Characterization of Structural Features That Mediate the Tethering of Caenorhabditis elegans Protein Kinase A to a Novel A Kinase Anchor Protein

INSIGHTS INTO THE ANCHORING OF PKAI ISOFORMS

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Caenorhabditis elegans protein kinase A (PKAI

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subunit of protein kinase A; AKAP, A kinase anchor protein; R, regulatory subunit of protein kinase A; IPTG, isopropyl-1-thio-D-galactopyranoside; GST, glutathione S-transferase; FPLC, fast protein liquid chromatography.

1 Mammalian PKA isomers are named according to their homomeric R subunits. Distinct genes encode RIα, RIβ, RIIα, and RIIβ.

2 The abbreviations used are: PKA, protein kinase A or cAMP-dependent protein kinase; PKA, A kinase anchor protein; R, regulatory subunit of protein kinase A; IPTG, isopropyl-1-thio-D-galactopyranoside; GST, glutathione S-transferase; FPLC, fast protein liquid chromatography.

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AKAPCE is a novel tethering protein that fulfills the specified criteria (37). Nevertheless, the cited studies suggest important principles: (1) the oversized supply of PKA catalytic subunits (34). Rescue of calcium flux by providing a properly localized supply of PKA catalytic subunits (34). Rescue of calcium channel regulation by immobilized PKAα is apparently explained by the discovery that high affinity RII-binding sites in certain AKAPs also ligate RII (PKAα). However, these AKAPs bind RIIα (PKAIIα) with 25–50-fold higher affinity than RIIα (34, 35). Only small amounts of PKAα are likely to be anchored by this mechanism in normal physiological contexts because most cells/tissues have concentrations of RIIα and/or RIIβ subunits that approach or exceed the level of RI (1–4, 36). Nevertheless, the cited studies suggest important principles: (a) RI dimers have a binding surface available for interactions with anchor proteins, (b) PKAα can be anchored in intact cells, and (c) properly targeted AKAP-PKAα complexes can be poised to phosphorylate a co-localized effector protein and thereby, selectively regulate a key, compartmentalized physiological process. A complementary but potentially incisive approach to advancing our understanding of the molecular basis and regulatory significance of PKA anchoring involves discovery and systematic characterization of RI-selective, prototype AKAPs. Ideally, AKAPs to be studied should engage RI (PKAI) with high affinity (Kd in the 1–100 nM range) and efficiently discriminate against RII subunits (e.g. Kd (RI):Kd (RII) > 100) in vitro and in cells. Recently, we discovered the first eukaryotic tethering protein that fulfills the specified criteria (37). AKAPCE is a novel Caenorhabditis elegans protein composed of 1280 amino acids (M, 144,000). The tethering site of AKAPCE binds the RIIα-like subunits (RCE) of C. elegans PKAI (PKAI) with a Kd of 7 nM (37). Moreover, high concentrations of RIIα and RIIβ subunits do not competitively inhibit formation of RCE-AKAPCE complexes in vitro or in transfected cells. The AKAPCE polypeptide includes a RING finger domain and other putative anchoring motifs3 that may mediate the routing of tethered PKAI to several intracellular docking sites (37). The catalytic subunit of PKAI is 82% identical with Ca2+, the predominant catalytic subunit of all mammalian PKA isoforms (38). RCE is closely related to mammalian RIs (~60% overall sequence identity) but not RII subunits (39). In C. elegans 60% of PKAI is tightly associated with organelles/cytoskeleton (39) and AKAPCE is the principal RCE-binding protein.3 Because pathways and mechanisms of signal transduction are highly conserved between C. elegans and mammals (40–42), it is probable that tethering and targeting of RCE subunits by AKAPCE adapts and diversifies PKAI for multiple functions in various compartments of C. elegans cells (39).

The discovery of a high affinity, RCE-selective anchor protein (AKAPCE) and availability of cDNAs, protein expression systems, and specific antibodies for AKAPCE and RCE (37–39) can be exploited to elucidate molecular mechanisms that govern the selective targeting/anchoring of PKAI. Several basic but fundamental questions merit immediate consideration: What structural features in AKAPCE are essential for avid ligation of RCE? Conversely, which amino acid side chains in RCE create a binding surface that docks with the tethering site in AKAPCE? Which residues in RCE and the anchor protein govern binding affinity? How are RII (PKAII) isoforms excluded from the tethering site of AKAPCE? Does the tethering domain of AKAPCE exhibit plasticity? That is, can substitution of one or a few amino acids generate a binding region that selectively sequesters RII subunits? Can the wild type RCE tethering site bind mammalian RIIα with high affinity? In this paper we address the central questions posed above and also report that the tethering module from AKAPCE may provide a molecular tool for manipulating the intracellular location of mammalian PKAI.

**EXPERIMENTAL PROCEDURES**

cDNAs and Expression Vectors—Complementary cDNAs encoding RCE, AKAP75, 5-AKAP84, and human RIIβ were cloned and characterized as described in previous papers (9, 19, 26). A cDNA clone encoding murine RIIα was generously provided by Dr. Robert Steinberg (Department of Biochemistry, University of Oklahoma). Full-length cDNAs for RCE and RIIβ were subcloned in the bacterial expression plasmid pET14b as described previously (26, 37). An analogous pET14b construct was created for expression of RIIa by following the strategy used for RCE cDNA (26). Phosphorylatable Ser residues were introduced into the pseudosubstrate sites of RCE and RIIα via site-directed mutagenesis, as described previously (37). This mutation has no effect on the properties of RCE or RIIα (43). However, it enables efficient phosphorylation of R subunits by incubation with Mgγ-[32P]ATP and the catalytic subunit of PKA, as previously reported (37). Cloning of cDNAs in pET14b enabled IPTG-inducible high level expression of soluble R subunits that contained an N-terminal, 20-residue fusion peptide. Included in the fusion peptide are six consecutive His residues (His tag) that enabled purification of recombinant RCE, RIIα, and RIIβ proteins to near homogeneity, as described by Li and Rubin (26). A fragment of AKAPCE cDNA that encodes amino acids 176–409 in the anchor protein was cloned into the expression plasmid pGEX-KG as described previously (37). This enabled synthesis of a GST-AKAPCE fusion protein in Escherichia coli DH5α that was transformed with recombinant plasmid and induced with IPTG. After induction, bacteria were disrupted in a French press, and the soluble GST-AKAPCE (176–409) protein was purified to near homogeneity by affinity chromatography on GST-Sepharose 4B beads (Amersham Pharmacia Biotech) as previously reported (44). The RCE tethering domain of AKAPCE encompassed residues 236–255 (37). Full-length AKAPCE was cloned in the mammalian expression vector pCI32 as described by Angelo and Rubin (37). Full-length RIIα cDNA was excised from a recombinant pBluescript SK plasmid by digestion with NotI and ApaI and ligated into the RoCMV mammalian expression vector (Invitrogen), which was cleaved with the same enzymes. This placed the cDNA downstream from a strong cytomegalovirus promoter and upstream from a polyadenylation signal. RIIα cDNA was also amplified by the polymerase chain reaction, using previously described conditions (44). The 5′ primer appended a BamHI restriction site to the amplified cDNA; the 3′ primer contained an SpeI cleavage site. Amplified DNA was digested with BamHI and SpeI and then cloned in the mammalian expression vector pEBG (45), which was cleaved with the same enzymes. (The pEBG plasmid was generously provided by Dr. R. A. Angelo and C. S. Rubin, unpublished observations.)
Joseph Avruch, Harvard Medical School.) This accomplished an in-frame fusion of RCE cDNA with an upstream GST gene. The fidelity of polymerase chain reaction-mediated amplification of RCE cDNA was verified by DNA sequencing. Transcription of the chimeric gene is driven by the strong, constitutively active EF1α promoter; an optimal polyadenylation signal follows the inserted RIα gene. Samples were then incubated for 12 h at 4 °C. Subsequently, 40 μl of a 50% suspension of protein A-Sepharose 4B beads was added, and the incubation was continued for 2 h. Beads containing IgG, RIα, and associated proteins were recovered by centrifugation at 3,000 × g for 1 min. Next, the beads were washed three times with 1 ml of lysis buffer by repeated resuspension and centrifugation. Finally, proteins bound to the beads were eluted by boiling in gel loading buffer and analyzed by denaturing electrophoresis and Western immunoblotting as described above.

**RESULTS AND DISCUSSION**

**The Tethering Site of AKAP CE Is a Mosaic Structure: RCE and RIα by AKAP CE**

Using deletion mutagenesis, we previously mapped the RCE-binding site to a segment of AKAP CE that encompasses amino acids 236–255 (38). To guide studies on individual amino acids that are crucial for the ligation of RCE, the sequence of the tethering region of AKAP CE was aligned with a prototype mammalian RII-binding domain (from AKAP75) and the binding region of S-AKAP84, which avidly complexes RII isoforms and binds RIα with low affinity (Fig. 1A). The sequences of the three tethering domains are divergent. However, like classical RII-binding regions (25), residues...
236–255 in AKAPCE are predicted to fold into an \( \alpha \)-helix that contains opposing hydrophobic and hydrophilic surfaces (Fig. 1B). The positions of Leu236, Ile248, and Leu252 in AKAPCE are invariably occupied by either Leu, Ile, Val, or Thr in previously characterized AKAPs (9, 19, 23, 52). Moreover, each large hydrophobic side chain of the corresponding amino acids in AKAP75 (Leu393, Ile405, and Ile409) (Fig. 1A) plays a key role in mediating high affinity binding of PKAII isoforms (19). The functional significance of Leu236, Ile248, and Leu252 (and other amino acids) in the AKAP CE tethering domain was evaluated by a combination of (a) site-directed mutagenesis, (b) AKAPCE fusion protein expression in \( E. \) coli, (c) purification of fusion proteins by affinity chromatography, and (d) biochemical assays for RCE binding activity and ligand specificity.

A cDNA fragment that encodes amino acids 176–409 in AKAPCE was cloned in the plasmid pGEX-KG to generate a template for mutagenesis and recombinant protein production. In the recombinant plasmid, the cDNA insert is preceded by a GST gene and a \( \text{tac} \) promoter. This arrangement enables IPTG-inducible, high level expression of wild type and mutant GST-partial AKAPCE polypeptides in \( E. \) coli (37). Wild type AKAPCE fusion protein (designated GST-AKAPCE 176/409) includes the complete tethering region (residues 236–255) and binds RCE with the same affinity and specificity as full-length AKAP CE (37). Effects of mutations on tethering activity were assessed by monitoring formation of \( 32^\text{P}-\text{RCE} \) complexes in a well-established, highly sensitive, overlay binding assay (49, 50). Substitution of Ala for Leu236 diminished the RCE binding activity of GST-AKAPCE 176/409 by \( \sim 90\% \) (Fig. 2). Replacement of either Ile248 or Leu252 suppressed RCE binding activity to an even greater extent. Under standard assay conditions, mutant fusion proteins containing Ala248 (Fig. 2) or Ala252 (Fig. 3) in the tethering site bound only 1–2\% of the amount of \( 32^\text{P}-\text{RCE} \) that was complexed by wild type GST-AKAPCE 176/409. The results suggest that the large, aliphatic side chains of Leu236, Ile248, and Leu252 contribute to a precisely configured binding pocket that accommodates a target nonpolar region in RCE dimers. Replacement of any of these large hydrophobic amino acids with Ala (which stabilizes \( \alpha \)-helices but possesses only a small nonpolar side chain) evidently alters the size and/or shape of the binding pocket so that it no longer provides a complementary surface for RCE ligation. In contrast to the preceding observations, replacement of Leu236, Val247, or both amino acids with Ala has no effect on formation of partial-AKAPCE-RCE complexes (Fig. 2 and Ref. 37). Thus, incorporation of Leu, Val, or Ile residues at nonconserved positions along the tethering site \( \alpha \)-helix results in side chain orientations that have little or no impact on the RCE-binding pocket.

Further analysis of the alignment of AKAPCE with AKAP75 and S-AKAP84 (Fig. 1) revealed distinctive features of the \( C. \) elegans anchor protein. The last two residues in the AKAP CE tethering domain are hydrophilic and carry positive charges; hydrophobic or neutral amino acids occupy these positions in classical RII-binding AKAPs. Mutation of His254-Arg255-Lys256 to the neutral tripeptide Glu-Ala-Ala caused a substantial (2–3-fold) increase in binding of RCE by AKAPCE (Fig. 3). Thus, the basic, C-terminal portion of the AKAP CE tethering site may negatively regulate affinity for RCE. An intrinsic limitation in RCE binding affinity could have significant physiological consequences. A substantial amount of PKAICE accumulates in the cytoplasm of \( C. \) elegans cells (39). By analogy with cytoplasmic...
Selective Tethering of RCE and RII by AKAP<sub>ce</sub>

![Diagram of AKAP<sub>ce</sub> tethering site.](Image)

AKAP<sub>ce</sub> tethering site.

**Fig. 2. Identification of amino acids in AKAP<sub>ce</sub> that are critical for high affinity binding of RCE.** Amino acids in the tethering site of AKAP<sub>ce</sub> were altered by site-directed mutagenesis as described under “Experimental Procedures.” Wild type and mutant GST fusion proteins (apparent M<sub>r</sub> = 54,000), which contain residues 176–409 from AKAP<sub>ce</sub>, were expressed in E. coli, purified by affinity chromatography, size-fractionated by denaturing electrophoresis, and transferred to an Immobilon P membrane (see “Experimental Procedures”). Equal amounts of purified protein (0.1 μg) were applied to each lane of the denaturing gel. Abilities of the wild type and mutant AKAP<sub>ce</sub> tethering domains to ligate <sup>32</sup>P-labeled RCE were assessed by performing overlay binding assays (“Experimental Procedures”) on the Western blots. Autoradiograms are shown. The experiment was repeated three times, and similar results were obtained in each replication. Lane 1 received a GST-AKAP<sub>ce</sub> fusion protein with the wild type (WT) binding domain; other fusion proteins contained AKAP<sub>ce</sub> tethering regions with the following mutations: lane 2, Leu<sup>236</sup> → Ala; lane 3, Ile<sup>246</sup> → Ala; lane 4, Leu<sup>248</sup> Val<sup>249</sup> → Ala Ala; lane 5, Phe<sup>243</sup> → Ala; lane 6, Phe<sup>243</sup> → Val; lane 7, Ser<sup>244</sup> → Asn; lane 8, Ser<sup>244</sup> → Asp. Only the relevant portion of the lanes is shown; no other bands were observed. Asterisks indicate amino acids that govern the affinity and/or R isoform selectivity of the tethering site; asterisks mark amino acids that are not directly involved in R<sub>ce</sub> ligation.

Physiological responses to signals carried by the second messenger.

A prominent and unique feature of AKAP<sub>ce</sub> is evident in the central core of the tethering domain. Classical RII-selective AKAPs contain a hydrophobic dipeptide composed exclusively of Leu, Val, or Ile residues (Leu-Val in AKAP75; Ile-Ile in S-AKAP84; Fig. 1A) that is crucial for the generation and maintenance of the RII-binding site (19, 52). The appearance of a Phe<sup>243</sup>-Ser<sup>244</sup> dipeptide in the corresponding region of the AKAP<sub>ce</sub> tethering domain represents a major deviation from the previously studied paradigm. Recently, we discovered that replacement of Phe<sup>243</sup> with Ala results in substantial suppression but not extinction of R<sub>ce</sub> binding activity (37). This raised the possibility that a side chain with aromatic hydrophobic character was required at this position in the tethering domain to ensure avid ligation RIs—like RCE—Retention of R<sub>ce</sub> binding activity upon substitution of Phe<sup>243</sup> with Trp and the unaltered binding activity of a Tyr<sup>237</sup> to Ala (Fig. 3) mutant are consistent with this idea. However, replacement of Phe<sup>243</sup> with Val yielded an AKAP<sub>ce</sub> fusion protein that bound R<sub>ce</sub> with high (but not maximal) affinity (Fig. 2). Thus, a large hydrophobic side chain at the first position in the dipeptide is sufficient to ensure R<sub>ce</sub> tethering; binding affinity may be further optimized by aromatic character in the side chain. Ser<sup>244</sup> introduces several properties that cannot be supplied by Leu, Ile, or Val residues incorporated into the core of the AKAP75/S-AKAP84 RI<sub>ii</sub>-binding regions (Fig. 1A). The Ser hydroxyl group is hydrophilic and polar, readily participates in hydrogen bonding, and can potentially undergo regulatory phosphorylation. Mutation of Ser<sup>244</sup> to a neutral hydrophilic residue (Asn) or Asp (to mimic phosphorylation) completely extinguished R<sub>ce</sub> binding activity (Fig. 2). Thus, Ser<sup>244</sup> is a unique and critical component of the tethering domain of AKAP<sub>ce</sub>.

**Characterization of Amino Acids in RCE That Govern Homodimerization and Affinity for AKAP<sub>ce</sub>—** Structural features of the AKAP<sub>ce</sub> regulatory subunit that mediate R<sub>ce</sub>-R<sub>ce</sub> dimerization and high affinity binding with AKAP<sub>ce</sub> were elucidated by further application of mutagenesis, protein expression, and binding assays. Wild type and mutant RCE polypeptides were expressed as His-tagged fusion proteins in E. coli and were purified to near homogeneity by affinity chromatography on nickel-chelate Sepharose 4B. An approximate location for the AKAP<sub>ce</sub>-binding domain in R<sub>ce</sub> was established by truncation
**Selective Tethering of RCE and RIα by AKAP<sub>CE</sub>**

**Analysis.** Neither deletion of 15 amino acids at the N terminus nor elimination of 277 residues (i.e. amino acids 100–376) that constitute the entire central and C-terminal portions of the cAMP-binding protein altered the ability of partial R<sub>CE</sub> proteins to dock with AKAP<sub>CE</sub> (Fig. 4A; R<sub>CE</sub> Δ15 and R<sub>CE</sub> 1–99). Further C-terminal deletion mutagenesis revealed that smaller fragments of R<sub>CE</sub>, which include amino acids 1–80 or 1–65, bind AKAP<sub>CE</sub> with approximately the same affinity (see competition binding assays in Fig. 5) as full-length R<sub>CE</sub> (376 amino acids, R<sub>CE</sub> wild type; Fig. 4A). Thus, the site in R<sub>CE</sub> that docks with the tethering domain of AKAP<sub>CE</sub> is generated from amino acids that lie between residues 16 and 64 at the N terminus.

A distinct domain, which promotes R-R homodimerization, is included in the corresponding N-terminal segments (amino acids 1–65) of mammalian RI and RI isoforms (26–29, 53). Moreover, mutations that prevent RIα or RIβ dimerization have a crucial secondary consequence: binding of RI with classical AKAPs is abolished (26–28). This is because two copies of a short, N-terminal segment of either RIα or RIβ must align (in an anti-parallel fashion) to create a single site that binds with AKAP tethering domains (29). By analogy, these observations suggested that R<sub>CE</sub> mutants, which are incapable of binding AKAP<sub>CE</sub>, may yield two levels of structural information. One subset of mutants may implicate certain amino acids in R<sub>CE</sub> oligomerization and the generation of the heterotetrameric holo-PKA<sub>CE</sub>; a second group of mutants could reveal individual amino acids that control the folding and/or affinity of the docking site for AKAP<sub>CE</sub> in the context of an R<sub>CE</sub> dimer. To correctly analyze and interpret experimental results, it was essential to determine whether mutated R<sub>CE</sub> proteins were dimers or monomers. This was accomplished by employing methodology illustrated in Fig. 5. Like RIα (54), R<sub>CE</sub> contains two N-terminal Cys residues (Cys<sup>23</sup> and Cys<sup>44</sup>) in R<sub>CE</sub> that engage in covalent, interchain cross-linking in the absence of reducing agents. Determination of the M<sub>r</sub> of dimeric regulatory subunits by denaturing electrophoresis will yield a value of 100,000 in the absence of reducing agent and 50,000 in the presence of 1 mM β-mercaptoethanol (Fig. 4, A and B). Monomeric R<sub>CE</sub> mutants will have a M<sub>r</sub> of 50,000 in both instances (Fig. 5B). In addition, analytical FPLC gel filtration on a column of Superose 12 readily resolved the peaks of R<sub>CE</sub> dimers and monomers (Fig. 5C). Using these approaches, all R<sub>CE</sub> mutants were characterized as dimers or monomers. Results are included in Fig. 4.

An extensive series of R<sub>CE</sub> variants was prepared by site-directed, scanning mutagenesis. Representative results that provide insights into the molecular basis for the docking of dimeric R<sub>CE</sub> with the tethering domain of AKAP<sub>CE</sub> are presented in Fig. 4 (B and C). (Numbering, alignments, and predicted secondary structure of the N-terminal regions of R<sub>CE</sub> and mammalian RIα and RIβ are given in Fig. 6). Replacement of the large, branched aliphatic side chain contributed by Ile<sup>27</sup> with the methyl group of Ala abrogates binding of R<sub>CE</sub> with AKAP<sub>CE</sub> (Fig. 4B). Likewise, substitution of Val<sup>27</sup> with Ala also severely compromises (but does not completely eliminate) the tethering of R<sub>CE</sub>. In contrast, mutation of Ile, Leu, or Val to Ala at positions 20, 35, 36, 40, and 41 has no impact on the formation of AKAP<sub>CE</sub>-R<sub>CE</sub> complexes (Fig. 4B). Thus, folding of the R<sub>CE</sub>-docking domain into a (predicted) α-helix (Fig. 6 and text below) differentially orients side chains of Val<sup>27</sup> and Ile<sup>27</sup> for interaction with a hydrophobic binding pocket in AKAP<sub>CE</sub>. Neither introduction nor removal of charged amino acids or exchange of a basic for acidic side chain in the region bounded by Glu<sup>105</sup> and Gln<sup>26</sup> (Fig. 6) affected docking of R<sub>CE</sub> with AKAP<sub>CE</sub> (Fig. 4, B and C). However, mutation of Cys<sup>23</sup> to Ala elicited a significant decline (~70%) in binding of R<sub>CE</sub> with the anchor protein (Fig. 4B), thereby demarcating the N-terminal end of the AKAP-binding region. Replacement of Cys<sup>44</sup> with Ala profoundly diminished coupling of R<sub>CE</sub> with the tethering region of AKAP<sub>CE</sub> and indicated that the R<sub>CE</sub>-docking domain spans at least 22 residues. R<sub>CE</sub> polypeptides that include single or double Cys to Ala mutations (Ala<sup>23</sup> and Ala<sup>44</sup>) dimerize normally (Fig. 4). Furthermore, the amounts of wild type and mutant R<sub>CE</sub> proteins complexed by the AKAP<sub>CE</sub> tethering domain were not altered when 0.1 mM dithiothreitol was added to the binding buffer. The indicated concentration of dithiothreitol reduces all Cys residues in native R subunit dimers (55). Thus, interchain disulfide bonds are not involved in shaping the configuration of the R<sub>CE</sub>-docking surface or stabilizing R<sub>CE</sub>-AKAP<sub>CE</sub> complexes. Cys sulfhydryl groups are also expected to be reduced in the intracellular environment. Under these conditions, Cys side chains could co-operatively promote (Cys<sup>23</sup>) or optimize (Cys<sup>44</sup>) tethering of PKAI<sub>CE</sub>
via hydrophobic interactions, involvement in hydrogen bonds, or (upon ionization) neutralization of charge.

Several mutations provided insights regarding amino acids that are crucial for the fundamental property of R CE homodimerization. Substitution of Phe 59 with Ala produced R CE monomers that are unable to bind AKAPCE (Fig. 4C). In mammalian RIIb, the corresponding mutation (Phe 36 to Ala; Fig. 6 and Ref. 26) has identical consequences. Moreover, alignment of residues 52–67 of RCE with homologous segments of R Iα and RIIα (Fig. 6) strongly indicates that this portion of the C. elegans regulatory subunit mediates dimerization. However, not all amino acids governing oligomerization are segregated in the predicted α-helix bounded by residues 52 and 67 (Fig. 6). N-terminal truncations that delete residues 1–40 or 1–25 from RCE preclude dimerization (Fig. 4A). Furthermore, scanning mutagenesis revealed that replacement of Tyr 26 with a small (Ala) or large (Val) aliphatic hydrophobic amino acid ablates RCE-RCE association (Fig. 4C). In contrast, a mutation that preserved aromatic character in the side chain (Tyr 26 to Phe) enabled dimerization. Thus, an aromatic amino acid (Tyr 26) embedded within the AKAP CE-docking region of R CE plays a critical role in stabilizing overall dimeric structure, which in

![Fig. 5. RCE dimers are resolved from monomers by denaturing electrophoresis and FPLC gel filtration.](https://example.com/fig5)

**FIG. 5.** RCE dimers are resolved from monomers by denaturing electrophoresis and FPLC gel filtration. A, samples (1 µg protein) of purified mouse RIα were denatured in gel loading buffer and subjected to electrophoresis in a 0.1% SDS-10% polyacrylamide gel. Positions of polypeptides within the gel were visualized by staining with Coomassie Blue. Samples were processed in the presence or absence of 1 mM β-mercaptoethanol (BME) as indicated. B, samples (1 µg) of purified RCE (lanes 1 and 2) and an RCE mutant (Phe59 to Ala) (lanes 3 and 4) were analyzed as described for A above. C, samples (0.9 mg in 0.2 ml of buffer A) of purified RCE and RCE Phe59 to Ala were applied to a calibrated column of Superose 12 (see “Experimental Procedures”). The column was eluted with buffer A at a rate of 0.2 ml/min. Protein concentration in the eluate was monitored by absorbance at 280 nm. Peaks of RCE (dimer) and the Phe59 to Ala RCE mutant (monomer) emerged at 43 and 47 min, respectively. A mixture of the two proteins was resolved into two peaks with the expected elution times (data not shown). All RCE mutants were characterized as dimers or monomers by both their electrophoretic mobility in nonreducing SDS-polyacrylamide gels and elution times during gel filtration, as illustrated by the examples shown.

![Fig. 6. Sequence alignment and structural properties of the AKAP binding (docking) and dimerization domains of RCE, RIα, and RIIα.](https://example.com/fig6)

**FIG. 6.** Sequence alignment and structural properties of the AKAP binding (docking) and dimerization domains of RCE, RIα, and RIIα. Structural and functional properties of amino acids and domains at the N terminus of murine RIIα were assigned on the basis of systematic mutagenesis/expression experiments (26–28) and determination of the solution structure of a fragment of RIIα (amino acids 1–44) (29). Correlations between domains in RIIα and murine RIα were established by Banky et al. (53). Amino acids shown in **bold** constitute a proximal α-helix that mediates docking with AKAPs; amino acids shown in **bold italics** comprise a distal α-helix that governs homodimerization. Ile5 (italics) in RIIα modulate the binding affinity for AKAP75/79 (27). RIIα lacks an N-terminal extension of the AKAP-binding helix (docking region) that is present in RCE and RIα (dotted underline). Residues in RCE that are essential for optimal binding with AKAP CE are marked with asterisks; amino acids essential for RCE dimerization are identified with pound signs.
selective tethering of RCE and RII by AKAP CE

![Diagram](https://via.placeholder.com/150)

**FIG. 7.** Differential binding of RCE with AKAP CE and classical mammalian anchor proteins. A Western blot was probed with [32P]-labeled RCE in an overlay binding assay ("Experimental Procedures"). Lane 1 received 0.1 μg of GST-AKAP CE 176/409; lane 2 contained 0.1 μg of purified His-tagged S-AKAP84 fusion protein (residues 205–451), which includes the RII tethering domain (9); and lane 3 was loaded with 0.1 μg of full-length AKAP75 (26). An autoradiogram is shown. Locations of proteins on the blot were determined by staining replicate lanes with Coomassie Blue.

The large aromatic side chain of Phe 243 makes a substantial contribution to binding site affinity and consequently promotes formation of stable AKAP CE-PKA complexes. Mutation of Phe 243 to Ala diminishes RCE binding activity by >90%, but isoform specificity is unchanged. However, replacement of Phe 243 with Val produces a mutant tethering site that selectively complexes RCE and exhibits only a modest decline in affinity. Thus, a bulky aliphatic amino acid at residue 243 will support high affinity RCE and RII (see below) binding in the context of Ser 244 or avid RIIa/RIIβ binding in the context of Ile 244, Leu 244, or Val 244 (e.g. Leu 244-Val 244 in the AKAP CE double mutant). In contrast, substitution of Ser 244 with alternative amino acids either disrupts binding of RCE or elicits inversion in specificity that produces an RII-binding protein. Therefore, Ser 244 governs the highly selective association of RI-like RCE isoforms with the AKAP CE tethering site. The Ser 244 side chain may also subserve exclusion of RII isoforms from the binding site, thereby further enhancing the degree of isoform selectivity. It is possible that the aromatic side chain of Phe 243 (which is absent in all RII binding, classical AKAPs) can potentiate RII exclusion by Ser 244 and thus contributes to RII isomeric specificity in an indirect manner. Conversely, substitution of Ser 244 in AKAP CE with Val introduces a bulky aliphatic side chain that promotes coupling with RII isoforms and sharply diminishes RCE ligation.

Hydrophobic residues in the wild type AKAP CE tethering site (Leu 236, Ile 248, and Leu 252) and their counterparts in classical RII-binding AKAPs apparently subserve similar functions. Their side chains collectively provide a nonpolar surface or scaffold that complements hydrophobic regions in RCE (R1α; see below) or RII dimers. The same array of conserved hydrophobic amino acids will promote either RCE (R1α) or RIIa/RIIβ ligation by AKAP CE or doubly mutated AKAP CE (Phe 243-Ser 244 → Leu-Val), respectively. Thus, it is probable that similarly configured hydrophobic subdomains of RCE, RIIα, and RIIβ are the binding targets. Multiple hydrophobic amino acids in the docking region of RII subunits have been rigorously implicated in binding with classical AKAPs (26–29). However, the identity of subsets of individual side chains that contribute (a) isoform-specific interactions and (b) binding surfaces shared by RCE, RIIa, and RII isoforms remains unknown. Our results and studies by Banky et al. (53) indicate that Cys 243, Ile 25, Val 32, and Cys 44 (in RCE) are candidate residues for coupling with the aliphatic hydrophobic binding pocket in the tethering region of AKAP CE. At present, the mechanism by which Ser 244 confers CYF/R1a selectivity is unknown. Future determination of identities of amino acids in RCE or R1α that interact with Ser 244 should illuminate this issue.

The observations on the roles of conserved hydrophobic residues and gatekeeper amino acids yield insights into the basic architecture and design of AKAP tethering domains. Folding of 15–20 residues into an α-helix and the disposition of three aliphatic hydrophobic side chains along one helical face generates a basic scaffold suitable for accommodating RI, RCE, or RII dimers. A reasonable speculation is that these properties typified a common ancestor of modern AKAPs. Isoform-specific binding selectivity could then be superimposed on the basic design by minimal changes. Subsequent mutation and selection pressure on one or two core (gatekeeper) amino acids would enable evolution of RI-RCE or RII-selective AKAPs. Additional mutual selection pressure may have been exerted by the parallel evolution of R subunits. For example, the high affinity R1α-binding AKAP CE (see below) co-evolved with RI-like RCE. In mammals, the emergence of RII subunits appears
to have played a dominant role in the selection of AKAP binding specificities. Most of the thoroughly characterized mammalian AKAPs exhibit highly preferential binding of RII (PKAII). However, RIα-selective and neutral (RIα ~ RII) binding sites were recently described in a mammalian germ cell anchor protein (56). This discovery and the characterization of RCE indicate that high affinity RIα-binding proteins are encoded in eukaryotic genomes. Further searches and analyses may yield more examples of such anchor proteins and further insights into function of anchored PKAI.

**AKAPCE Is a High Affinity, RIα-binding Protein**—Alignment of the sequences of the docking region of RCE (residues 23–47; Fig. 6) and the corresponding segment of mouse RIA (residues 18–42; Fig. 6) revealed 15 identities and 5 conservative substitutions (interchange of Arg for Lys, Asp for Glu, Thr for Ile, and Val for Leu) over a span of 25 contiguous amino acids. The high level of sequence similarity indicated that the AKAPCE tethering module might accommodate mammalian RIα dimers. Competitive solution binding assays revealed that RIα inhibited binding of 32p-RCE with the same concentration dependence as nonradioactive RCE (Fig. 8). Thus, the KD for an AKAPCE·RIα complex is approximately 10 nM. To directly characterize RIα-AKAP CE interactions, an Ala residue in the pseudosubstrate site of RIα was mutated to Ser, thus generating a PKA phosphorylation site. This modification does not alter the ability of RIα to bind cAMP, catalytic subunits, RIα (dimerization), or AKAPCE (43. 32p-RIα is avidly sequestered by AKAPCE in an overlay binding assay (Fig. 8A). Moreover, both RCE and nonradioactive RIα potently inhibit binding of the radiolabeled ligand (Fig. 8, A and B). Thus, the nematode (RCE) and mammalian (RIα) regulatory subunits seem to be tethered at the same site. To confirm these observations, solution binding assays were also performed with wild type or phosphorylated RIα, and AKAPCE·RIα complexes were detected by protein staining or immunochemistry. Direct, stable binding of RIα by the AKAPCE tethering module was evident (a representative subset of results is shown in Fig. 8, C and D).

**AKAPCE Binds RIα in Intact Cells**—The *in vitro* binding data suggest that the AKAPCE tethering module might ultimately provide a novel molecular tool for manipulating the intracellular location and/or function of mammalian PKAIα. The feasibility of such experimentation depends upon the demonstration that AKAPCE·RIα complexes are generated in intact cells. Thus, hamster AV12 cells were co-transfected with a full-length AKAPCE transgene and expression plasmids encoding either RIα or GST-RIα. Both precipitation with anti-RIα IgGs and binding of GST-RIα to GSH-Sepharose 4B beads yielded complexes that contain substantial amounts of co-purifying AKAPCE (Fig. 9). Thus, the anchor protein stably sequesters RIα (PKAIα) in the context of the environment of intact cells.

**Conclusions, Insights, and Implications for Current Models**—We have identified and characterized crucial structural features that ensure stable association of RCE dimers (PKAIC1α) with a *C. elegans* anchor protein (AKAPCE). Elucidation of these structural properties suggested further experimentation, which (a) revealed that the tethering module of AKAPCE binds mouse RIα (PKAIα) with high affinity in vitro and in intact cells and (b) demonstrated that one or two core amino acids govern the differential binding of either RI-like RCE or RII isoforms by AKAPs. Concepts underlying a current structural model for the tethering of PKAα are derived from detailed studies on properties associated with the N termini of RIα and RIβ subunits (26–29). Specific functions subserved by discrete N-terminal regions of mammalian RI isoforms are indicated in Fig. 6. Short segments of polypeptide that govern subunit dimerization and create a docking surface for AKAPs are located within residues 1–50 of RII isoforms (26–28). A turn–helix–turn–helix motif divides the N-terminal portion of RI subunits into three functional regions (29). Two sets of tandem Pro residues precede distinct α-helices that mediate RII–RII homodimerization (Fig. 6, helix II residues 29–45 in RIα) or
generate a core hydrophobic binding site (helix I, residues 10–25 in RIα) for AKAPs. Groups of aromatic residues and amino acids with large aliphatic side chains contribute essential functional properties in helices I and II of RIα andRIβ (26–28). Ile residues upstream from the first Pro-Pro turn modulate the affinity ofRII dimers for AKAPs (27). A structural model derived from solution NMR analysis (29) indicates that coalescence of the hydrophobic surfaces of the RII-docking site and the AKAP tethering region results from the formation of many specific, intermolecular hydrophobic contacts.

The N-terminal segment ofRCE includes two predicted α-helices and shares several critical properties with corresponding regions in RIIα andRIβ. The structure and function of the dimerization helix (residues 52–67 in RCE, Fig. 6) are highly conserved between RCE andRIα/RIβ. Substitution of Ala for Phe (29) in otherwise full-length, wild type RCE abolishes dimerization and concomitantly ablates binding with AKAPCE. These results illustrate basic principles of R subunit architecture that are conserved from nematodes to mammals: in the distal helix, individual amino acids with aromatic side chains (Phe) are indispensable for maintaining overall dimeric structure of R subunits, and monomeric R subunits cannot generate a functional AKAP-docking surface from a single wild type copy of the AKAP-binding region (Fig. 6). Like RIα/RIβ, RCE has a proximal helix that mediates high affinity binding with the tethering domain of an isoform-selective anchor protein. Robust hydrophobic interactions promote the stable tethering of both RII- and RI-like RCE dimers. Side chains from 6 of the 9 large hydrophobic amino acids in the docking (AKAP-binding) region of RIα engage in hydrophobic contacts with core apolar residues in the tethering domains of classical mammalian AKAPs (29). For RCE, side chains derived from Cys23, Val27, Ile32, and Cys44 apparently assemble a hydrophobic surface that couples with a complementary, nonpolar binding pocket derived from Leu236, Ile248, and Leu252 in the AKAPCE tethering site.

Striking differences between the docking regions of RCE andRIα are also evident. The segment of RCE that engages the anchor protein is 50% longer than the corresponding domain in RIα. In contrast to the conserved dimerization regions, amino acid sequences of the RCE- andRIII-docking regions are not homologous (only ~20% identical residues). A high proportion (66%) of large hydrophobic amino acids in the proximal helix of RIα directly participate in tethering, whereas side chains from Leu20, Leu21, Val25, Ile40, and Val44 in RCE are not essential for high affinity ligation with AKAPCE. Optimal alignment of the docking regions (Fig. 6) places only two critical residues from RCE (Ile32 and Cys44) in register with hydrophobic amino acids that mediate binding of RIα with classical AKAPs. Three other amino acids that evidently play central roles in tethering PKA-Iα dimers are not conserved between RCE and mammalian RI isoforms make substantial contributions toward the stabilization of RCE-AKAPCE complexes. Docking sites in RI isoforms are devoid of Cys. Reduced Cys sulphydryls can participate in hydrogen bonds or undergo ionization, but these are atypical and infrequently observed properties. It is more likely that Cys23 and Cys44 side chains promote tethering by their incorporation into a hydrophobic binding surface. Thus, RCE and RII-docking domains differ substantially in overall size, configuration, amino acid sequence, and the positions and identities of critical residues along the helix. This sharp divergence in N-terminal structural features accounts (in part) for RCE-selective tethering and RI exclusion by AKAPCE.

Recent investigations disclosed that several classical mammalian AKAPs bind both RII isoforms and RIα at the same tethering site (34, 35). However, RI isoforms are bound 25–500-fold more avidly than RIα. Banky et al. (53) thoroughly characterized properties in RIα that mediate the lower affinity binding with DAKAP-1 (also known as S-AKAP84/AKAP121 (9, 46)), Val, Ile, and Cys residues in RIα, which align with Val27, Ile32, and Cys44 in RCE (Fig. 6), control coupling with DAKAP-1. Thus, several structural properties that drive high affinity binding of RCE with AKAPCE are highly conserved from C. elegans to mammals and are sufficient to enable lower affinity binding with an AKAP optimized for tethering RII isoforms. The conservation of the RCE/RII-docking region in the distant nematode and mammalian branches of evolution supports the idea that PKAα may be adapted and targeted for specific functions by anchor proteins in a broad spectrum of eukaryotes.

Binding of RCE and RIα with their cognate anchor proteins declines precipitously upon substitution of critical Val or Ile...
residues (Val$^{27}$ and Ile$^{32}$ in R$_{CE}$) (Fig. 6) with Ala. Thus, the extended hydrophobic side chains of these amino acids are essential for diversifying functions of both PKA1 isoforms. In contrast, tethering of R$_{CE}$ is more stringently dependent on Cys residues than immobilization of R$_{Io}$. Mutation of Cys$^{44}$ to Ala in R$_{CE}$ severely impairs binding with AKAP$_{CE}$ (Fig. 4), whereas the corresponding mutation in R$_{Io}$ has a minimal effect on the formation of complexes with DAKAP-1 (53). Binding of R$_{Io}$ with DAKAP-1 declined substantially only when Cys$^{37}$ was replaced with His, which contains a markedly different side chain. Moreover, replacement of Cys$^{23}$ with Ala reduces docking of R$_{CE}$ with AKAP$_{CR}$ by $>$50%; the analogous mutation in R$_{Io}$ has no impact on coupling with DAKAP-1 (53). Thus, Cys residues in the proximal N-terminal helix of R$_{CE}$ may be involved in cooperatively maximizing the stability of AKAP$_{CR}$R$_{CE}$ complexes in concert with interactions mediated by Val$^{27}$ and Ile$^{32}$. The roles of Cys$^{23}$ and Cys$^{44}$ are evidently magnified in the context of the high affinity R$_{CE}$/R$_{Io}$-binding site of AKAP$_{CR}$.

As this manuscript reached completion, Miki and Eddy (57) reported an analysis of the binding of R$_{Io}$ and R$_{IIo}$ subunits with two sites on a germ cell anchor protein known as FSC1 (56) or AKAP82 (58). Domain B of FSC1 selectively complexes R$_{Io}$; domain A binds both R$_{II}$ and R$_{Io}$ with similar affinities. Binding activities and specificities were assessed after Ala or Val scanning mutagenesis. In addition, mutations were introduced into a classical AKAP, Ht31 (57), to probe an R$_{II}$-selective tethering site. The investigation focused on 10 central amino acids in the cited binding regions: domain B (Y$^{DN}$QV$^{ASDM}$), domain A (Y$^{VN}$RL$^{LSL}$VI), and H31 (AG$^{SA}$RI$^{VD}$I). These residues align with amino acids $239-248$ of AKAP$_{CR}$ (Fig. 1). Three important determinants were discerned in these regions and rules for RI-selective, R$_{II}$-selective, and dual binding specificities were suggested. Our current results and previous studies (9, 19, 23, 37, 52, 59) are in partial accord with these suggestions. However, other aspects of our observations suggest distinctly different interpretations. Miki and Eddy (57) propose that a large hydrophobic side chain at position 10 (Met, Ile, and Ile for FSC1-A, FSC1-B, and Ht31, respectively) is essential (but nonselective) for R$_{Io}$ dimer binding. This suggestion is consistent with the previously published demonstration that conversion of the corresponding (Ile) residues in AKAP75, AKAP-KL, DAKAP200, and AKAP$_{CR}$ to Ala eliminates binding activity (19, 37, 52, 59). A second, critical insight is that residue 6 (Ala, Ser, and Val in A, B, and H31) determines isofrom specificity of the tethering site. It is proposed that Ala is required for selective tethering of R$_{Io}$; Ser enables dual binding of R$_{Io}$ and R$_{IIo}$ with similar affinities, and Ile, Val, or Leu promotes R$_{II}$ binding. The last suggestion is consistent with previously published alignments and conclusions indicating that Leu, Ile, and Val invariably occupy these positions in RI-selective binding proteins (9, 23, 24, 37, 52), and mutation of these residues to Ala compromises RI$_{II}$ binding activity in all cases (19, 37, 52, 59). Mutagenesis/expression studies on AKAP$_{CR}$ document Ser$^{244}$ or Val$^{244}$ (corresponding to position 6) are crucial determinants for R$_{Io}$ isofrom selectivity. The suggested rules predict that Ser at this position is characteristic of a dual specific AKAP (57). In contrast, AKAP$_{CR}$ is an exceptionally selective R$_{CE}$ (and R$_{Io}$) anchor protein. The ratio of R$_{CE}$/RI$_{II}$ binding is 50; a large excess of RI$_{Io}$ or RI$_{II}$ does not efficiently inhibit R$_{CE}$ binding with AKAP$_{CR}$ in competition assays; R$_{Io}$ and R$_{II}$ compete for the AKAP$_{g}$ tethering site with high affinity ($K_{D} = 10$ nM); and AKAP$_{CR}$ binds R$_{Io}$ but not RI$_{II}$ in the context of intact cells. The binding properties of the wild type tethering domain of AKAP$_{CR}$ contravene another proposed rule (57). Occupancy of position 6 by Ala is not required for establishing R$_{Io}$-selective binding activity. Finally, it is proposed that Ala at position 2 is critical for optimal binding of either RI or RI$_{II}$ isoforms (57). However, replacement of the corresponding Ala in AKAP75, AKAP-KL, and AKAP$_{CR}$ with Ser has no effect on tethering (19, 37, 52).

Several factors may have contributed to the indicated differences in results and interpretations. We used specified concentrations of purified $^{32}$P-R$_{CE}$, $^{32}$P-R$_{Io}$, or $^{32}$P-RI$_{II}$ and equal amounts of nearly homogeneous wild type and mutant AKAPs in all in vitro binding studies. This enabled direct assessment of AKAP-R complex formation and facile comparison of binding of R$_{CE}$, R$_{Io}$, and RI$_{II}$ with the tethering domain. High affinity binding of R$_{CE}$ and RI$_{II}$ was established by direct equilibrium binding and competition assays in solution. In studies on FSC1 A and B domains, crude testis extracts were employed as the source of RI$_{Io}$ and RI$_{II}$ subunits (57). Thus, the concentrations of the two ligands were not precisely known or normalized. The possibility that substantial amounts of RI$_{Io}$ and R$_{Io}$ were complexed with endogenous AKAPs in the extracts was not excluded. In addition, RI$_{Io}$ and RI$_{II}$ binding activities of AKAPs were monitored in a highly indirect manner: via Western immunoblot analysis. Marked discrepancies between actual amounts of RI$_{Io}$ and RI$_{II}$ bound and chemiluminescence signals can be introduced by differences in affinities of the primary antibodies. Additional limitations in quantitative analysis may have arisen from the use of different secondary antibodies and the narrowly limited linear range of chemiluminescence signals. No information was provided regarding either quantitative calibration of the indirect, antibody-based methodology used for assessment of binding activities or $K_{D}$ values of the various tethering domain complexes with RI$_{Io}$ and RI$_{II}$. Finally, an essential criterion for establishing the significance of the various tethering domain variants involves the demonstration that stable, isofrom-selective tethering occurs (as predicted) in the internal environment of intact cells. The model proposed by Miki and Eddy (57) was not evaluated by data obtained from intact cells.

Despite the caveats and substantial differences in results and interpretation regarding requirements for individual amino acid residues, our current and previous (19, 37, 52, 59) results and the study of Miki and Eddy (57) underscore the importance of position 10 in controlling binding affinity. Immunochemistry studies on RI$_{Io}$ and RI$_{II}$ (as predicted) in the internal environment of intact cells. The significance of the various tethering domain variants involves the demonstration that stable, isofrom-selective tethering occurs (as predicted) in the internal environment of intact cells. The model proposed by Miki and Eddy (57) was not evaluated by data obtained from intact cells.

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