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

Lily Jun-shen Huang‡§, Kyle Durick‡¶, Joshua A. Weiner‖**, Jerold Chun‖, and Susan S. Taylor‡ ‡‡

From the ‡Department of Chemistry and Biochemistry and the ‖Department of Pharmacology and Neuroscience Program, School of Medicine, University of California, San Diego, La Jolla, California 92093-0654

Compartmentalization of cAMP-dependent protein kinase is achieved in part by interaction with A-kinase anchoring proteins (AKAPs). All of the anchoring proteins identified previously target the kinase by tethering the type II regulatory subunit. Here we report the cloning and characterization of a novel anchoring protein, D-AKAP1, that interacts with the N terminus of both type I and type II regulatory subunits. A novel cDNA encoding a 125-amino acid fragment of D-AKAP1 was isolated from a two-hybrid screen and shown to interact specifically with the type I regulatory subunit. Although a single message of 3.8 kilobase pairs was detected for D-AKAP1 in all embryonic stages and in most adult tissues, cDNA cloning revealed the possibility of at least four splice variants. All four isoforms contain a core of 526 amino acids, which includes the R binding fragment, and may be expressed in a tissue-specific manner. This core sequence was homologous to S-AKAP84, including a mitochondrial signal sequence near the amino terminus (Lin, R. Y., Moss, S. B., and Rubin, C. S. (1995) *J. Biol. Chem.* 270, 27804–27811). D-AKAP1 and the type I regulatory subunit appeared to have overlapping expression patterns in muscle and olfactory epithelium by *in situ* hybridization. These results raise a novel possibility that the type I regulatory subunit may be anchored via anchoring proteins.

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 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 pseudosubstrate site, while R^{II} contains a phosphorylatable substrate site at this region. The N terminus of each R subunit 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^{II}, 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).

[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAP, A-kinase anchoring protein; R subunit, cAMP-dependent protein kinase regulatory subunit; C subunit, cAMP-dependent protein kinase catalytic subunit; R^I, type Iα regulatory subunit of cAMP-dependent protein kinase; R^{II}, type IIα regulatory subunit of cAMP-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PBS, phosphate-buffered saline.

In most cells, the type I holoenzyme appears not to be anchored and is typically cytoplasmic; however, there are cases where R^I is compartmentalized. For example, R^I 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 R^I and the R^{II} subunits of PKA.
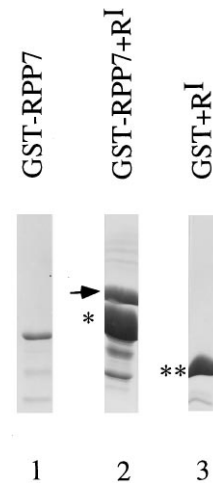
EXPERIMENTAL PROCEDURES

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 λEXlox 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-indoyl β-D-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 at 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 $R^I\alpha$ 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 approximately 2 million transformants, 10 survived nutritional selection and were β-galactosidase-positive specifically for the R^I portion of Ret/ptc2. The library plasmids from these co-transformants were isolated, and cDNA sequences were determined by the procedure of Sanger *et al.* (19). Two of these encoded fragments of $R^I\alpha$ corresponding to residues 12–120 and 17–117. Four of the remaining eight contained an identical cDNA, coding for a protein fragment designated RPP7.

Expression and Purification of Recombinant R Subunit-binding Proteins—To determine whether the protein coded for by RPP7 binds to R^I *in vitro*, the RPP7 cDNA was subcloned from the pVP16 plasmid into both pGEX-KG, to make a GST fusion protein, and into pRSETb-*Not*I, to make a polyhistidine fusion protein. pRSETb-*Not*I was constructed by ligating a linker containing a *Not*I site into the *Nde*I and *Hin*dIII sites of pRSETb. These RPP7 fusion proteins, designated GST-RPP7 and His$_6$RPP7, respectively, were expressed in *Escherichia coli* BL21(DE3) at 37 °C and purified to near homogeneity using either glutathione resin for GST-RPP7, as described previously (20), or nickel-NTA resin for His$_6$RPP7. In short, bacterial cell lysates containing His$_6$RPP7 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. His$_6$-RPP7 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—R^I was expressed in BL21(DE3) cells and purified on a DE52 ion exchange column (21). His$_6$R^{II} and His$_6$R^I(Δ63–379) were purified on nickel-NTA resin as described previously. R^{II}(Δ46–400) was expressed and purified as a polyhistidine-tagged fusion protein. After removing the polyhistidine tag with factor X (22), the R subunit was further purified to homogeneity by gel filtration using Sephedex 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 R^I and/or R^{II} 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-Protean II electrophoresis system (Bio-Rad). SDS-PAGE reagents


FIG. 1. **GST-RPP7 binds to R^I.** *Lane 1* shows purified GST-RPP7. GST-RPP7 or GST were immobilized on glutathione resin as described under "Experimental Procedures." After removal of nonspecific proteins, 200 μg of R^I subunit were added to the resin and incubated for 2 h 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 2* and *3*, R^I only associates with GST-RPP7 but not GST. *, GST-RPP7; **, GST; *arrow*, R^I.

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 ^{32}P-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 × 10^6 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 λEXlox was screened with ^{32}P-labeled RPP7 cDNA. The cDNA fragment RPP7 was excised from the two-hybrid library plasmid pVP16, using the *Not*I restriction endonuclease, and purified on agarose gel. This purified cDNA was then labeled with [α-^{32}P]dCTP using random prime labeling. Approximately 1.6 million plaques were screened, and positive clones were plaque-purified. Positive phage clones were converted into plasmids by infection of hosts expressing the P1 cre recombinase, which recognizes the loxP site on the λEXlox vectors and forms the plasmids by site-specific recombination. Plasmids were isolated, and the cDNA inserts were then subcloned into the *Eco*RI and *Hin*dIII sites of pBluescriptII KS(+). DNA sequences were determined by the dideoxynucleotide chain termination procedure (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 N1 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 His$_6$RPP7—To prepare antibodies against purified His$_6$RPP7, 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 His$_6$RPP7
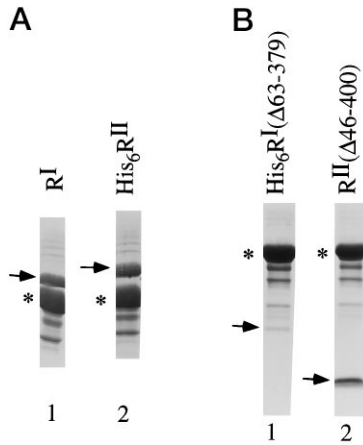
FIG. 2. **GST-RPP7 binds to R^I and R^{II} subunits.** Bacterial cell lysates containing GST-RPP7 were incubated with glutathione-agarose resin for 2 h at 4 °C in binding buffer as described. 100–200 μg of different forms of R^I or R^{II} were then added to the resin and incubated for 2 h at 4 °C. After 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*, R^I and His_6R^{II} interact with GST-RPP7. *B*, $His_6R^I(\Delta63$–$379)$ and $R^{II}(\Delta46$–$400)$ interact with GST-RPP7.
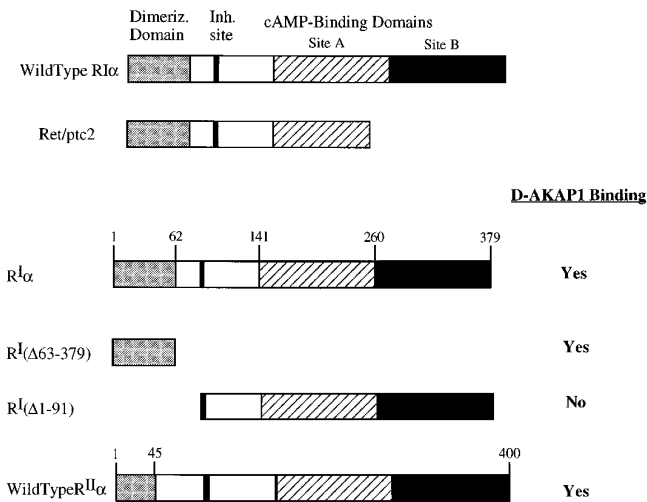

FIG. 3. **RPP7 interacts with the N terminus of the R^I subunit.** The *schematic diagram* indicates the binding capacity of various truncation mutants of R^I to GST-RPP7. The N-terminal dimerization domain, inhibitory site, and two cAMP-binding domains are indicated on the wild type $R^I\alpha$. The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

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 His_6RPP7 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. RIα 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.
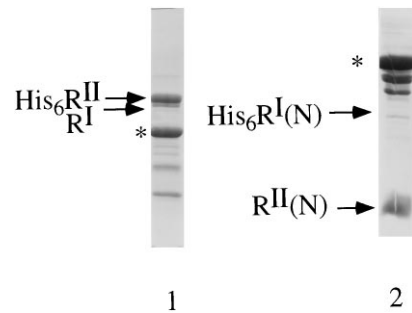
FIG. 4. **GST-RPP7 preferentially interacts with the full-length R^{II} subunit or its N terminus.** Equal molar ratios of either full-length R^I and His_6R^{II} or $His_6R^I(\Delta63$–$379)$ and $R^{II}(\Delta46$–$400)$ were added in the assay. GST-RPP7 preferentially interacted with the full-length R^{II} subunit (*lane 1*) or its N terminus (*lane 2*). *, GST-RPP7. *Arrows* mark the different R subunits in each sample. In *lane 2*, $His_6R^I(\Delta63$–$379)$ is labeled as *$His_6R_I(N)$*, and $R^{II}(\Delta46$–$400)$ is labeled as *$R_{II}(N)$*.
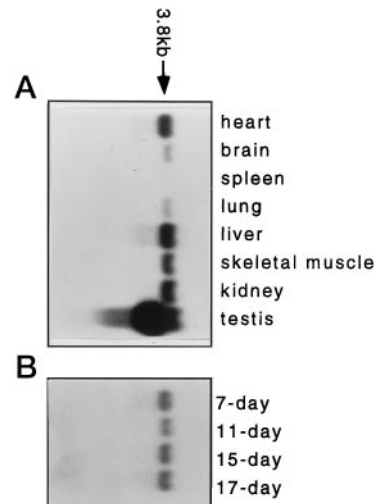
FIG. 5. **Size and abundance of D-AKAP1 mRNA in mouse tissues and various embryonic stages.** Blots containing 2 μg of mRNAs of selected adult tissues (*A*) or total mRNA at different embryonic stages (*B*) were probed with ^{32}P-radiolabeled RPP7 cDNA for 16 h at 42 °C in 5 × SSC, 5 × Denhardt's reagent, 0.5% SDS, and 50% formamide. After washing under 0.1 × SSC and 0.1% SDS at 68 °C, hybridizing signals were detected by autoradiography.

RESULTS

Identification of a Novel R^I-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 $R^I\alpha$ subunit of PKA (17). Ret/ptc2 begins with the first 235 amino acids of RIα, which include the dimerization domain, the C subunit inhibitor 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 R^I 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 R^I *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 then expressed and tested for its ability to bind R^I 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 incu-

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   1  AAGATGGCAATCCAGTTGCGTTCGCCCTTCCCCTTGGCGTTGCCCGGAATGCTGGCCCTCCTTGGCTGGTGGTGG
         M   A   I   Q   L   R   S   P   F   P   L   A   L   P   G   M   L   A   L   L   G   W   W   W   -24

  76  TTTTTCTCTCGTAAAAAAGATCGGCTCAGCAGCAGTGATAAGCAGGTGGAGACACTGAAGGTTGGCCCTGCCATC
         F   F   S   R   K   K   D   R   L   S   S   S   D   K   Q   V   E   T   L   K   V   G   P   A   I   -49

 151  AAGGACCGACGGCTCAGTGAAGAGGCCTGTCCTGGAGTTCTGTCTGTGGCCCCCACTGTCACACAGCCTCCTGGA
         K   D   R   R   L   S   E   E   A   C   P   G   V   L   S   V   A   P   T   V   T   Q   P   P   G   -74

 226  AGGGAAGAGCAGCGCTCTGTGGACAAGCCTTCTACAGAGCCCCTGGCCTTGCCGAGGACTCGCCAGGTTCGACGA
         R   E   E   Q   R   S   V   D   K   P   S   T   E   P   L   A   L   P   R   T   R   Q   V   R   R   -99

 301  AGATCAGAGTCCTCAGGCAACCTCCCCAGCGTTGCAGACACGAGGTCGCAGCCAGGACCGTGCAGAGATGAGATC
         R   S   E   S   S   G   N   L   P   S   V   A   D   T   R   S   Q   P   G   P   C   R   D   E   I   -124

 376  GCCAAAGTGGAACTCTCCCTGATGGGGGACAAAGCCAAATCTATTCCTCTTGGATGTCCGCTTCTCCCAAAGGAT
         A   K   V   E   L   S   L   M   G   D   K   A   K   S   I   P   L   G   C   P   L   L   P   K   D   -149

 451  GCGTCCTTCCCCTATGAAGCAGTGGAAAGGTGTAAGCAGGAGTCCGCACTGGGCAAGACTCCTGGAAGAGGCTGG
         A   S   F   P   Y   E   A   V   E   R   C   K   Q   E   S   A   L   G   K   T   P   G   R   G   W   -174

 526  CCAAGCCCGTATGCGGCCTCTGGAGAGAAAGCGAGAGAGACAGGTGGGACAGAGGGGACTGGAGATGCTGTGTTG
         P   S   P   Y   A   A   S   G   E   K   A   R   E   T   G   G   T   E   G   T   G   D   A   V   L   -199

 601  GGGGAAAATGTATCTGAGGAAGGCCTATTGTCCCAGGAGTGTGTCTCAGAAGTGGAGAAGATTGAGTTTCCAATC
         G   E   N   V   S   E   E   G   L   L   S   Q   E   C   V   S   E   V   E   K   I   E   F   P   I   -224

 676  CTGGCCCCCGGGGGAGGTGAGGGAGAAGAGGTGAGCCATGGCCCCCCACAGGTAGCTGAACTTTTAAAGAAGGAA
         L   A   P   G   G   G   E   G   E   E   V   S   H   G   P   P   Q   V   A   E   L   L   K   K   E   -249

 751  GAATATATTGTTGGGAAGTTGCCGAGTAGCTTTGTGGAGCCAGTTCACTCAGAGCCGGTTAAGGACGAGGATGCG
         E   Y   I   V   G   K   L   P   S   S   F   V   E   P   V   H   S   E   P   V   K   D   E   D   A   -274

 826  TTGGAACCCCAGGTCAAAGGTAGCAGCAATACTTCGGATAGAGACCTGGCTGGAGAGCTGGACAAAGACGAGACC
         L   E   P   Q   V   K   G   S   S   N   T   S   D   R   D   L   A   G   E   L   D   K   D   E   T   -299

 901  GTGCCTGAAAATGACCAGATTAAGCAGGCTGCCTTCCAGCTCATCTCCCAGGTGATCTTGGAAGCAACTGAAGAG
         V   P   E   N   D   Q   I   K   Q   A   A   F   Q   L   I   S   Q   V   I   L   E   A   T   E   E   -324

 976  TTGCGGGCCACCACAGTGGGCAAGACTGTGGCACAAGTGCACCCAATCTCGGCCACTCAGCCTAAGGGGAAGGAG
         L   R   A   T   T   V   G   K   T   V   A   Q   V   H   P   I   S   A   T   Q   P   K   G   K   E   -349

1051  GAGAGCTGTGTTCCAGCCAGCCAGGAAACTAGCTTGGGACAAGACACCTCAGATCCTGCTTCCACCAGAACAGGT
         E   S   C   V   P   A   S   Q   E   T   S   L   G   Q   D   T   S   D   P   A   S   T   R   T   G   -374

1126  GCCACTGCCAGCCCTTCAGCAGAAGCTCTGCCACCAAAGACCTATGTAAGCTGTCTCAGCAGCCCTCTGTCAGGC
         A   T   A   S   P   S   A   E   A   L   P   P   K   T   Y   V   S   C   L   S   S   P   L   S   G   -399

1201  CCCACCAAGGACCAGAAGCCAAAGAACTCTGCACATCACATCTCCCTGGCTCCCTGCCCACCGCCAGTCACCCCC
         P   T   K   D   Q   K   P   K   N   S   A   H   H   I   S   L   A   P   C   P   P   P   V   T   P   -424

1276  CAGAGGCAGTCTCTGGAGGGGGGCAAGTAACCCGAGAGGTGATGACAACTTTGTCGCCTGTATGGCCAACAACAGC
         Q   R   Q   S   L   E   G   A   S   N   P   R   G   D   D   N   F   V   A   C   M   A   N   N   S   -449

1351  CAGAGTGTCCTTTCAGTTAGCTCCTTGGGGCAGTGCTCAGATCCTGTCAGTACTTCGGGGCTTGAAGACTCTTGC
         Q   S   V   L   S   V   S   S   L   G   Q   C   S   D   P   V   S   T   S   G   L   E   D   S   C   -474

1426  ACAGAGACCATCTCAAGCTCCGGAGACAAAGCTATCACCCCACCACTGCCAGTCAGTACTCAGCCCTTCAGCAAC
         T   E   T   I   S   S   S   G   D   K   A   I   T   P   P   L   P   V   S   T   Q   P   F   S   N   -499

1501  GGGGTGCTGAAGGAGGAGCTGTCAGACTTAGGGACCGAGGATGGATGGACCATGGATACAGAAGCAGATCACTCA
         G   V   L   K   E   E   L   S   D   L   G   T   E   D   G   W   T   M   D   T   E   A   D   H   S   -524

1576  GGAGGT
         G   G   -526
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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*.

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 R^I. 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 R^I 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 R^{II}. As seen in Fig. 2A, RPP7 also interacted with His_6R^{II} in the assay. Thus, in contrast to other AKAPs described so far, RPP7 is the first member of this family demonstrated to bind both R^I and R^{II} with high

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 R^I, a series of deletion mutants, summarized in Fig. 3, were used. As seen in Fig. 2B, GST-RPP7 associated with $His_6R^I(\Delta63–379)$, which contains only the first 62 residues of R^I. In contrast, $R^I(\Delta1–91)$, 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 R^{II} (17, 25, 26). To determine whether RPP7 also bound to the N terminus of R^{II}, several deletion mutants of R^{II} were tested for their ability to bind GST-RPP7. As shown in Fig. 2B, GST-RPP7 interacted with $R^{II}(\Delta46–400)$, a construct containing only the N-terminal 45 residues of R^{II}. GST alone does not interact with any of the
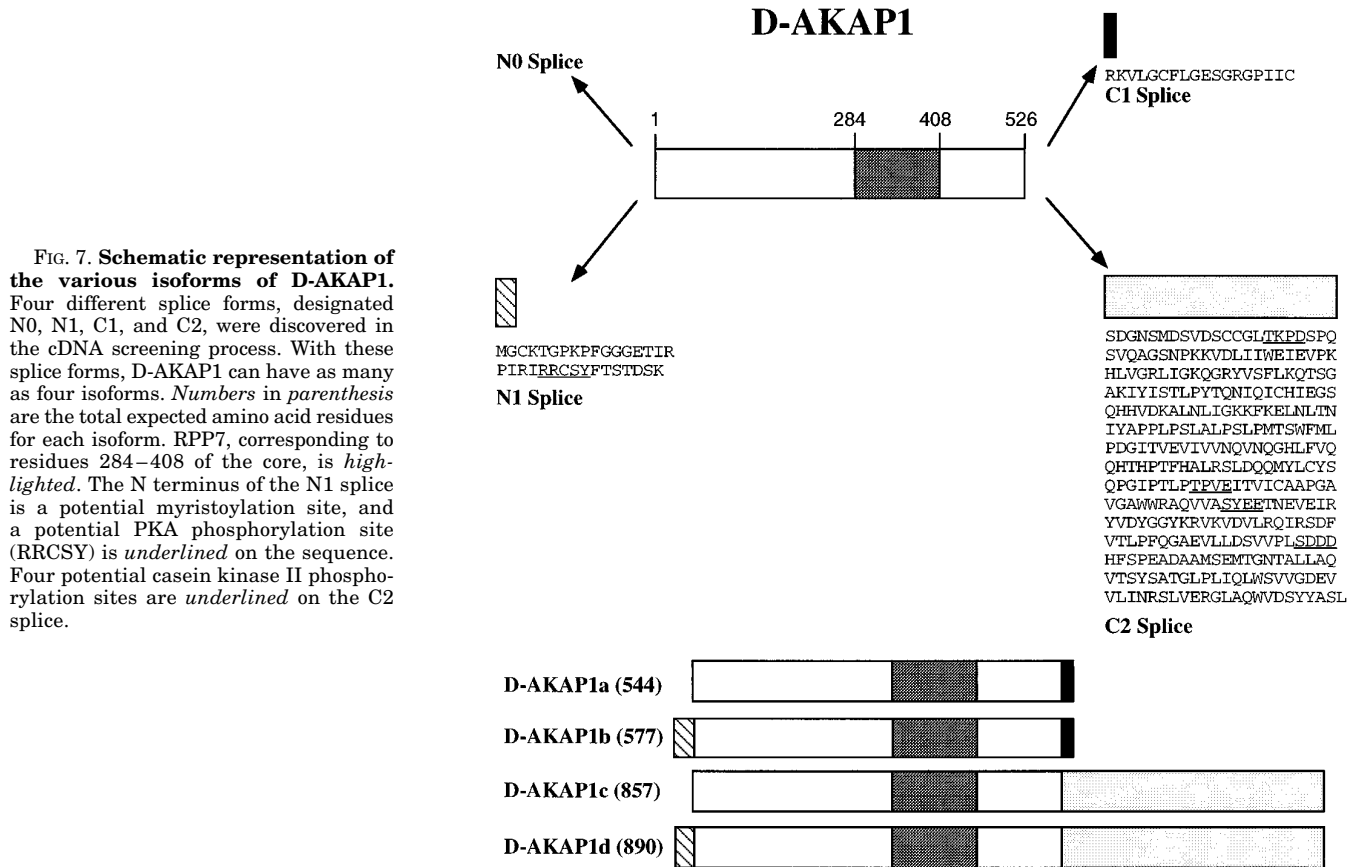
D-AKAP1

FIG. 7. **Schematic representation of the various isoforms of D-AKAP1.** Four different splice forms, designated N0, N1, C1, and C2, were discovered in the cDNA screening process. With these splice forms, D-AKAP1 can have as many as four isoforms. *Numbers in parenthesis* are the total expected amino acid residues for each isoform. RPP7, corresponding to residues 284–408 of the core, is *highlighted*. The N terminus of the N1 splice is a potential myristoylation site, and a potential PKA phosphorylation site (RRCSY) is *underlined* on the sequence. Four potential casein kinase II phosphorylation sites are *underlined* on the C2 splice.

R subunits we have tested. Thus, the N terminus of R^I or R^{II} 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 R^I or R^{II} was pulled down by GST-RPP7 based on SDS-PAGE (Fig. 2A); however, when incubated with both R subunits, GST-RPP7 preferentially bound R^{II}. As shown in Fig. 4, when an equal molar ratio of either full-length R^I and His_6R^{II} or $His_6R^I(\Delta63–379)$ and $R^{II}(\Delta46–400)$ were added in the assay, GST-RPP7 preferentially bound to the two R^{II} subunit constructs. These results indicate that the binding regions of R^I and R^{II} on D-AKAP1 are partially, if not completely, overlapping. More detailed mapping of the binding sites on R^I, R^{II}, and D-AKAP1 is now under way. Preliminary results using surface plasmon resonance indicated that the affinity between R^I and GST-RPP7 is at most 25-fold lower than that for R^{II} 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 μg of poly(A)$^+$ RNA from different adult mouse tissues or different embryonic stages were probed with ^{32}P-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. The 3.8 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 3.2-kb mRNA was not detected in the embryonic samples. Since RPP7 was isolated from a mouse embryonic library, the 3.8-kb signal was predicted to represent the full-length message for D-AKAP1.

Cloning of D-AKAP1—To obtain a full-length clone of D-AKAP1, a mouse embryonic cDNA library was screened using RPP7 cDNA as a probe. After radiolabeling of the RPP7 probe, 13 positive clones containing overlapping partial cDNA sequences were isolated from approximately 1.6 million recombinants. After sequence analysis, three cDNA inserts were identified. These included two, designated C1 and C2, that differed only in their 3' region, and a third, designated N1, in which the 3' end overlapped with the 5' ends of C1 and C2. Both C1 and C2 have identical 5' sequences of 1581 base pairs but different 3' termini. N1 has additional 5' sequence including a consensus Kozak start site (27). The 5' end of N1 was amplified using polymerase chain reaction to generate an additional probe to rescreen the library. Three positive clones were identified and sequenced, and they yielded 810 base pairs of a novel 5' sequence, N0, containing a consensus Kozak sequence and an in-frame stop codon 60 base pairs upstream. This precluded the possibility of using an alternative ATG further upstream.

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. 6). In addition to this core, cDNAs coding for two N-terminal and two C-terminal splice variants were discovered (Fig. 7). 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 His_6RPP7 fusion protein as the antigen and purified on a antigen column. This antibody recognized both His_6RPP7 and GST-RPP7 specifically in crude cell lysates, as shown in Fig. 8*A*. Proteins were extracted from different mouse tissues, separated on SDS-PAGE gel, Western blotted, and probed with the antibody. As seen in Fig. 8*B*, 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 R^I, *in situ* hybridization was performed using probes for both D-AKAP1 and R^I 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. 9*A*). 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 $R^I\alpha$ on the adjacent embryonic section. Riboprobes were made from residues 18–169 of $R^I\alpha$, including the N-terminal region and the beginning of the first cAMP binding site. This fragment of R^I was used because it has the least similarity in DNA sequence to R^{II}. When comparing the *in situ* patterns, overlapping expression for D-AKAP1 and $R^I\alpha$ 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 R^I 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
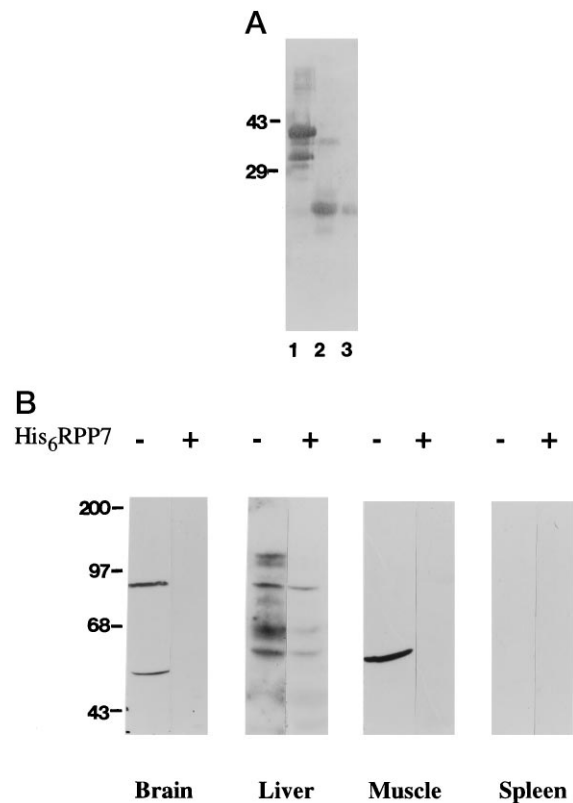
FIG. 8. **D-AKAP1 protein is present in brain, liver, and muscle.** *A*, affinity-purified His_6RPP7 antibodies specifically recognize bacterial lysates containing 0.2 μg of GST-RPP7 (*lane 1*), His_6RPP7 (*lane 2*), or 0.2 μg of purified His6RPP7. Signals were visualized with a 1:10,000 dilution of 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-His_6RPP7 antibodies. Enhanced chemiluminescence was used for visualization of the signals. Samples that were preincubated with purified His6RPP7, and the primary antibodies are indicated (+).

identified as a fragment from a yeast-two hybrid screen based on specific interaction with the R^I portion of Ret/ptc2. As demonstrated in an affinity precipitation assay, this fragment, RPP7, includes most, if not all, of the R^I/R^{II} 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 R^I and R^{II} 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}. Since the N-terminal dimerization domain is also proposed to be a key requirement for interaction between other AKAPs and R^{II} (9, 25, 32), the amino acid sequences at the N terminus of R^I and R^{II} were aligned to identify conserved residues. When aligned, the N-terminal regions of R^I and R^{II} show the least sequence identity. However, conserved Leu^{29} and Phe^{52} on R^I were identified at positions equivalent to Leu^{13} and Phe^{36} of R^{II}. Substitution of Ala for these two residues generates monomeric $R^{II}\beta$ subunits that cannot bind AKAP75 (32). Leu^{36} in R^I is located at a position that is equivalent to Val^{20} in R^{II}. An $R^{II}\beta$ mutant where Val^{20}-Leu^{21} were replaced by Ala-Ala still dimerized but was unable to bind to AKAPs (32). Whether mutations of these corresponding residues on R^I will disrupt dimerization and/or interaction with D-AKAP1 is still under investigation.
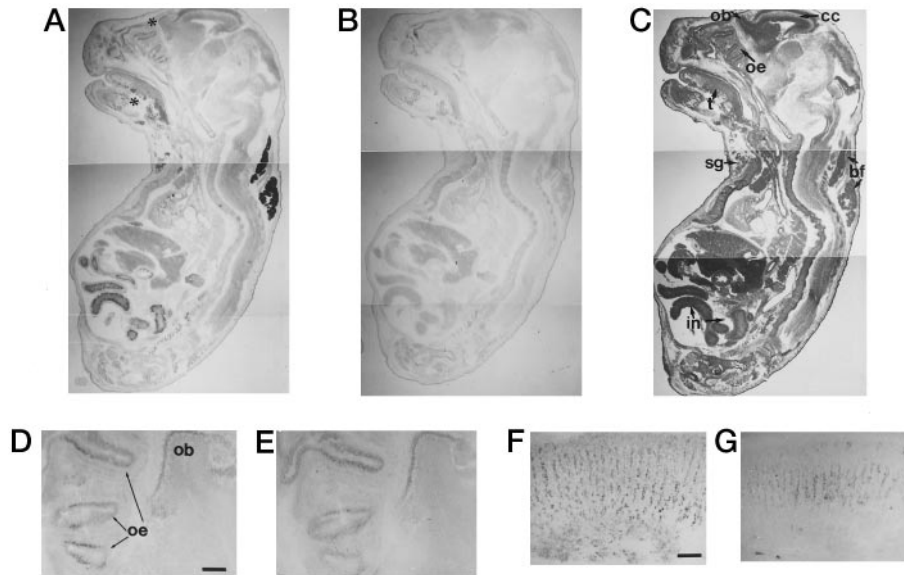
FIG. 9. **In situ hybridization pattern of D-AKAP1, which shows overlapping regions for $R^I\alpha$.** RNA probes of D-AKAP1 were derived from the cDNA sequence of isoform D-AKAP1c. $R^I\alpha$ probes were derived from residues 18–169. Hybridization was carried out on adjacent 20-μm cryostat sections 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. *ob*, olfactory bulb; *oe*, olfactory epithelium; *cc*, cerebral cortex; *t*, tongue; *sg*, submandibular gland; *bf*, brown fat; *in*, intestine. *D* and *E*, messages of D-AKAP1 and $R^I\alpha$, respectively, at the olfactory bulb and olfactory epithelium region. *Bar*, 200 μm. *F* and *G*, messages of D-AKAP1 and $R^I\alpha$, 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*).

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DAK1 MAIQLRSPFPLALPGMLALLGWWWFFSRKKDRLSSSDKQ-VET----LKVGPAIKDRRLSE
AK84 MAIQFRSLFPLALPGMLALLGWWWFFSRKKGHVSSHDEQQVEAGAVQVRADPAIKEPLPVE

DAK1 EACPGVLSVAPTVTQPPGREEQRSVDKPSTEPLALPRTRQVRRRSESSGNLPSVADTRSQP
AK84 DVCPKVVSTPPSVTEPPEKE-LSTVSKLPAEPPALLHPHPPCRRSESSGILPNTTDMRLRP

DAK1 GPCRDEIAKVELSLMGDKAKSIPLGCPLL-PKDASFPYEAVERCKQESALGKTPGRGWPSP
AK84 GTRRDDSTKLELALTGGEAKSIPLECPLSSPKBVLFSSKSAEVCKQDSPFSRVPRKVQPGY

DAK1 YAA------SGEKARETGGTEGTGDAVLGENVSEEGLLSQECVSEVEKIEFPILAPGGGEG
AK84 PVVPAEKRSSGERARETGGAEGTGDAVLGEKVLEEALLSREHVLELENSKGPSLASLEGEE

DAK1 EEVSHGPPQVAELLKKEEYIVGKLPSSFVEPVHSEPVKDEDALEPQV--------------
AK84 DKGKSSSSQVVGPVQEEEYVAEKLPSRFIESAHTELAKDDAAPAPPVADAKAQDRGVEGEL

DAK1 --KGSS--NTSDRDLAGE--------LDKDETVPE-NDQIKQAAFQLISQVILEATEELRA
AK84 GNEESLDRNEEGLDRNEEGLDRNEESLDRNEEGLDRNEEIKRAAFQIISQVISEATEQVLA
             •        •        •        •      •

DAK1 TTVGKTVAQVHPISATQPKG-KEESCVPASQETSLGQDTSDPASTRTGATAS---------
AK84 TTVGKVAGRVCQ--ASQLQGQKEESCVPVHQKTVLGPDTAEPATAEAAVAPPDAGLPLPGL

DAK1 PSAEALPPKTYVSCLSSPLSGPTKDQKPKNSAHHISLAPCPPPVTPQRQSLEGASNPRGDD
AK84 PAEGSPPPKTYVSCLKSLLSSPTKDSKPNISAHHISLASCLALTTPSEELPDRAGILVEDA

DAK1 NFVACMANNSQSVLSVSSLGQCSDPVSTSGLEDSCTETISSSGDKAITPPLPVSTQPFSNG
AK84 TCVTCMSDSSQSVPLVASPGHCSDSFSTSGLEDSCTETSSSPRDKAITPPLPESTVPFSNG

DAK1 VLKEELSDLGTEDGWTMDTEADHSGGS
AK84 VLKGELSDLGAEDGWTMDAEADHSGVA
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Since binding competition between R^I and R^{II} showed that R^{II} can compete with R^I and is actually preferentially bound by GST-RPP7, the regions on RPP7 that are responsible for binding R^I or R^{II} are likely to be partially, if not completely, overlapping. However, we cannot rule out the possibility that R^I and R^{II} bind at distinct but interacting sites. Although D-AKAP1 preferentially binds R^{II} *in vitro*, since R^I and R^{II} differ in their tissue expression, subcellular localization, and temporal expression during development, the actual microenvironment for D-AKAP1 with respect to R^I and/or R^{II} is not understood at this point. These results raise the possibility that, in addition to R^{II}, R^I 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

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 indicated these S-AKAP84 RNA transcripts may arise from a single gene. It is therefore likely that the different isoforms of D-AKAP1 emerged from splicing variants as well. Interestingly, however, S-AKAP84 was not found to interact with R^I. Amino acid sequence comparison between the two proteins revealed the greatest similarity in the core section of the protein, which includes a mitochondria target/signal region at the N terminus and an R^{II} binding domain, as described by Rubin and co-workers (33) (Fig. 10). The putative R^{II} 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 R^{II} binding domain is indeed the R^I and/or R^{II} 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 Schwoch *et al.* (34) also documented that R^I 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 R^I and D-AKAP1 in embryonic day 16 mouse embryos. These data showed that R^I 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 $R^I\alpha$. 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 R^{II}, R^I can potentially be specifically anchored at various subcellular locations via AKAPs.

Note Added in Proof—During the review of our manuscript, Trendelenburg and co-workers published a report of a related protein that has a C2 splice similar to D-AKAP1 (Trendelenburg, G., Hummel, M., Riecken, E., and Hanski, 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.

REFERENCES

1. Scott, J. D., and McCartney, S. (1994) *Mol. Endocrinol.* **8,** 5–11
2. Keller, B. U., Hollmann, M., Heinemann, S., and Konnerth, A. (1992) *EMBO J.* **11,** 891–896
3. Montminy, M. R., Gonzalez, G. A., and Yamamoto, K. K. (1990) *Trends Neurosci.* **13,** 184–188
4. Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971) *J. Biol. Chem.* **246,** 1986–1995
5. Deviller, P., Vallier, P., Bata, J., and Saez, J. M. (1984) *Mol. Cell. Endocrinol.* **38,** 21–30
6. Wen, W., Harootunian, A. T., Adams, S. R., Feramisco, J., Tsien, R. Y., Meinkoth, J. L., and Taylor, S. S. (1994) *J. Biol. Chem.* **269,** 32214–32220
7. Scott, J. D., and Carr, D. W. (1992) *News Physiol. Sci.* **7,** 143–148
8. Harper, J. F., Haddox, M. K., Johanson, R., Hanley, R. M., and Steiner, A. L. (1985) *Vitam. Horm.* **42,** 197–252
9. Hausken, Z. E., Coghlan, V. M., Hastings, C. A. S., Reimann, E. M., and Scott, J. D. (1994) *J. Biol. Chem.* **269,** 24245–24251
10. Leiser, M., Rubin, C. S., and Erlichman, J. (1986) *J. Biol. Chem.* **261,** 1904–1908
11. Coghlan, V., Perrino, B. A., Howard, M., Langeberg, L. K., Hicks, J. B., Gallatin, W. H., and Scott, J. D. (1995) *Science* **267,** 108–111
12. Klanck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S., and Scott, J. D. (1996) *Science* **271,** 1589–1592
13. Faux, M. C., and Scott, J. D. (1996) *Cell* **85,** 9–12
14. Rubin, C. S., Erlichman, J., and Rosen, O. M. (1972) *J. Biol. Chem.* **247,** 6135–6139
15. Skalhegg, B. S., Tasken, K., Hansson, V., Huitfeldt, H. S., Jahnsen, T., and Lea, T. (1994) *Science* **263,** 84–87
16. Vojtek, A. B., Hollenburg, S. M., and Cooper, J. A. (1993) *Cell* **74,** 205–214
17. Luo, Z., Shafit-Zagardo, B., and Erlichman, J. (1990) *J. Biol. Chem.* **265,** 21804–21810
18. Durick, K., Wu, R. Y., Gill, G. N., and Taylor, S. S. (1996) *J. Biol. Chem.* **271,** 12691–12694
19. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
20. Adams, J. A., McGlone, M. L., Gibson, R., and Taylor, S. S. (1995) *Biochemistry* **34,** 2447–2454
21. Saraswat, L. D., Filutowics, M., and Taylor, S. (1988) *Methods Enzymol.* **159,** 325–336
22. Nagai, K., and Thogersen, H. C. (1984) *Nature* **309,** 810–812
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685
24. Chun, J. M., Schatz, D. G., Oettinger, M. A., Jaenisch, R., and Baltimore, D. (1991) *Cell* **64,** 189–200
25. Scott, J. D., Stofko, R. E., McDonald, J. R., Comer, J. D., Vitalis, E. A., and Mangili, J. A. (1990) *J. Biol. Chem.* **265,** 21561–21566
26. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Bishop, S. M., Acott, T. S., Brennan, R. G., and Scott, J. D. (1991) *J. Biol. Chem.* **266,** 14188–14192
27. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8148
28. Glantz, S. B., Amat, J. A., and Rubin, C. S. (1992) *Mol. Biol. Cell* **3,** 1215–1228
29. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Cone, R. D., and Scott, J. D. (1992) *J. Biol. Chem.* **267,** 16816–16823
30. Carr, D. W., Hausken, Z. E., Fraser, I. D. C., Stofko-Hahn, R. E., and Scott, J. D. (1992) *J. Biol. Chem.* **267,** 13376–13382
31. Coghlan, V. M., Langeberg, L. K., Fernandez, A., Lamb, N. J. C., and Scott, J. D. (1994) *J. Biol. Chem.* **269,** 7658–7665
32. Li, Y., and Rubin, C. S. (1995) *J. Biol. Chem.* **270,** 1935–1944
33. Lin, R.-Y., Moss, S. B., and Rubin, C. S. (1995) *J. Biol. Chem.* **270,** 27804–27811
34. Schwoch, G., Trinczek, B., and Bode, C. (1990) *Biochem. J.* **270,** 181–188
35. Boekhoff, I., and Breer, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 471–474
36. Heldman, J., and Lancet, D. (1986) *J. Neurochem.* **47,** 1527–1533
37. Boekhoff, I., Schleicher, S., Strotmann, J., and Breer, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11983–11987