

## D-AKAP2, a novel protein kinase A anchoring protein with a putative RGS domain

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**ABSTRACT** Subcellular localization directed by specific A kinase anchoring proteins (AKAPs) is a mechanism for compartmentalization of cAMP-dependent protein kinase (PKA). Using a two-hybrid screen, a novel AKAP was isolated. Because it interacts with both the type I and type II regulatory subunits, it was defined as a dual specific AKAP or D-AKAP1. Here we report the cloning and characterization of another novel cDNA isolated from that screen. This new member of the D-AKAP family, D-AKAP2, also binds both types of regulatory subunits. A message of 5 kb pairs was detected for D-AKAP2 in all embryonic stages and in all adult tissues tested. In brain, skeletal muscle, kidney, and testis, a 10-kb mRNA was identified. In testis, several small mRNAs were observed. Therefore, D-AKAP2 represents a novel family of proteins. cDNA cloning from a mouse testis library identified the full length D-AKAP2. It is composed of 372 amino acids which includes the R binding fragment, residues 333–372, at its C-terminus. Based on coprecipitation assays, the R binding domain interacts with the N-terminal dimerization domain of R<sup>I</sup>α and R<sup>II</sup>α. A putative RGS domain was identified near the N-terminal region of D-AKAP2. The presence of this domain raises the intriguing possibility that D-AKAP2 may interact with a Gα protein thus providing a link between the signaling machinery at the plasma membrane and the downstream kinase.

Protein phosphorylation is one of the most important mechanisms for enzyme regulation and signal transduction in eukaryotic cells. cAMP-dependent protein kinase (PKA), one of the first protein kinases discovered, mediates a variety of hormonal and neurotransmitter responses by phosphorylating serine and threonine residues on various substrate proteins. The observation of preferential phosphorylation of specific target substrates by PKA, despite its broad specificity, may be explained by the discovery of the anchoring proteins of PKA. These A kinase anchoring proteins (AKAPs) function as targeting units and tether the kinase to specific subcellular localizations. Compartmentalization of PKA therefore serves as a potential regulatory mechanism that increase the selectivity and intensity of a cAMP-mediated hormonal response.

Recently, it was shown that anchoring proteins may also act as adapters for assembling multiprotein complexes. For example, Scott and coworkers (1, 2) showed that AKAP79, in addition to binding tightly to PKA, also interacts with calcineurin and protein kinase C. Therefore, the targeting of AKAP79 to neuronal postsynaptic densities brings enzymes with opposite catalytic activities together in a single transduction complex. This adds another level of intracellular organization for PKA and also facilitates the diversity of the cAMP-mediated signal transduction pathways (3).

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In the absence of its activating ligand, cAMP, PKA exists as an inactive holoenzyme containing two regulatory (R) and two catalytic subunits. The two classes of R subunits, R<sup>I</sup> and R<sup>II</sup>, based on their elution from the DEAE cellulose, define the two types of holoenzymes. Because a significant proportion of the type II holoenzyme associates with the particulate fraction of cell homogenates, most previous studies focused on identification of AKAPs that anchor the kinase through R<sup>II</sup> (4, 5). Recently, using a yeast two-hybrid screen, a new member of the anchoring protein family, D-AKAP1, was identified that binds both R<sup>I</sup> and R<sup>II</sup>. This raises the possibility that like R<sup>II</sup>, R<sup>I</sup> may also serve to compartmentalize PKA. There are, in fact, indications that R<sup>I</sup> is also compartmentalized in some case in a dynamic way. For example, R<sup>I</sup> subunits in human erythrocytes are tightly bound to the plasma membrane (6). R<sup>I</sup>α is also enriched in the neuromuscular junctions (7). Type I holoenzyme is also depleted from the cytoplasm and accumulates at the "cap" site when lymphocytes are stimulated with anti-CD3 antibodies (8). R<sup>I</sup> was also found to associate with activated B cell receptors (9).

Here we report the identification and characterization of another potential A kinase anchoring protein, D-AKAP2. In addition to an R binding domain, D-AKAP2 contains a putative RGS domain characteristic of proteins that interact with the Gα subunits and possess GTPase activating protein-like activity (10–12). The presence of these two motifs in a single molecule provides a previously unrecognized direct link between two major components of the cAMP signaling pathway.

### EXPERIMENTAL PROCEDURES

**Materials.** All vectors for the yeast two-hybrid system were from S. Hollenberg (Vollum Institute, Oregon). The following reagents were purchased as indicated: Mouse testis cDNA library (CLONTECH), Mouse Multiple Tissue Northern Blot and Mouse Embryonic Northern Blot (CLONTECH), Ready-to-Go DNA labeling kit (Pharmacia), Genius labeling system (Boehringer Mannheim), ATP, phenylmethylsulfonyl fluoride, benzamidine, Triton X-100 and glutathione S-transferase (GST) agarose resin (Sigma), standard saline citrate (SSC) buffer (5 Prime–3 Prime Inc.), nickel-nitrilotriacetic acid resin (Qiagen, Chatsworth, CA), 5-bromo-4-chloro-3-indoyl β-D-galactoside and enzymes used for DNA manipulations (Life Technologies, Grand Island, NY), DNA sequencing kit (United States Biochemical). All oligonucleotides were synthesized

Abbreviations: PKA, cAMP-dependent protein kinase; AKAP, A kinase anchoring protein; R, PKA regulatory subunit; R<sup>I</sup> and R<sup>II</sup>, type I and II regulatory subunit; GST, glutathione S transferase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF021833).

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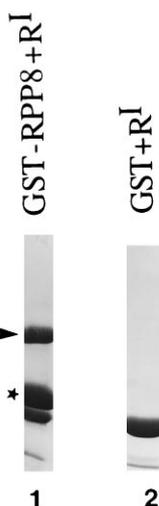


FIG. 1. GST-RPP8 binds to R<sup>I</sup>α. Lane 1 shows purified GST-RPP8. GST-RPP8 or GST were immobilized on glutathione resin as described in *Experimental Procedures*. After removal of nonspecific proteins, 200 μg of R<sup>I</sup>α-subunit were added to the resin and incubated for 2 hr at 4°C. After washing the resin extensively, proteins associated with either GST-RPP7 or GST were eluted from the glutathione resin by boiling in SDS gel-loading buffer and analyzed on SDS/PAGE. As shown in lanes 1 and 2 R<sup>I</sup>α only associates with GST-RPP8 but not GST. \*, GST-RPP8; >, R<sup>I</sup>α.

at the Peptide and Oligonucleotide Facility at the University of California, San Diego.

**Two-Hybrid Screen.** A yeast two-hybrid screen was used to isolate proteins that associate with the Ret/ptc2 oncoprotein, where the C-terminal domain of the Ret receptor tyrosine kinase, is fused to the N terminus of the R<sup>I</sup>α subunit of PKA (13). Using Ret/ptc2 to screen a mouse embryonic cDNA library, several interacting clones were isolated. Eight of these cDNA clones coded for three novel protein fragments, designated RPP7, RPP8, and RPP9, that associated specifically with the R<sup>I</sup> portion of Ret/ptc2. RPP7 was later identified to be part

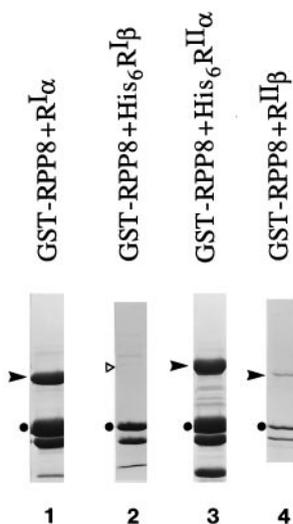


FIG. 2. Specificity of GST-RPP8. Bacterial cell lysates containing GST-RPP8 were incubated with glutathione agarose resin for 2 hr at 4°C in binding buffer as described. One to two hundred micrograms of different forms of R<sup>I</sup> or R<sup>II</sup> were then added to the resin and incubated for 2 hr at 4°C. After washing the resin extensively, proteins associated with the GST-RPP8 were eluted from the GST resin by boiling in SDS gel-loading buffer and analyzed on SDS/PAGE. >, The different R subunits in each sample. In lane 2, > indicates where His<sub>6</sub>R<sup>I</sup>β should run on this gel. Lanes: 1, R<sup>I</sup>α; 2, His<sub>6</sub>R<sup>I</sup>β; 3, His<sub>6</sub>R<sup>II</sup>α; 4, R<sup>II</sup>β.

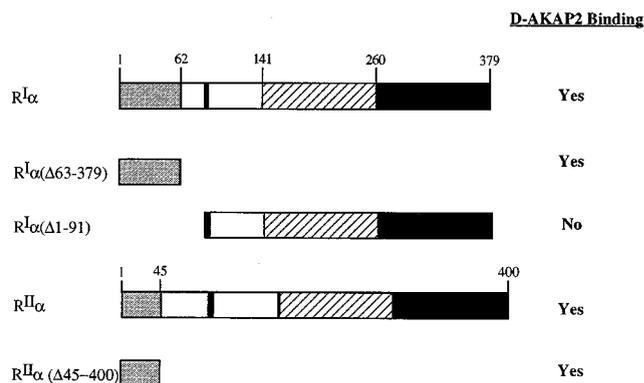


FIG. 3. RPP8 interacts with the N terminus of the R<sup>I</sup>-subunit. Schematic diagram indicating the binding capacity of various truncation mutants of R<sup>I</sup> to GST-RPP8. The N-terminal dimerization domain, inhibitory site, and two cAMP-binding domains are indicated on the wild-type R<sup>I</sup>α.

of a novel anchoring protein of PKA, D-AKAP1 (14). Here we report the cloning of full-length RPP8.

**Expression and Purification of Recombinant R Binding Proteins.** To determine whether the protein coded by RPP8 binds to the R<sup>I</sup>-subunit *in vitro*, the RPP8 cDNA was subcloned from the pVP16 plasmid into pGEX-KG to make a GST-fusion protein. This RPP8 fusion protein, designated GST-RPP8, was expressed in *Escherichia coli* BL21(DE3) at 37°C and purified to near homogeneity using glutathione resin as described earlier (15). The BL21(DE3) cell strain was a gift from Bill Studier (Brookhaven National Laboratories, Brookhaven, NY).

**Construction of Mutants of R Subunits.** Site-directed mutations of the R<sup>I</sup>α-subunit were generated according to Kunkel *et al.* (16). The R<sup>I</sup>α mutant with Cys-16 changed to His was designated R<sup>I</sup>(C16H), and mutant with Cys-37 changed to His or Ala were designated R<sup>I</sup>(C37H) and R<sup>I</sup>(C37A), respectively. A double mutant with both Cys-16 and Cys-37 changed to Ala was designated as R<sup>I</sup>(C16A-C37A). In addition, Val-20 and Ile-25 were mutated to Ala on R<sup>I</sup>α, respectively, to make R<sup>I</sup>(V20A) and R<sup>I</sup>(I25A).

**Expression and Purification of R Subunits.** R<sup>I</sup> and the various R<sup>I</sup> mutants were either expressed in BL21(DE3) cells and purified on a DE52 ion exchange column (17) or by cAMP

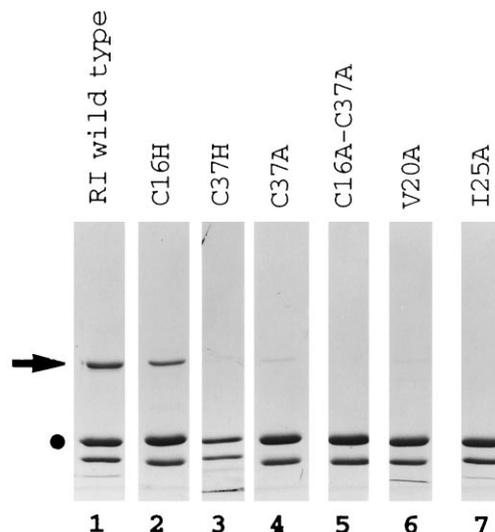


FIG. 4. Specific residues on R<sup>I</sup>α that are necessary to interact with D-AKAP2. Equal amounts of the various R<sup>I</sup>α-subunit mutants were tested in the binding assay. ●, GST-RPP8; >, Various R<sup>I</sup>α mutants.

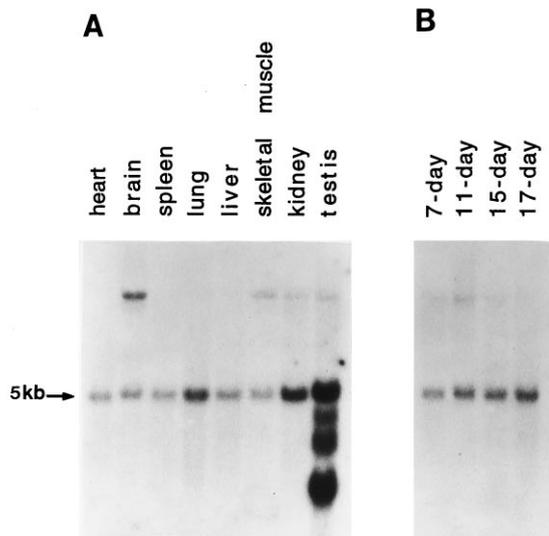


FIG. 5. Size and abundance of D-AKAP2 mRNA in mouse tissues and various embryonic stages. Blots containing 2  $\mu$ g of mRNAs from selected adult tissues (A) or total mRNA at different embryonic stages (B) were probed with  $^{32}$ P-radiolabeled RPP8 cDNA for 16 hr at 42°C in 5 $\times$  SSC, 5 $\times$  Denhardt's reagent, 0.5% SDS, and 50% formamide. After washing with 0.1 $\times$  SSC and 0.1% SDS at 68°C, hybridizing signals were detected by autoradiography.

resin (18). His<sub>6</sub>R<sup>II</sup>, His<sub>6</sub>R<sup>I</sup>( $\Delta$ 63–379), and R<sup>II</sup>( $\Delta$ 46–400) were purified as described (14).

**In Vitro Binding Assay.** Bacterial cell lysates containing GST-RPP8 were incubated with glutathione resin for 2 hr at 4°C in PBS (10 mM potassium phosphate, 150 mM NaCl, pH 7.4) with 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5 mM benzamide and 5 mM 2-mercaptoethanol, and then washed extensively with the same buffer. Wild type and mutants (100–200  $\mu$ g) of R<sup>I</sup> and/or R<sup>II</sup> were added to the resin and incubated for 2 hr at 4°C. After washing the resin extensively with PBS, proteins associated with the GST-RPP8 were eluted by boiling in SDS gel-loading buffer and analyzed by SDS/PAGE. All electrophoresis was performed using Mini-Protean II electrophoresis system (Bio-Rad). SDS/PAGE reagents were prepared according to Laemmli (19). Proteins were visualized by Coomassie blue staining.

**Northern Blot Analysis.** Blots containing 2  $\mu$ g immobilized samples of mRNAs from selected adult tissues or total mRNA at different embryonic stages were probed with  $^{32}$ P-radiolabeled RPP8 cDNA. Nitrocellulose filters were prehybridized in 5 $\times$  SSC (750 mM sodium chloride, 75 mM sodium citrate, pH 7.0), 5 $\times$  Denhardt's reagent (0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% acetylated bovine serum albumin), 0.5% SDS, and 50% formamide for 6 hr at 42°C, and then hybridized to 1.5  $\times$  10<sup>6</sup> cpm/ml of denatured radiolabeled cDNA probe in the same buffer. Hybridization was performed at 42°C for 16 hr and nonhybridized probe was removed with 0.1 $\times$  SSC, 0.1% SDS at 68°C. Hybridizing mRNA signals were detected by autoradiography.

**Screening of cDNA Libraries.** An adult mouse testis cDNA library in  $\lambda$ gt10 was screened with  $^{32}$ P-labeled RPP8 cDNA. The cDNA fragment RPP8 was excised from the two-hybrid library plasmid pVP16 using the *NotI* restriction endonuclease, and purified on agarose gel. This purified cDNA was then labeled with [ $^{32}$ P] $\alpha$ dCTP using random-prime labeling. Approximately 1.6 million plaques were screened, and positive clones were plaque-purified. Positive phage cDNAs were then isolated and digested with *EcoRI*, then the inserts were subcloned into the *EcoRI* site of pBluescriptII KS(+). All DNA sequences were determined by the dideoxynucleotide chain termination procedure (20). All sequences were ana-

lyzed using PCGENE software (IntelliGenetics) and the BLAST program provided by the National Center for Biotechnology Information server at the National Library of Medicine, National Institute of Health.

**In Situ Hybridization.** RNA probes of D-AKAP2 were derived from cDNA sequence 1–1469. R<sup>I</sup> $\alpha$  probes were derived from residues 18 to 169. These cDNA fragments were first subcloned into pBluescriptII KS(+), and digoxigenin-labeled riboprobes were transcribed in the sense and antisense orientations following template linearization. Probe synthesis employed the Genius labeling system, and hybridization was carried out on 20- $\mu$ m cryostat sections of the testis of adult BALB/c mice as previously described by Chun and coworkers (21, 22). Hybridized probe was visualized by alkaline phosphatase histochemistry.

## RESULTS

**Identification of a Novel R<sup>I</sup> Binding Protein.** Using Ret/*ptc2* to screen a mouse embryonic library, three novel cDNAs were isolated that coded for proteins designated RPP7, RPP8, and RPP9, which interacted specifically with the R<sup>I</sup> $\alpha$  portion of the bait. RPP7 was further characterized and identified as a fragment of a novel PKA anchoring protein, D-AKAP1 (14). Here we describe the characterization of RPP8 and the cloning of the full length cDNA from which it was derived. RPP8 coded for a 40-amino acid protein fragment.

To determine whether RPP8 binds R<sup>I</sup> $\alpha$  *in vitro*, the fragment corresponding to RPP8 was expressed as a fusion protein to GST in *E. coli* from a pGEX vector encoding GST fused to the RPP8 cDNA. GST-RPP8 was then expressed and tested for its ability to bind R<sup>I</sup> $\alpha$  in an affinity precipitation assay as described earlier (14). Cell lysates containing either GST or GST-RPP8 were incubated with glutathione resin. Bacterially expressed R<sup>I</sup> $\alpha$ -subunit was then added. Proteins associated with the resin after stringent washing were analyzed by SDS/PAGE. As seen in Fig. 1, this construct codes for a stable protein that can specifically pull down near stoichiometric amounts of the R<sup>I</sup> $\alpha$ -subunit. GST alone does not interact with R<sup>I</sup> $\alpha$ . These results confirmed the results of the two-hybrid screen, and established furthermore that no other factors are required for the interaction between R<sup>I</sup> $\alpha$  and RPP8 *in vitro* and *in vivo*.

**Specificity of RPP8.** All AKAPs previously identified bind only to R<sup>II</sup> with the exception of D-AKAP1 that was isolated from the same screen and was shown to be a D-AKAP based on its ability to bind both R<sup>I</sup> $\alpha$  and R<sup>II</sup> $\alpha$ . Therefore, the binding specificity of RPP8 toward the various isoforms of the R subunits was examined. As seen in Fig. 2, in addition to R<sup>I</sup> $\alpha$ , RPP8 also interacted with His<sub>6</sub>R<sup>II</sup> $\alpha$  and R<sup>II</sup> $\beta$  in the assay. However, GST-RPP8 was not capable of binding His<sub>6</sub>R<sup>I</sup> $\beta$ . Thus, RPP8 also belongs to the AKAP family that binds both R<sup>I</sup> and R<sup>II</sup> with high affinity. For this reason, we designate RPP8 as an active fragment of D-AKAP2.

**Localization of D-AKAP2 Binding Site.** To localize more precisely the RPP8 binding site on R<sup>I</sup> $\alpha$  and R<sup>II</sup> $\alpha$ , a series of deletion mutants, summarized in Fig. 3, were used. As seen in Fig. 3, GST-RPP8 associated with His<sub>6</sub>R<sup>I</sup> $\alpha$ ( $\Delta$ 63–379), which contains only the first 62 residues of R<sup>I</sup>. In contrast, R<sup>I</sup> $\alpha$ ( $\Delta$ 1–91), which lacks the N-terminal 91 residues of R<sup>I</sup>, was not precipitated with GST-RPP8. All AKAPs for R<sup>II</sup>, including D-AKAP1, bound the N terminus of R<sup>II</sup> $\alpha$  (13, 23, 24). To determine whether RPP8 also bound R<sup>II</sup> $\alpha$  through the N terminus, several deletion mutants of R<sup>II</sup> $\alpha$  were tested for their ability to bind GST-RPP8. As shown in Fig. 3, GST-RPP8 interacted with R<sup>II</sup> $\alpha$ ( $\Delta$ 46–400), a construct containing only the N-terminal 45 residues of R<sup>II</sup> $\alpha$ . GST alone does not interact with any of the R subunits we have tested. Thus, the N terminus of R<sup>I</sup> $\alpha$  or R<sup>II</sup> $\alpha$  is sufficient for the interaction with D-AKAP2.

**Specific Residues on R<sup>1</sup> $\alpha$  That Are Necessary to Interact with D-AKAP2.** To determine specific residues on R<sup>1</sup> $\alpha$  that are necessary to interact with D-AKAP2, a series of site-directed mutants of R<sup>1</sup> $\alpha$  were also tested to identify specific amino acids on the N-terminal dimerization domain that are essential for interaction with R<sup>1</sup> $\alpha$  and D-AKAP2. As seen in Fig. 4, a mutant of R<sup>1</sup> $\alpha$  where Cys-16 was mutated to His, R<sup>1</sup>(C16H), interacted with GST-RPP8 like wild-type R<sup>1</sup> $\alpha$  in this assay. Mutants of Cys-37 to Ala, R<sup>1</sup>(C37A), and Val-20 to Ala, R<sup>1</sup>(V20A), had dramatic decreased affinity for GST-RPP8. Mutation of Cys-37 to His, R<sup>1</sup>(C37H), Ile-25 to Ala, R<sup>1</sup>(I25A), or both Cys-16 and Cys-37 to Ala, R<sup>1</sup>(C16A-C37A), completely lost the ability to interact with GST-RPP8 although all of these proteins were functional dimers (unpublished data).

**Tissue Distribution of D-AKAP2 mRNA.** To investigate the tissue and developmental expression patterns of D-AKAP2, Northern blots containing 2  $\mu$ g of poly(A<sup>+</sup>)RNA from dif-

ferent adult mouse tissues or different embryonic stages were probed with <sup>32</sup>P-labeled RPP8 cDNA. As shown in Fig. 5A, a 5kb mRNA was detected in all tissues with equal intensity. A weak signal at 10 kb was detected in brain, skeletal muscle, kidney, and testis. In addition, mRNA of 3.4 kb, 2.8 kb, and 1.8 kb were detected only in testis. The 5-kb transcript was detected in all embryonic stages with comparable intensity (Fig. 5B), indicating that the mRNA expression level in the embryo was the same throughout different developmental stages. The other mRNAs were not detected in the embryonic samples.

**Cloning of D-AKAP2.** To obtain a full-length clone of D-AKAP2, a mouse testis cDNA library was screened using RPP8 cDNA as a probe. After radiolabeling of the RPP8 probe, positive clones were isolated from  $\approx$ 1.6 million recombinants. After sequence analysis, one cDNA clone was identified that contained a full-length cDNA of D-AKAP2. The

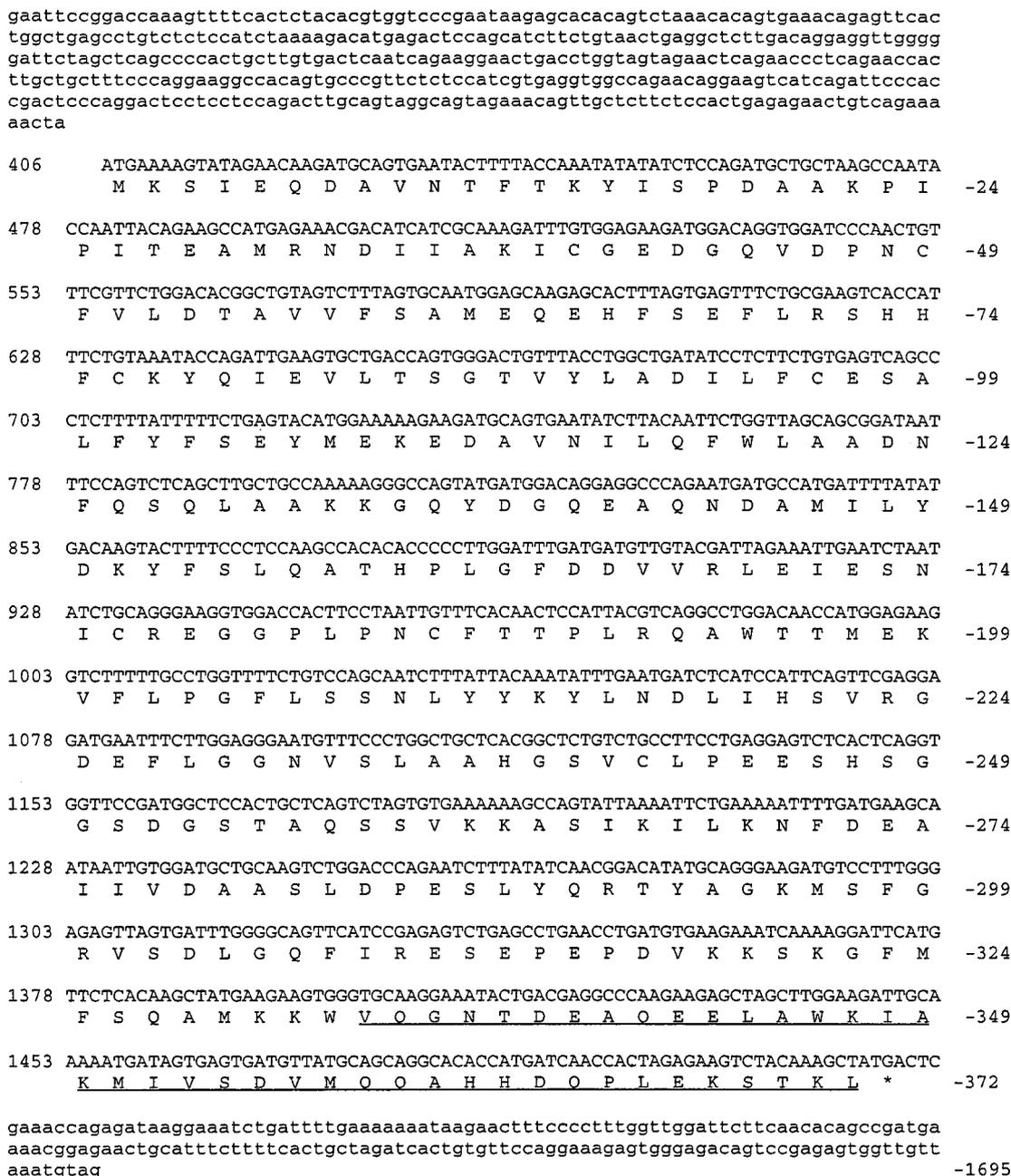


Fig. 6. cDNA and deduced amino acid sequences for D-AKAP2 core. Numbers on the left are for cDNA sequence, and numbers on the right indicate amino acid position. RPP8 sequence is underlined.

DNA sequence and the deduced amino acid are shown in Fig. 6. This cDNA fragment is likely to represent a full-length clone of D-AKAP2 because it contained a proper initiation sequence and stop codons followed by the polyA tail (25). Stop codons were also identified in frame upstream from the initiation site, precluding any possible use of another ATG upstream.

D-AKAP2 contains 372 amino acids. The D-AKAP2 protein sequence was compared with the GenBank database and there was no overall homology to known protein sequences. Surprisingly, however, residues 70–215 showed homology to the RGS domain found in RGS proteins.

**In Situ Hybridization.** To determine the expression pattern of D-AKAP2 in comparison to  $R^I\alpha$ , *in situ* hybridization was performed using probes for both D-AKAP2 and  $R^I\alpha$  in mouse testicle sections. As shown in Fig. 7, D-AKAP2 and  $R^I\alpha$  were expressed in many of the same regions in testis.

## DISCUSSION

D-AKAP2, named for its potential dual specificity, was first identified as a fragment from a yeast two-hybrid screen based on specific interaction with the  $R^I$  portion of Ret/ptc2. In that screen, another anchoring protein, D-AKAP1, was also identified and was found to bind both  $R^I$  and  $R^{II}$ . As demonstrated in an affinity precipitation assay, this fragment of D-AKAP2, RPP8, includes most, if not all, of the  $R^I/R^{II}$  binding domain. Secondary structure predictions indicate that RPP8 has an amphipathic  $\alpha$ -helix at its N terminus, and this is consistent with the proposed model for other AKAPs where predicted

amphipathic helices are hallmarks for R binding domains (24, 26–28). RPP8 was shown to bind  $R^I\alpha$ ,  $R^{II}\alpha$ , and  $R^{II}\beta$ , but not  $R^I\beta$ . This isoform selectivity of AKAPs is consistent with results published earlier. Leiser *et al.* (5) showed that  $R^I\alpha$  had a 6-fold preference for microtubule-associated protein-2 (MAP2) over  $R^{II}\beta$ , whereas  $R^{II}\beta$  had a two fold preference for AKAP75 over  $R^I\alpha$ . Follicle stimulating hormone treatment of rat granulosa cells also induces an 80-kDa  $R^I\alpha$  selective AKAP (29).

Using this functional R binding fragment, RPP8, the interaction regions on both  $R^I\alpha$  and  $R^{II}\alpha$  were localized to their N termini. This N-terminal region corresponds to the first 62 amino acids in  $R^I$  and the first 45 amino acids in  $R^{II}\alpha$ . The actual microenvironment for D-AKAP2 with respect to  $R^I\alpha$  and/or  $R^{II}\alpha$  is not understood at this point. Further characterization will be required to predict the interaction patterns between D-AKAP2 and both types of R subunits *in vivo*.

To further characterize the novel interaction between D-AKAP2 and  $R^I\alpha$ , various mutations were made in the N-terminal region of  $R^I\alpha$  and tested for their effects on D-AKAP2 binding. Mutating Cys-16 to His had no effect, however, changing Cys-37 to His totally abolished binding. Therefore, although Cys-16 is disulfide bonded to Cys-37 in the  $R^I\alpha$  dimer, they exhibited different roles in contributing to the binding interface for D-AKAP2. Mutation at Val-20 had dramatically decreased binding, whereas mutation at Ile-25 totally abolished binding, indicating that these residues are important, either directly or indirectly, for binding D-AKAP2. Mutation of Cys-37 to Ala had dramatically decreased binding and a double mutant with both cysteines changed to alanines totally abolished binding to GST-RPP8. Whether the better tolerance of an Ala than a His at position 37 is due to disruption of a hydrophobic interaction surface or due to the disruption of a specific conformation is still unclear.

A complicated mRNA expression pattern was detected for D-AKAP2, therefore it could represent a novel family of proteins. In addition to the 5-kb mRNA that was expressed in all tissues tested, and also throughout all embryonic stages, brain, skeletal muscle, and kidney had an additional 10-kb message. Moreover, in testis, there are total of five mRNA species. The expression of D-AKAP2 in testis was further confirmed by *in situ* hybridization. D-AKAP2 and  $R^I\alpha$  appeared to be expressed in many of the same regions in adult mouse testicle sections.

Sequence comparison revealed that D-AKAP2 contained a potential RGS domain in its N-terminal region. RGS proteins are a new family of “regulator of G-protein signaling” proteins, which down-regulate signaling by heterotrimeric G proteins. The first biochemical studies of RGS proteins have shown that they accelerate the GTPase activities of G-protein  $\alpha$  subunits,

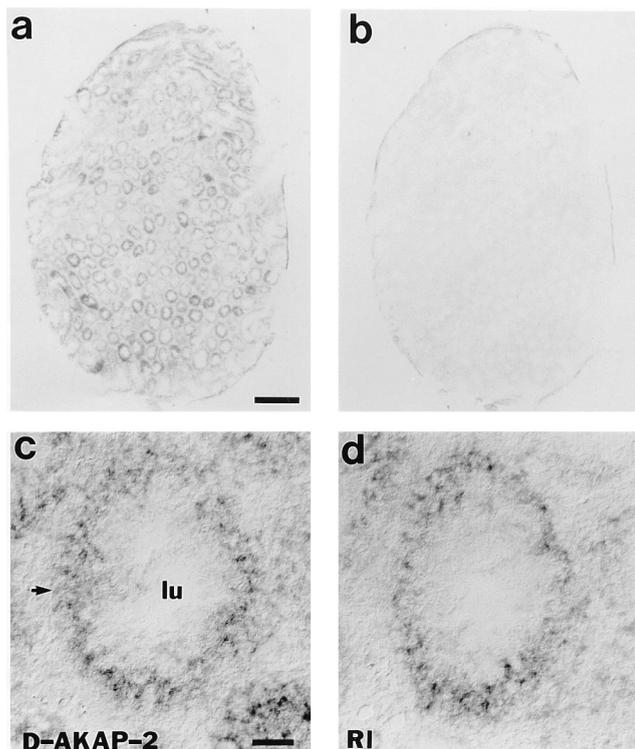


FIG. 7. *In situ* hybridization pattern of D-AKAP2 that shows overlapping regions for  $R^I\alpha$ . RNA probes of D-AKAP2 were derived from the cDNA sequence of 1–1469.  $R^I\alpha$  probes were derived from cDNA coding for residues 18–169. Hybridization was carried out on adjacent 20- $\mu$ m cryostat sections of adult mouse testis and signals were visualized by alkaline phosphatase histochemistry. (a) D-AKAP1 mRNA is present in various regions of testis. (b) Signal of the sense strand as negative control. (c) High magnification of signal for D-AKAP2 on one of the testicular tube cross sections. (d) High magnification of signal for  $R^I\alpha$  on one of the testicular tube cross sections. (Bar = 100  $\mu$ m.)

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RGS1  LSAAEVMQWSQS---LEKLLANQTGQNVFGSFLKSEFSEENIEFWLACEDYKKT
RGS2  PSPEEAQLWSEA---FDELLASKYGLAAFRALFKSEFCEENIDFWLACEDFKKT
RGS3  PTSEEAALKWGES---LEKLLVHKYGLAVFQAFRLRTEFSEENLEFWLACEDFKKV
RGS4  VSQEEVKKWAES---LENLINHECGLAFAFKFLKSEYSEENIDFWLISCEEYKKI
DAK2  LRSHHFCYQIE (8) LADILFCESALFYFSEYMEKEDAVNLLQFWLAADNFQSQ

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RGS1  --ES-DLLPCKAEETKAFVHSDAAKQINIDFRTRESTAKKIKAP---TPTCF
RGS2  --KSPQKLSKARKIYTDFTIEKEAPKEINIDFQTKTLIAQNIQEA---TSGCF
RGS3  --KSQSKMASKAKKIFAETIAIQACKEVNLDSTREHTKIDNLQSV---TRGCF
RGS4  --KSPSKLSPKAKKIYNEFISVQATKEVNLDSTREETSRLMLEP---TITCF
DAK2  (7) YDQQAQNDAIMILYDKYFSLQATHPLGFDDVVRLIEISNICREGGPLNCF

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RGS1  DEAQKVIYTLMEKDSYPRFLKSDIYLNLL
RGS2  TTAQKRIVYSLMENNNSYPRFLESEFYQDLG
RGS3  DLAQKRIFGLMEKDSYPRFLRSDLYLDLI
RGS4  DEAQKIFNLMKDSYRRLKSRFYLDLT
DAK2  TTPLRQAWTMEKVFLPGFLSSNLYKYL

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FIG. 8. D-AKAP2 contains a potential RGS domain. Sequence alignment of the putative RGS domain in D-AKAP2 with other RGS proteins. ●, Conserved residues.

thus driving G proteins into their inactive GDP-bound forms. These RGS domains therefore function as GTPase-activating proteins of the heterotrimeric G proteins (10–12). Alignment of this region of D-AKAP2 to some of the known RGS domains from RGS proteins showed striking similarity (Fig. 8). All conserved hydrophobic residues proposed to be important for maintaining the folding of the domain were highly conserved. Surprisingly, not all of the charged residues conserved in RGS proteins reported so far were conserved in D-AKAP2. In contrast to the hydrophobic residues, these charged amino acids lie on the surface. The lack of conservation of these charge residues suggests that if D-AKAP2 is a functional RGS, it may not interact with  $G_i\alpha$  as most of the known RGSs do, but with other  $G\alpha$  isoforms.

We report here the identification of another novel anchoring protein family of PKA, D-AKAP2, that binds to both  $R^I$  and  $R^{II}$ . In addition to an R binding domain, D-AKAP2 contains a potential RGS domain that was recently shown to interact with  $G\alpha$  subunits and possess GAP-like activity. The presence of these two motifs in a single molecule provides a previously unrecognized direct link between two major components of the cAMP signaling pathway.

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