B). The presence of the dimeric peak was also salt-dependent. When the column was equilibrated with high salt (1 M NaCl), the ratio of the dimer to monomer changed with the dimer peak being enhanced (data not shown). These combined results indicate that there are critical amino acids between residues 17 and 22 that mediate dimerization. Their removal significantly reduced the equilibrium between the two protomers. Given that the interaction is enhanced at higher ionic strength, the nature of the interaction of the monomers is likely to be predominantly hydrophobic in nature. We thus believe that the main “core” for mediating dimerization of RIα lies within the region flanked by residues 17 and 37, and we refer to this disulfide-bonded core subsequently as subdomain 1 (Fig. 4).

Alanine-scanning Mutagenesis of RIα—The contribution of particular amino acids to dimerization of RIα was evaluated by alanine-scanning mutagenesis. Since the two cysteines in the dimerization domain, Cys16 and Cys37, form interchain and anti-parallel disulfide bonds, it was essential to analyze these mutants in the absence of any covalent linkage. Earlier, Leon et al. (39), had shown that the removal of one Cys, namely C37H, abolished both disulfide bonds thus supporting the original prediction for anti-parallel arrangement of the two protomers (39, 46). However, the dimer remained intact, demonstrating that the disulfide bonds were not essential for dimerization. All additional mutants for this study of the dimerization determinants were made in addition to the C37H mutation. The mutants were classified into two categories that reflect two “subdomains,” the disulfide-bonded core mutants (subdomain 1) and the “C-terminal helix” mutants (subdomain 2) (Fig. 4). Based on the deletion mutants “subdomain 1” is essential for dimerization. Since salt-dependence of dimerization was indicative of hydrophobic interactions, we speculated that hydrophobic amino acids are key players at the dimer interface, and the mutagenesis was set up to test this hypothesis. The C-terminal helix or “subdomain 2” is based on earlier secondary structure predictions done by CDESTIMA as reported earlier (39) as well as sequence alignments and inference from NMR secondary structure analysis that had been done on the dimerization domain of RII (3). The boundaries of the subdomains and the various mutants are summarized in Fig. 4.

Each mutant protein was analyzed for its oligomerization state by analytical gel filtration and non-denaturing gel electrophoresis. Surprisingly, none of the mutations in the core affected the oligomerization state of RIα. As shown in Fig. 5A all the core mutants as well as the single site cysteine mutants migrated like the wild type RIα subunit dimer under native conditions. Moreover, they eluted with the same retention volume on an analytical gel filtration column (Fig. 2D). The only mutations that affected dimerization were RIα(C37H/F47A)...

**Fig. 3.** **Gel filtration analysis of (Δ1–22)RIα.** A, concentration dependence. (Δ1–22)RIα was loaded at a concentrations of 1 mg/ml (left), 0.5 mg/ml (middle), and 0.25 mg/ml (right). The Superdex 200 column was run in 150 mM potassium chloride, 20 mM MOPS, pH 7.0, 1 mM dithiothreitol at a flow rate of 0.4 ml/min. Column effluent was monitored at an absorbance of 280 nm. Arrows indicate the position of the dimeric and monomeric R subunits. (Δ1–22)RIα migrates as two distinct species, one with the molecular weight corresponding to dimeric RIα and the other corresponding to monomeric RIα. B, establishment of equilibrium. The dimeric species of (Δ1–22)RIα re-equilibrates. The ascending portion of the first peak was collected, concentrated, and reloaded on the Superdex 200 column. VM, retention volume of dimer; V_M, retention volume of monomer.

**Fig. 4.** **Subdomains within the RIα DD domain.** The DD domain of RIα is comprised of two subdomains. Subdomain 1 is flanked by the cysteines, and subdomain 2 begins at residue 45 and is predicted to be helically based on secondary structure algorithms. The residues within subdomain 1 that were mutated are marked with asterisks, and those in subdomain 2 are marked with an arrow. F/A marks the two single-site alanine mutant that were monomeric.
and RIα(C37H/F52A). As shown in Fig. 5B their mobility on a non-denaturing gel was clearly shifted as compared with the dimeric RIα subunit. Assuming that this mutation has not affected the charge to mass ratio, the mobility is indicative of a change in apparent molecular weight of this protein (44). Analytical gel filtration analysis of these mutants further confirmed that they are monomeric (Figs. 5C and 2D). Additionally, once these mutants were analyzed on SDS-PAGE, it was confirmed that the integrity of the molecule was retained, and the change seen in apparent molecular weight was not simply due to degradation (Fig. 5D).

Localization of the RIα-binding Surface—To localize further the D-AKAP1-binding site on RIα, the N-terminal deletion mutants discussed above were tested for their ability to bind the R-binding region of D-AKAP1(RPPTP). His9-RPPTP was able to pull down (Δ1–17)RIα and (Δ1–22)RIα. However, (Δ1–45)RIα and (Δ1–91)RIα were not pulled down in the binding assay. Residues 22–45 are thus necessary for interaction with D-AKAP1. Since deletion of the first 45 residues also abolished dimerization, it is likely that a dimer is necessary for interaction with D-AKAP1 (Fig. 6, A and B). To characterize further the anchoring properties of RIα, a series of site-directed mutants were also tested to identify specific amino acids that are essential for interaction with D-AKAP1. The Cys residues that form disulfide bonds and some of the hydrophobic amino acids were targeted in particular. The single site mutants were then characterized for their ability to bind His9-RPPTP.

The anti-parallel disulfide bonding between Cys16 and Cys37 is one of the features that distinguishes RIα from RIβ. Although these disulfide bonds are not essential for dimerization, they are extremely stable even under highly reducing conditions (39). To test if they contributed to D-AKAP1 interaction, Cys16 was mutated to His, and Cys37 was mutated to both His and Ala. A double mutant with both Cys16 and Cys37 changed to Ala was also engineered. Although RIα(C16H) showed no reduction in binding to D-AKAP1, D-AKAP1 binding was nearly abolished when Cys37 was replaced with His. Therefore, even though Cys16 forms an intermolecular disulfide bond with Cys37 and neither mutation interfered with dimerization, the mutations had different effects upon D-AKAP1 binding. As shown in Fig. 6C, RIα(C37A) and the double mutant RIα(C16A,C37A) also were unable to bind tightly to D-AKAP1.

Since long chain aliphatic residues were shown to be essential for RIβ-AKAP interaction (44), Val20 and Ile25 were also mutated to Ala individually and tested for their ability to interact with D-AKAP1. As shown in Fig. 6C, both mutations resulted in the total loss of ability to associate with D-AKAP1.

D-AKAP1 Interaction with Chimeric Proteins of RIα and RIβ—Although D-AKAP1 tethers both RIα and RIβ through their N termini, these domains are among the least conserved regions in the two R subunits. The dimerization domain boundaries are defined within residues 12–61 of RIα and the first 45 residues of RIβ (39, 47, 48). To characterize further the requirements for conveying D-AKAP1 binding at the N terminus of the two classes of R subunits and to determine whether the dimerization domain was sufficient for anchoring, we tested the ability of two chimeric proteins, (His6)RIα/RIβ and (His6)RIβ/RIα, to bind GST-RPPTP. These two proteins were engineered so that only the N-terminal dimerization domains were swapped. Specifically, the RIα/RIβ chimera contained the dimerization domain of RIα and the C terminus of RIβ, whereas the RIβ/RIα chimera contained the dimerization domain of RIβ and the C terminus of RIα (Fig. 1B). As shown in Fig. 6D, GST-RPPTP bound to both chimeras but had lower affinity for His9RIα/RIβ. Based on these preliminary qualitative findings, the first 61 amino acids of RIα are probably not sufficient to generate an optimal binding site for D-AKAP1.

Effect of Mutations at the Inhibitor Site of RIα for Anchoring D-AKAP1—The inhibitor site in RIα that is responsible for recognition by the C subunit is localized C-terminally to the dimerization domain. This site, Arg94–Arg95–Gly-Ala-Ile98, is
thought to bind in the active site of the C subunit in the holoenzyme, therefore rendering the enzyme inactive. A double mutant with the two arginines changed to Ala was also tested for its ability to bind D-AKAP1. RIα(R94A,R95A) showed decreased affinity for D-AKAP1 relative to wild type RIα. These data are schematically summarized in Fig. 6E.

**Isoform Specificity for D-AKAP1**—To investigate further the specificity of D-AKAP1 for the various R subunit isoforms, RIα, His6-RIβ, His6-RIIα, and RIIβ were tested in parallel for their ability to bind to the R-binding fragment of D-AKAP1, RPP7. As shown in Fig. 7, RIα, His6-RIIα, and RIIβ were all pulled down by the GST-fused R-binding fragment of D-AKAP1 and GST-RPP7, but His6-RIβ was not.

**DISCUSSION**

The dimerization domain at the N terminus of RIα is a very stable domain that is also essential for RIα binding to a novel family of dual specificity AKAPs (1, 39). To emphasize the multiple functions of this domain, it will subsequently be referred to as the dimerization/docking (DD) domain. A series of mutants were engineered to probe the structural features of this domain and to identify regions as well as residues that contribute to dimerization and/or docking.

![Fig. 6](http://www.jbc.org/Downloaded_from http://www.jbc.org/Downloaded_from.png)
By using deletion and site-specific mutants, the boundaries required for dimerization were defined. Deletion of up to 17 residues had no effect on dimerization, whereas the deletion of five more residues (Δ1–22) generated a concentration-dependent dimer. Since dimerization of (Δ1–22)RIα was enhanced by high salt, the hydrophobic residues in this region, Leu18, Tyr19, and Val20, are potential contributors to the hydrophobic dimer interface. Single site replacement of Val20 to Ala, however, did not abolish dimer formation. Deletion of the first 37 residues abolished dimerization. Therefore, the region flanked by the two cysteines, the “disulfide-bonded core” or subdomain 1, is essential for dimerization of RIα.

Single site mutations of Cys16 or Cys37 to Ala or His in the disulfide-bonded core were not sufficient to abolish dimerization; however, two other mutations did abolish dimerization, RIα(C37H,F47A) and RIα(C37H,F52A). These aromatic residues, both localized in subdomain 2, are clearly contributing to the dimer interface either directly or by folding back on to subdomain 1. Thus, although residues 17–37 are essential for dimerization, they are not sufficient.

The anchoring of RIα to D-AKAP1 also depended on the dimerization state of RIα. Deletion of the region between residues 22 and 45 abolished both dimerization and anchoring, whereas (Δ1–22)RIα was able to bind D-AKAP1. Thus, the surface created by the dimer is probably as critical for anchoring to D-AKAP1 as it is for anchoring to other AKAPs (48, 49). Single site mutations, C37H, V20A, and I25A identified at least three critical residues for D-AKAP1 interaction. Although these mutations did not abolish dimerization, they did reduce the affinity of RIα for D-AKAP1, suggesting that the requirements for dimerization and docking are distinct yet overlapping. The effect of the C37H mutation was particularly striking since the C16H replacement had no effect on binding to D-AKAP1 and suggested that the specific surface flanking Cys37 might be a “hot spot” for RIα and D-AKAP1 interaction. Furthermore, even though the two cysteines are involved in an interchain disulfide bond, their role in anchoring appears to be asymmetric: Cys37 is essential and Cys16 is not.

The construction of RIα/RIIα chimeras shed further light on the differences in anchoring requirements between RIα and RIIα. The apparent affinity of D-AKAP1 for RIIα (50 nM) is higher than that for RIα (1 μM) based on surface plasmon resonance (50). Since the RIα/RIα chimera that contained only the DD domain of RIIIα also bound tightly to D-AKAP1, this domain is sufficient for conveying high affinity binding to D-AKAP1. In contrast, the parallel chimera that contained only the DD domain of RIα did not bind tightly to D-AKAP1, indicating that additional regions peripheral to the DD domain are required for RIα to bind optimally to D-AKAP1. Further evidence to support this hypothesis came from another RIα mutant where the inhibitor site that docks to the active site cleft of the C subunit in the holoenzyme complex was mutated. Specifically, Arg94 and Arg95 that dock to the P-3 and P-2 recognition sites in the catalytic subunit were replaced with Ala. This mutant RIα(R94A,R95A) also did not bind efficiently to D-AKAP1, suggesting that these residues either interact directly with D-AKAP1 or contribute to the domain organization of RIα so that the DD domain is poised for interaction with D-AKAP1. Quantitation of binding is necessary to determine whether full-length RIκ binds more tightly to D-AKAP1 than the DD domain alone. Based on our results, however, the dimerization domain of RIΙκ alone, but not RIα, is necessary and sufficient for conveying high affinity binding to AKAPs.

Since previous AKAPs have been shown to interact specifically with RIΙκ, it was of particular interest to compare the anchoring properties of D-AKAP1 to the different isoforms of

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**Fig. 7. Isoform selectivity of D-AKAP1.** RIα, His9 RIIIα, and RIIβ were pulled down by GST-RPP7 in the binding assay, whereas His9 RIβ was not. Closed circles indicate GST-RPP7. Closed triangles indicate RIα, His9 RIIIα, and RIIβ, and the open triangle indicates where His9 RIβ should run on the SDS-PAGE.

**Fig. 8. Comparative analysis of RIα and RIΙκ DD domain.** Sequence alignment of RI and RIΙκ are shown. Conserved residues are highlighted in red. Red arrows denote mutations that have affected dimerization. Blue arrows indicate mutations that do not affect dimerization. Asterisks indicate mutations that have disrupted anchoring. Below each R subunit the boundaries of secondary structure are indicated. In the case of RIα subunit it is based on secondary structure algorithms (39), and in the case of RIΙκ subunit it is based on NMR analysis (3).
the R subunits. D-AKAP1 binds RIα, RIβ, and RIγ, but it does not bind RIβ unlike the sequence similarities between RIα and RIγ. Sequence comparison of the two indicates that within the region flanked by the two cysteines, subdomain 1, there are two nonconservative mutations (K22L and A24G). Given that these mutations lie within a region predicted to be critical for D-AKAP1 interaction, it might be sufficient that the absence of a charged group or a polar residue is enough to perturb the binding surface required for D-AKAP1 binding. In the subdomain 2, there are four non-conservative mutations (T34I, A38S, A46K, and A60N). These mutations would also perturb the binding surface indirectly by altering the dimer interface. Moreover, the actual boundaries of dimerization and the nature of disulfide linkages have not been mapped out in RIβ; there are two additional cysteines that, depending on their oxidation states, could affect the structural orientation of the dimeric interface and subsequently the binding surface for D-AKAP1.

The mutations described here also allow for a rigorous comparison of RIα and RIα subunits in terms of both functions of the N-terminal domain, namely dimerization and docking. The alignment shown in Fig. 8 reveals that the similarities within this domain are more extensive than previously thought. Functional similarities are further supported by the mutations when our results are compared with previous biochemical and functional studies on RIα and RIβ (43, 44).

Based on site-directed mutagenesis and deletions, the dimerization domain of RI, encompassing the first 45 residues, is necessary for its interaction with previously identified AKAPs. Dimerization, in fact, is a prerequisite for AKAP binding (22–24). In analogy, Huang et al. (1) also showed that the DD domain of RIα is sufficient for D-AKAP1 interaction. Since all regulatory subunits of PKA are dimers, except that of the RI(DD) has been probed by similar techniques. An understanding of these mutations in the context of their role in the secondary structures of these domains and ultimately tertiary and quaternary structure will help delineate the exact mechanisms of homodimerization as well as anchoring of RI and RI'.
Dimerization/Docking Domain of the Type Iα Regulatory Subunit of cAMP-dependent Protein Kinase: REQUIREMENTS FOR DIMERIZATION AND DOCKING ARE DISTINCT BUT OVERLAPPING
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