Identification of a Novel Protein Kinase A Anchoring Protein That Binds Both Type I and Type II Regulatory Subunits*

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Compartmentalization of cAMP-dependent protein kinase (PKA), 1 one of the first protein kinases discovered, mediates a variety of hormonal and neurotransmitter responses by phosphorylating different substrate proteins in the cell. Although PKA is a multifunctional enzyme with a broad substrate specificity, activation of this kinase permits preferential phosphorylation of specific target substrates (1). For example, phosphorylation of membrane-bound ion channels modulates the flow of ions into the cell (2), while phosphorylation of CREB, a nuclear transcription factor, alters the expression of cAMP-responsive genes (3). While the importance of PKA in regulating many cellular processes has long been apparent, the potential importance of compartmentalization for the function and regulation of PKA has only recently been recognized.

In the absence of its activating ligand, cAMP, PKA, PKA exists as an inactive holoenzyme of two regulatory (R) and two catalytic (C) subunits. The two classes of R subunits, R I and R II, based on their elution from the DEAE cellulose (4), define the two types of holoenzymes. A significant proportion of the type II holoenzyme associates with the particulate fraction of cell homogenates, while the type I holoenzyme appears to be mostly cytoplasmic (5). Following an increase in intracellular cAMP, the R subunits bind cAMP, resulting in the dissociation of the holoenzyme and the release of free active C subunits. The free C subunit can then either phosphorylate cytoplasmic substrates or translocate into the nucleus by passive diffusion and phosphorylate nuclear substrates (6). In addition to the C subunit migrating between compartments, the holoenzyme itself can be anchored to specific sites via interactions of its regulatory subunits with specific anchoring proteins. This may allow for activation of localized pools of the kinase (7, 8).

Both classes of R subunits contain two tandem cAMP binding sites at the carboxyl terminus that account for approximately two-thirds of the protein. Both R subunits also contain a site that mimics a substrate or inhibitor and lies in the active site cleft of the C subunit in the holoenzyme complex. R I contains a very stable dimerization domain. This domain, which is the region of the least sequence identity between the two R subunits, is thought to be responsible for interaction with the anchoring proteins (9). Several AKAPs (A-kinase anchoring proteins) have been characterized, and all bind specifically with very high affinity to the type II regulatory subunit (R II) (1, 10).

Recently, it was shown that anchoring proteins may also act as adapters for assembling multiprotein complexes. For example, Scott and co-workers (11, 12) showed that AKAP79, in addition to binding tightly to R I, also interacts with the calcium and calmodulin-dependent protein phosphatase 2B (calcineurin) and protein kinase C. Targeting AKAP79 to neuronal postsynaptic densities would therefore bring 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 pathway (13).

cAMP-dependent protein kinase (PKA), 1 one of the first protein kinases discovered, mediates a variety of hormonal and neurotransmitter responses by phosphorylating different substrate proteins in the cell. Although PKA is a multifunctional enzyme with a broad substrate specificity, activation of this enzyme with a broad substrate specificity, activation of this

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In most cells, the type I holoenzyme appears not to be anchored and is typically cytoplasmic; however, there are cases where RII is compartmentalized. For example, RII subunits in human erythrocytes are tightly bound to the plasma membrane (14). Type I holoenzyme is also depleted from the cytoplasm and accumulates at the "cap" site of lymphocytes when stimulated with anti-CD3 antibodies (15). Here we report the identification and characterization of a potential dual specificity protein kinase A anchoring protein, D-AKAP1, which binds to both the RII and the RIII subunits of PKA.

**EXPERIMENTAL PROCEDURES**

**Materials**—All vectors for the yeast two-hybrid system were from Dr. Stan Hollenberg (Vollum Institute). The following reagents were purchased as indicated: mouse 16-day embryonic cDNA library in λEEXlox vector (Novagen); mouse multiple tissue northern blot and mouse embryonic Northern blot (Clontech); Ready-to-Go DNA labeling kit (Pharmacia Biotech Inc.); Genius labeling system (Boehringer Mannheim); Affi-Gel 15 (Bio-Rad); ECL kit (Amersham Corp.); ATP, phenylmethylsulfonyl fluoride, benzamidine, Triton X-100, and GST-agarose resin (Sigma); SSC buffer (5 Prime → 3 Prime, Inc., Boulder, CO); nickel-NTA resin (Qiagen); 5-bromo-4-chloro-3-indolyl β-ν-galactoside and enzymes used for DNA manipulations (Life Technologies, Inc.); and the DNA sequencing kit (U.S. Biochemical Corp.). Antibodies were generated in female rabbits at Cocalico Corp. All oligonucleotides were synthesized with the Peptide and Oligonucleotide Facility at the University of California, San Diego.

**Two-hybrid Screen**—A yeast two-hybrid screen was performed according to Vojtek et al. (16). Briefly, cDNA coding for the Ret/ptc2 oncogene, which consists of the N-terminal two-thirds of R1’s fused to the c-Ret tyrosine kinase domain (17), was subcloned into the pBTM116 LexA fusion vector. L40 yeast transformed with this construct was used to screen an embryonic mouse random-primed cDNA library (18). From the c-Ret tyrosine kinase domain (17), was subcloned into the pBTM116 vector. RII restriction endonuclease, and purified with the GST-RPP7 was constructed by ligating a linker containing a NolI site into the NdeI site and HindIII sites of pSETb. These RPP7 fusion proteins, designated GST-RPP7 and HisRPP7, respectively, were expressed in Escherichia coli BL21(DE3) at 37 °C and purified to near homogeneity using either glutathione resin, GST-RPP7, or nickel-NTA resin for HisRPP7. In short, bacterial cell lysates containing HisRPP7 were incubated with nickel-NTA resin in PBS (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 at 4 °C for 1 h and then washed with the same buffer with 5 mm imidazole to remove nonspecific proteins. HisRPP7 was then eluted from the resin with PBS containing 100 mm imidazole. The BL21(DE3) cell strain was a gift from Bill Studier (Brookhaven National Laboratories).

**Expression and Purification of R Subunits**—R1 was expressed in BL21(DE3) cells and purified on a DE52 ion exchange column (21). HisR1 and HisR1NH3(36–379) were purified on nickel-NTA resin as described previously. R1NH3(46–400) was expressed and purified as a polystyrene-tagged fusion protein. After removing the polystyrene tag with factor X (22), the R subunit was further purified to homogeneity by gel filtration using Sephadex 75.

**In Vitro Binding Assay**—Bacterial cell lysates containing GST-RPP7 were incubated with glutathione resin for 2 h at 4 °C in PBS with 0.1% Triton X-100, 1 mm phenylmethylsulfonyl fluoride, 1 mm EDTA, 5 mm benzamidine, and 5 mm β-mercaptoethanol and then washed extensively with the same buffer. Full-length and deletion mutants (100–200 μg) of R1 and/or RII were added to the resin and incubated for 2 h at 4 °C. After washing the resin extensively with PBS, proteins associated with the GST-RPP7 were eluted by boiling in SDS gel-loading buffer and analyzed by SDS-PAGE. All electrophoresis was performed using Mini-Protein II electrophoresis system (Bio-Rad). SDS-PAGE reagents were prepared according to Laemmli (23). Proteins were visualized by Coomassie Staining.

**Northern Analysis**—Blots containing 2 μg of immobilized samples of mRNAs from selected adult tissues or total mRNA at different embryonic stages were probed with 32P-radiolabeled RPP7 cDNA. Nitrocellulose filters were prehybridized in 5 × SSC (750 mm sodium chloride, 75 mm sodium citrate, pH 7.0), 5 × Denhardt’s reagent (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% acetylated bovine serum albumin), 0.5% SDS, and 50% formamide for 6 h at 42 °C and then hybridized to 1.5 × 106 cpm/ml of denatured radiolabeled cDNA probe in the same buffer. Hybridization was performed at 42 °C for 16 h, and nonhybridized probe was removed with 0.1 × SSC, 0.1% SDS at 68 °C. Hybridizing mRNA signals were detected by autoradiography.

**Screening of cDNA Libraries**—A 16-day mouse embryonic cDNA library in vector λEEXlox was screened with 32P-labeled RPP7 cDNA. The cDNA fragment RPP7 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 [α-32P]dCTP using random prime labeling. Approximately 1.6 million plaques were screened, and positive clones were plaque-purified. Positive phage clones were cloned into plasmids by infection of hosts expressing the P1 cre recombinase, which recognizes the loxP site on the λEEXlox vectors and forms the plasmids by site-specific recombination. Plasmids were isolated, and the cDNA inserts were then subcloned into the EcoRI and HindIII sites of pBluescriptII KS(+) (19). DNA sequence analysis revealed three cDNA sequences, including one 5′ sequence and two 3′ sequences after composition. These cDNAs all had identical overlapping DNA sequences and were designated N0 splice, C1 splice, and C2 splice, respectively. The 5′ 545 base pairs of the 5′ clone, corresponding to DNA sequence 5–549 of the N1 splice, were then amplified by polymerase chain reaction and used as a probe to screen the same library. This round of screening yielded a novel 5′ sequence with the proper Kozak start site and an upstream stop codon, designated N0 splice. The composite cDNA sequences revealed the possibility of four isoforms, and the deduced amino acid sequences were named D-AKAP1a, D-AKAP1b, D-AKAP1c, and D-AKAP1d, respectively, as will be discussed later (Fig. 4). All sequences were analyzed using PCGENE-IntelliGenetics software and the BLAST program provided by the NCBI server at the National Library of Medicine, National Institutes of Health.

**Production and Purification of Antibodies against HisRPP7**—To prepare antibodies against purified HisRPP7, the protein was expressed and purified on NTA resin to near homogeneity and then run on an SDS-PAGE gel. Fusion protein was then excised from SDS-PAGE and used as the antigen. The subsequent preparation of rabbit antibodies was carried out at Cocalico Biological Co. according to established procedures. Affinity-purified polyclonal antibodies against HisRPP7 were used to detect the protein in Western blots and to screen the two-hybrid cDNA library for HisRPP7 binders.
were obtained from 4 ml of serum on a 1-ml Affi-Gel 15 antigen column (10 mg/ml resin).

**Western Immunoblot Analysis**—Proteins from different mouse tissues were extracted, separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. Blots were blocked overnight in 5% powdered non-fat milk and then incubated with affinity-purified His6RPP7 antibodies at a 1:1000 dilution. After extensive washing with TTBS buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween, pH 7.5), D-AKAP1 was visualized by 1:10,000 dilution alkaline phosphatase-conjugated secondary antibodies or enhanced chemiluminescence with a 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibodies.

**In Situ Hybridization**—RNA probes of D-AKAP1 were derived from cDNA sequence 523–3514 of isoform D-AKAP1d. RetR probes were derived from residues 18–169. 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-μm cryostat sections of embryonic day 16 Balb/c mice as described previously by Chun et al. (24). Hybridized probe was visualized by alkaline phosphatase histochemistry.

**RESULTS**

Identification of a Novel Rα-binding Protein—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 Ret/ptc2 (17). Ret/ptc2 begins with the first 235 amino acids of Rα, which include the dimerization domain, the inhibitory site, and most of the first cAMP binding domain. Using Ret/ptc2 to screen a mouse embryonic cDNA library, several interacting clones were isolated (18). Eight of these cDNA clones coded for three novel protein fragments, designated RPP7, RPP8, and RPP9, that associated specifically with the Rα portion of Ret/ptc2. Four of the clones coded for the same protein, RPP7, which was 125 residues in length.

To determine whether RPP7 bound Rα in vitro, RPP7 was expressed as a fusion protein to GST in E. coli from a pGEX vector containing GST fused to the RPP7 cDNA. GST-RPP7 was expressed and tested for its ability to bind Rα in an affinity precipitation assay. The expressed protein had an apparent molecular mass on SDS-PAGE of 40 kDa, consistent with a protein of 13 kDa fused to GST. GST-RPP7 was fully soluble.

Cell lysates containing either GST or GST-RPP7 were incubated with glutathione-agarose resin for 2 h at 4 °C in binding buffer as described. 100–200 μg of different forms of Rα or RII were then added to the resin and incubated for 2 h at 4 °C. After resin was washed extensively, proteins associated with the GST-RPP7 were eluted from the GST resin by boiling in SDS gel-loading buffer and analyzed on SDS-PAGE. * GST-RPP7. Arrows mark the different R subunits in each sample. A, Rα and His6RI interact with GST-RPP7. B, His6Rα(Δ63–379) and RII(Δ46–400) interact with GST-RPP7.

**FIG. 2.** GST-RPP7 binds to Rα and RII subunits. Bacterial cell lysates containing GST-RPP7 were incubated with glutathione-agarose resin for 2 h at 4 °C in binding buffer as described. 100–200 μg of different forms of Rα or RII were then added to the resin and incubated for 2 h at 4 °C. After resin was washed extensively, proteins associated with the GST-RPP7 were eluted from the GST resin by boiling in SDS gel-loading buffer and analyzed on SDS-PAGE. * GST-RPP7. Arrows mark the different R subunits in each sample. A, Rα and His6RI interact with GST-RPP7. B, His6Rα(Δ63–379) and RII(Δ46–400) interact with GST-RPP7.

**FIG. 3.** RPP7 interacts with the N terminus of the Rα subunit. The schematic diagram indicates the binding capacity of various truncation mutants of Rα to GST-RPP7. The N-terminal dimerization domain, inhibitory site, and two cAMP-binding domains are indicated on the wild type Rα. The Rα portion in Ret/ptc2 is labeled as Ret/ptc2.

**FIG. 4.** GST-RPP7 preferentially interacts with the full-length RII subunit or its N terminus. Equal molar ratios of either full-length Rα and His6RII or His6Rα(Δ63–379) and RII(Δ46–400) were added in the assay. GST-RPP7 preferentially interacted with the full-length Rα subunit (lane 1) or its N terminus (lane 2). * GST-RPP7. Arrows mark the different R subunits in each sample. In lane 2, His6Rα(Δ63–379) is labeled as His6Rα(N), and RII(Δ46–400) is labeled as RII(N).

**FIG. 5.** Size and abundance of D-AKAP1 mRNA in mouse tissues and various embryonic stages. Blots containing 2 μg of mRNAs of selected adult tissues (A) or total mRNA at different embryonic stages (B) were probed with 32P-radiolabeled RPP7 cDNA for 16 h at 42 °C in 5 x SSC, 5 x Denhardt’s reagent, 0.5% SDS, and 50% formamide. After washing under 0.1 x SSC and 0.1% SDS at 68 °C, hybridizing signals were detected by autoradiography.
bated with glutathione resin. Bacterially expressed R I was then added. Proteins associated with the resin after stringent washing were analyzed on SDS-PAGE. As seen in Fig. 1, this construct codes for a stable protein that can specifically pull down nearly stoichiometric amounts of RI. GST alone does not interact with R I. These results confirmed the results of the two-hybrid screen and established furthermore that no other factors are required for the interaction between RI and RPP7 in vitro and in vivo.

Specificity of RPP7—AKAPs identified until now have all bound specifically to the type II regulatory subunit. We therefore tested whether RPP7 could bind to RII. As seen in Fig. 2A, GST-RPP7 associated with His6RI(D63–379), which contains only the first 62 residues of R I. In contrast, R I(D1–91), which lacks the N-terminal 91 residues of R I, was not precipitated with GST-RPP7 (data not shown). Most AKAPs have been shown to specifically bind the N terminus of RII (17, 25, 26). To determine whether RPP7 also bound to the N terminus of RII, several deletion mutants of RII were tested for their ability to bind GST-RPP7. As shown in Fig. 2B, GST-RPP7 interacted with RII(D46–400), a construct containing only the N-terminal 45 residues of RII. GST alone does not interact with any of the affinity. For this reason, we designate RPP7 as an active fragment of D-AKAP1, for dual specificity AKAP1.

Localization of D-AKAP1 Binding Site—To localize more precisely the RPP7 binding site on RI, a series of deletion mutants, summarized in Fig. 3, were used. As seen in Fig. 2B, GST-RPP7 associated with His6RI(D63–379), which contains only the first 62 residues of R I. In contrast, R I(D1–91), which lacks the N-terminal 91 residues of R I, was not precipitated with GST-RPP7 (data not shown). Most AKAPs have been shown to specifically bind the N terminus of RII (17, 25, 26). To determine whether RPP7 also bound to the N terminus of RII, several deletion mutants of RII were tested for their ability to bind GST-RPP7. As shown in Fig. 2B, GST-RPP7 interacted with RII(D46–400), a construct containing only the N-terminal 45 residues of RII. GST alone does not interact with any of the

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**Fig. 6.** cDNA and deduced amino acid sequences for D-AKAP1 core. Numbers on the left are for the cDNA sequence, and numbers on the right are for the amino acid sequence. The RPP7 sequence is *underlined*. 

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R subunits we have tested. Thus, the N terminus of RI or RII is sufficient for the interaction with D-AKAP1.

To further characterize the interaction between the two types of R subunits and RPP7, competition experiments were performed. When assayed individually, a nearly stoichiometric amount of RI or RII was pulled down by GST-RPP7 based on SDS-PAGE (Fig. 2A); however, when incubated with both R subunits, GST-RPP7 preferentially bound RII. As shown in Fig. 4, when an equal molar ratio of either full-length RI and His6RII or His6RI(D63–379) and RII(D46–400) were added in the assay, GST-RPP7 preferentially bound to the two RII subunit constructs. These results indicate that the binding regions of RI and RII on D-AKAP1 are partially, if not completely, overlapping. More detailed mapping of the binding sites on RI, RII, and D-AKAP1 is now under way. Preliminary results using surface plasmon resonance indicated that the affinity between RI and GST-RPP7 is at most 25-fold lower than that for RII and GST-RPP7 (data not shown).

Tissue Distribution of D-AKAP1 mRNA—To investigate the tissue and developmental expression patterns of D-AKAP1, Northern blots containing 2 \( \mu \)g of poly(A)\(^+\) RNA from different adult mouse tissues or different embryonic stages were probed with 32P-labeled RPP7 cDNA. As shown in Fig. 5A, a 3.8-kb mRNA was detected in all tissues except the spleen. D-AKAP1 mRNA expression is highest in heart, liver, skeletal muscle, and kidney. In addition, a strong signal at 3.2 kb was detected only in the testis sample. More detailed mapping of the binding sites on RI, RII, and D-AKAP1 is now under way. Preliminary results using surface plasmon resonance indicated that the affinity between RI and GST-RPP7 is at most 25-fold lower than that for RII and GST-RPP7 (data not shown).

Sequence analysis of these cDNAs revealed a core open reading frame of 526 residues. RPP7 is included within this protein and corresponds to amino acid residues 284–408 in the core sequence (Fig. 5). In addition to this core, cDNAs coding for two N-terminal and two C-terminal splice variants were discovered (Fig. 6). At the 5' end, one splice generated a message coding for 33 residues before the core, designated isoform N1. These additional 33 amino acids include a potential myristoylation site and a potential PKA phosphorylation site. N1 begins its coding region with its first ATG, because the 5' sequence of N1 did not contain another Kozak sequence. The other 5' variant, designated N0, has an in-frame stop codon upstream from its start site, which corresponds to the start site of the core. The two 3' variants, designated C1 and C2, had an additional 18 or 331 residues 3' from the core open reading frame. Therefore, although the mRNA for D-AKAP1 appeared as a single species...
of 3.8 kb in mouse embryo by Northern analysis, cDNA cloning has identified at least four isoforms, designated as D-AKAP1a, D-AKAP1b, D-AKAP1c, and D-AKAP1d. These potential isoforms contain 544, 577, 857, and 890 amino acid residues, respectively.

**Western Blot Analysis**—Antiserum against D-AKAP1 was raised in rabbits using the His6RPP7 fusion protein as the antigen and purified on an antigen column. This antibody recognized both His6RPP7 and GST-RPP7 specifically in crude cell lysates, as shown in Fig. 8A. Proteins were extracted from different mouse tissues, separated on SDS-PAGE gel, Western blotted, and probed with the antibody. As seen in Fig. 8B, two protein bands with apparent molecular masses of 86 and 57 kDa were detected in the brain extract, a 64-kDa protein was detected in the muscle extract, and a doublet of 132 kDa was detected in the liver sample. Since the message of D-AKAP1 was not found in the spleen in the Northern analysis, extracts from the spleen were used as a negative control. These bands were not detected in the spleen and, therefore, were predicted to represent D-AKAP1 proteins in the tissue samples. In addition, preincubation of the antigen with the antibody abolished these bands, suggesting that the signal is specific for D-AKAP1. The existence of different protein isoforms of D-AKAP1 is consistent with the identification of various cDNA splice variants. The sizes of 132 and 86 kDa were confirmed by in vitro translation of the cDNAs coding for the C1 and C2 splice isoforms of D-AKAP1 (data not shown). In the tissue extracts and also the in vitro translation, the apparent molecular masses were higher than the calculated ones. This discrepancy has also been observed for many other AKAPs (27, 28). Antibodies specific for each isoform are currently being raised to further establish whether the isoforms are expressed in a tissue-specific manner.

**In Situ Hybridization**—To determine the expression pattern of D-AKAP1 in comparison with RII, in situ hybridization was performed using probes for both D-AKAP1 and RII in whole embryonic day 16 mouse embryo sections. Riboprobes were derived from the C2 splice of D-AKAP1. Expression was most prominent in brown fat surrounding the trapezius muscle. Other skeletal muscle, intestine, olfactory epithelium, and numerous regions of the central nervous system (CNS) also showed a significant hybridization signal (Fig. 9A). Interestingly, in brain, the regions of early cerebral cortex and basal ganglia that showed the greatest signal were zones containing postmitotic neurons. Similar results have also been shown for AKAP150 (28). The sense strand control showed no hybridization.

In situ patterns were also investigated for RIIα on the adjacent embryonic section. Riboprobes were made from residues 18–169 of RIIα, including the N-terminal region and the beginning of the first cAMP binding site. This fragment of RIIα was used because it has the least similarity in DNA sequence to RIIβ. When comparing the in situ patterns, overlapping expression for D-AKAP1 and RIIα were found in muscle, such as the tongue, but the most striking overlap patterns came from the olfactory bulb and olfactory epithelium. As shown in Fig. 9, D-G, the RII and D-AKAP1 messages appear to be localized in many of the same regions of these structures.

**DISCUSSION**

Anchoring of PKA through the regulatory subunit is proposed to localize the kinase at specific subcellular sites. All AKAPs documented so far interact specifically with the type II regulatory subunit. Here we report a novel PKA anchoring protein, D-AKAP1, that binds and potentially targets both the type I and the type II regulatory subunits.

D-AKAP1, named for its potential dual specificity, was first identified as a fragment from a yeast-two hybrid screen based on specific interaction with the RII portion of Ret/ptc2. As demonstrated in an affinity precipitation assay, this fragment, RPP7, includes most, if not all, of the RII/RII binding domain. Secondary structure predictions indicate that RPP7 has an amphipathic α-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 (26, 29–31).

Using this functional R-binding fragment, RPP7, the interaction regions on both RII and RII′ were localized to their N termini. This N-terminal region corresponds to the first 62 amino acids in RII and the first 45 amino acids in RII′. Since the N-terminal dimerization domain is also proposed to be a key requirement for interaction between other AKAPs and RII′ (9, 25, 32), the amino acid sequences at the N terminus of RII′ and RII′ were aligned to identify conserved residues. When aligned, the N-terminal regions of RII and RII′ show the least sequence identity. However, conserved Leu29 and Phe52 on RII′ were identified at positions equivalent to Leu13 and Phe36 of RII. Substitution of Ala for these two residues generates monomeric RIIβ subunits that cannot bind AKAP75 (32). Leu36 in RII is located at a position that is equivalent to Val29 in RII′. An RIIβ mutant where Val29-Leu21 were replaced by Ala-Ala still dimerized but was unable to bind to AKAPs (32). Whether mutations of these corresponding residues on RII will disrupt dimerization and/or interaction with D-AKAP1 is still under investigation.

![Fig. 8. D-AKAP1 protein is present in brain, liver, and muscle.](image-url)

A, affinity-purified His6RPP7 antibodies specifically recognize bacterial lysates containing 0.2 μg of GST-RPP7 (lane 1), His6RPP7 (lane 2), or 0.2 μg of purified His6RPP7. Signals were visualized with a 1:10,000 dilution of the alkaline phosphatase-conjugated secondary antibodies. The expected sizes of GST-RPP7 and His6RPP7 are 40 and 18 kDa, respectively. B, 60 μg of solubilized extracts from mouse brain, muscle, and spleen and 120 μg of solubilized extracts from mouse liver were separated on an SDS-PAGE gel and probed with a 1:1000 dilution of the purified anti-His6RPP7 antibodies. Enhanced chemiluminescence was used for visualization of the signals. Samples that were preincubated with purified His6RPP7, and the primary antibodies are indicated (+).
Since binding competition between R1 and R11 showed that R1 can compete with R11 and is actually preferentially bound by GST-RPP7, the regions on RPP7 that are responsible for binding RI or RII are likely to be partially, if not completely, overlapping. However, we cannot rule out the possibility that R1 and R11 bind at distinct but interacting sites. Although D-AKAP1 preferentially binds R11 in vitro, since R1 and R11 differ in their tissue expression, subcellular localization, and temporal expression during development, the actual microenvironment for D-AKAP1 with respect to RI and/or R11 is not understood at this point. These results raise the possibility that, in addition to R11, R1 may also be a target for compartmentalization. Further characterization will be required to predict the interaction patterns between D-AKAP1 and both types of R subunits in vivo.

Further potential for diversity is indicated by the fact that there are multiple splice variants of D-AKAP1 resulting in a family of different isoforms. Western analysis using antibodies against RPP7 detected various protein bands of different molecular masses in extracts from various tissues. Two protein bands with apparent molecular masses of 86 and 57 kDa were detected in the brain extract, a 64-kDa protein was detected in the muscle extract, and a doublet at 132 kDa was detected in the liver sample. From two N-terminal splice variants and two C-terminal splice variants identified in cDNA cloning, four of the isoforms were identified. Each of these splice variants

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FIG. 9. In situ hybridization pattern of D-AKAP1, which shows overlapping regions for R1α. RNA probes of D-AKAP1 were derived from the cDNA sequence of isoform D-AKAP1c. R1α probes were derived from residues 18–169. Hybridization was carried out on adjacent 20-μm cryostat sections of embryonic day 16 (E16) Balb/c mice, and signals were visualized by alkaline phosphatase histochemistry. A, D-AKAP1 mRNA is present in the brown fat surrounding trapezius muscle, skeletal muscle, intestine, olfactory epithelium, and numerous regions of the central nervous system. Regions with asterisks are shown magnified in D and F. B, signal of the sense strand as negative control. C, cresyl violet cellular stain of a nearly adjacent section. D, and E, messages of D-AKAP1 and R1α, respectively, at the olfactory bulb and olfactory epithelium region. Bar, 200 μm. F and G, messages of D-AKAP1 and R1α, respectively, in the tongue muscle. Bar, 100 μm.

FIG. 10. Alignment of the core amino acid sequence of D-AKAP1 (DAK1) with S-AKAP84 (AK84). The amino acid sequence of S-AKAP84 (bottom) and the core amino acid sequence of D-AKAP1 (top) are aligned. Boxed residues 1–30 indicate the mitochondria signal/anchor region, and shaded residues 317–338 indicate the R-binding domain, as suggested by Rubin et al. (33). The leucines in the leucine zipper of S-AKAP84 are indicated (dots).
contains distinct features. For example, the N1 splice contains a potential myristoylation site and a potential PKA phosphorylation site, while the C1 splice variant contains several potential casein kinase II phosphorylation sites (Fig. 7). Since different protein isoforms were detected in different tissues using the antibodies against RPP7, these isoforms may be expressed in a tissue-specific manner. Whether these splice variants have distinct physiological functions in the particular tissues that express them is still unclear. This heterogeneity in the isoform expression pattern of D-AKAP1 also introduces an additional mechanism for regulating the compartmentalization of PKA. During the cloning of D-AKAP1, data base comparison identified a new AKAP protein, S-AKAP84, sharing homologous amino acid sequence with D-AKAP1 (33). Unlike D-AKAP1, which is expressed in most tissues, S-AKAP84 is a PKA anchoring protein expressed principally in the male germ cell lineage. It is likely that the 3.2-kb transcript detected in the testis sample is indeed the mouse homolog of S-AKAP84. For S-AKAP84 there was also evidence of multiple mRNA isoforms.

A simple S-AKAP84 genomic pattern was described by Rubin and co-workers (33) that indicates these S-AKAP84 RNA transcripts may arise from a single gene. It is therefore likely that the 3.2-kb transcript detected in the section of the protein, which includes a mitochondria target/signal region at the N terminus and an RII binding domain, as described by Rubin and co-workers (33) (Fig. 10). The putative RII binding domain in S-AKAP84 is contained within RPP7, the R binding fragment of D-AKAP1. The question of whether the specific segment homologous to the RII binding domain is indeed the R and/or RII binding site of D-AKAP1 and the specific side chains required for interactions are still under investigation. Since the core of D-AKAP1 contains the potential mitochondrial anchor region at the N terminus, it is likely that at least some of the D-AKAP1 isoforms are targeted to the mitochondria. This is consistent with results indicating that the message of this protein was found predominantly in tissues with high mitochondria content, such as cardiac or skeletal muscle and liver. Results by Schwob et al. (34) also documented that RII holoenzyme anchored in the inner membrane of mitochondria. Immunofluorescence microscopy will further determine the subcellular localization of the D-AKAP1 isoforms.

D-AKAP1 does not contain the leucine zipper motif that was identified in S-AKAP84. Further investigation to identify additional partners for the D-AKAP1-PKA or the S-AKAP84-PKA complex will give more information on the physiological function of this family of anchoring proteins and especially the function of the leucine zipper motif of S-AKAP84.

In situ hybridization experiments were performed to determine the expression patterns of RII and D-AKAP1 in embryonic day 16 mouse embryos. These data showed that RII and D-AKAP1 have overlapping expression patterns in muscle cells and olfactory epithelium. In the olfactory system, D-AKAP1 appeared to be expressed in the same regions as RII. It has been demonstrated that the odorant-induced cAMP response in the olfactory system is attenuated by PKA, possibly through phosphorylation of the odorant receptors (35–37). D-AKAP1 could therefore function to anchor PKA near the target receptor.

We report here for the first time the use of the yeast two-hybrid system to identify a CAMP-dependent protein kinase anchoring protein and raise the novel possibility that like RII, RII can potentially be specifically anchored at various subcellular locations via AKAPs.

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**Note Added in Proof**—During the review of our manuscript, Tredelenburg and co-workers published a report of a related protein that has a C2 splice similar to D-AKAP1 (Tredelenburg, G., Hummel, M., Riecken, E., and Hanksi, C. (1996) Biochem. Biophys. Res. Commun. 225, 313–319). These authors pointed out that the C2 splice region contains a KH domain which is a potential RNA-binding motif. This KH motif corresponds to residues 38–87 in the C2 splice of D-AKAP1.