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Cloning and Characterization of A-kinase Anchor Protein 100 (AKAP100)

A PROTEIN THAT TARGETS A-KINASE TO THE SARCOPLASMIC RETICULUM*

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Differential localization of the type II cAMP-dependent protein kinase (PKA) is achieved by interaction of the regulatory subunit (RII) with A-kinase anchor proteins (AKAPs). Anchoring is a likely means to adapt PKA for regulation of cAMP-responsive events through colocalization of the kinase with preferred substrates. Using an interaction cloning strategy with an RIIα protein probe, we have identified a 655-amino acid protein (named AKAP100). Recombinant AKAP100, expressed in *Escherichia coli*, binds RIIα in a solid-phase overlay assay. The cellular and subcellular distribution of AKAP100 was analyzed by various methods. Northern blot analysis with the AKAP100 cDNA as a probe detected an 8-kilobase message in some human tissues including various brain regions; however, the message was predominately expressed in cardiac and skeletal muscle. Anti-AKAP100 antibodies confirmed expression in the rat cardiac and skeletal muscle cell lines, H9c2 and L6P, whereas immunohistochemical analysis revealed that AKAP100 was localized to the sarcoplasmic reticulum of both cell types. RII was also detected in these regions. AKAP100 was detected in preparations of RII purified from L6P cell extracts by cAMP-agarose affinity chromatography. Collectively, these results suggest that AKAP100 functions to maintain the type II PKA at the sarcoplasmic reticulum.

Considerable research has focused on determining the role of cAMP in signal transduction since it was discovered as an intracellular second messenger of hormone action (1–2). Cyclic AMP is released from distinct locations at the cell membrane by hormonal activation of adenylate cyclase and diffuses or is transported to its site of action. The predominant effect of cAMP is to activate a cAMP-dependent protein kinase (PKA).[1] Four molecules of cAMP bind each dormant PKA holoenzyme, activating the kinase by releasing the catalytic (C) subunits from the regulatory (R) subunit-cAMP complex (2). Active PKA potentiates the hormonal response by phosphorylating key enzymes and structural proteins, thus modifying their function. Although the mechanics of PKA activation are well understood, it is still unclear how individual hormones that utilize this ubiquitous cAMP signaling pathway are able to exert localized and specific effects (3–5). To accommodate these pleotrophic properties, PKA must be localized in a microenvironment with the adenylate cyclase complex or in a cellular microcompartment that preferentially concentrates cAMP. Therefore, selective activation of compartmentalized PKA is a plausible mechanism to ensure the selectivity of a hormonal response, and, accordingly, the cellular location of the kinase must be highly regulated (3, 6).

The cellular location of PKA is dictated by the regulatory (R) subunit (7). Two classes of R subunit exist, RI and RII, which form the type I and type II PKA holoenzymes, respectively (8, 9). The RI isoforms (RIα and RIβ) are thought to be primarily cytoplasmic, although RI is membrane bound in erythrocytes (10) and may associate with the T-cell receptor in activated lymphocytes (11). In contrast, a significant proportion of the RII isoforms (RIIα and RIIβ) are particulate, and up to 75% of the cellular RII pool associates with the plasma membrane, cytoskeletal components, endoplasmic reticulum, secretory granules, or nuclei (9, 12–16). The subcellular localization of RII is maintained through high affinity interaction with a family of adapter proteins called A-kinase anchor proteins (AKAPs) (12, 17–19). RII dimerization is required for AKAP binding (20–21). More recently, the first 5 amino acids of each RII protomer have been shown to be critical for AKAP interaction, specifically isoleucines at position 3 and 5 (22). The acceptor site on the AKAPs for PKA is more defined; anchoring proteins contain a conserved amphipathic helix of 14–18 amino acids, which must be maintained in the correct conformation to permit binding to the kinase (19, 23, 24). Synthetic peptides patterned after the amphipathic helix bind RII and the type II PKA holoenzyme with nanomolar affinity and can block interaction with AKAPs (24). These "anchoring inhibitor peptides" have been used to disrupt the RII-AKAP interaction in neurons and uncouple the regulation of AMPA-kainate receptors by PKA (25). In accordance with the targeting subunit hypothesis (26), each AKAP must contain additional binding sites responsible for targeting to cellular structures and/or interaction with other proteins. In an effort to identify AKAP binding proteins, complementary DNAs that encode proteins that associate with AKAP79, a neuronal anchor protein (24), were isolated using the yeast two-hybrid system (27, 28). A cDNA for the phosphatase 2B, calcineurin, was isolated and is consistent with earlier observations that PKA and calcineurin copurify through a number of chromatography steps (29). Biochemical and immunological studies have confirmed that both PKA and calcineurin are targeted to subcellular sites by association with AKAP79, possibly to regulate the phosphorylation state of key neuronal substrates (28).

In this report, we describe the cloning and characterization

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U17195.

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAP, A-kinase anchor protein; bp, base pair(s); PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

of a novel AKAP, AKAP100, which is selectively expressed in the brain, cardiac, and skeletal muscle. Biochemical and immunochemical analyses suggest that AKAP100 and RII associate *in vivo* at the sarcoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Cloning Strategy—Human cDNA expression libraries were screened by direct overlay with ^{32}P-labeled RIIα as a probe with modifications to the method of Lohmann *et al.* (17) as described in Ref. 18. Plaques were lifted onto nitrocellulose filters as described (30). Phosphorylation of RIIα by the C subunit of PKA was performed with [γ-^{32}P]ATP as described (31). All sequencing reactions were performed by the dideoxy chain termination method of Sanger *et al.* (32).

RNA Analysis—Filters containing immobilized samples of mRNAs of selected human tissues (Clontech) and of human brain regions (provided by Dr. Jeff Arriza, Vollum Institute, Portland, OR) were probed with a ^{32}P-radiolabeled 1400-base pair fragment encompassing the 3′-coding region of the AKAP100 cDNA. Radiolabeling of the AKAP100 cDNA probe was achieved by the random priming method described in Ref. 33 using [α-^{32}P]dCTP. Nitrocellulose filters were prehybridized in 400 mM sodium phosphate, pH 6.6, 1 mM EDTA, 5% SDS, 1 mg/ml BSA, 50% formamide for 2 h at 42 °C. The radiolabeled cDNA probe was denatured by heating at 100 °C for 10 min and then added directly to the nitrocellulose filters. Hybridization was performed in the same buffer at 42 °C overnight with gentle agitation. Non-hybridized probe was removed by washing (three times) in excess 0.1 × SSC, 0.1% SDS, and 1 mM EDTA at 53 °C for a total of 2 h. ^{32}P-Radiolabeled β-actin cDNA was used to probe both of these blots under similar conditions. Hybridizing mRNA species were detected by autoradiography.

Expression of Recombinant AKAP100—Construction of the AKAP100 expression vector pET11d AKAP100kfc was performed by simultaneous ligation of two DNA fragments into the bacterial expression vector pET11dkfc (34). Initially, a 1398-bp *Eco*RI-*Bam*HI fragment was excised from the original cDNA clone. Second, an 821-base pair fragment encompassing the 5′-end of the AKAP100 coding region was amplified by polymerase chain reaction. Primers were designed to create an *Nco*I site at the 5′-end (5′-TCCCAAA**CCATGG**CCTTTACTGGCAG-3′) and the T3 site at the 3′-end (5′-ATTAACCCTCACTAAAG-3′). Digestion of the polymerase chain reaction product with *Nco*I and *Eco*RI removed a 597-bp fragment. The expression plasmid pET11d AKAP100kfc was constructed by ligating the 1398-bp *Eco*RI-*Bam*HI and 597-bp *Nco*I-*Eco*RI fragments into an *Nco*I-*Bam*HI cut vector. The fidelity of the AKAP100 coding region was confirmed by nucleotide sequencing. Expression of AKAP100kfc protein was achieved in *Escherichia coli* pLysS cells as described (35). Soluble Kfc protein was purified by affinity chromatography on calmodulin-agarose as described (34). Protein was concentrated by ultrafiltration (Amicon).

RII Overlays—Overlay assays were performed by the method of Lohmann *et al.* (18), and quantitative overlays were performed as described (22). Binding was detected by autoradiography and was measured by densitometry after scanning into a computer and analyzed by the National Institutes of Health Image 1.55 program. In control experiments, ^{32}P-labeled RII probe was pre-incubated with Ht31-(493–515) anchoring inhibitor peptide (0.5 mM) as described (36).

Western Blot Analysis—Proteins were separated by SDS-PAGE as described (37, 38) and electrotransferred to polyvinylidene difluoride membrane (Immobilon, Millipore). Rabbit polyclonal antibodies to AKAP100 (produced by Bethyl Laboratories, Inc., Montgomery, TX) were affinity purified using AKAP100 protein coupled to Affi-Gel 15 (Bio-Rad) and used at 1:250 dilution. Anti-RII antibodies, raised in rabbits (36), were affinity purified and used at 1:5000 dilution.

Preparation of H9c2 and L6P Cell Extracts—Rat cardiac muscle and skeletal muscle cell lines H9c2 and L6P were grown in 75-cm^2 tissue culture flasks (NUNC) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum until confluent. The cells were washed three times with phosphate-buffered saline (PBS), pH 7.4, and scraped from the tissue culture flasks. The cell pellet was concentrated by centrifugation at 3000 × *g* for 5 min at 4 °C, resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100 0.05 mM dithiothreitol, 0.5 mM $MgCl_2$, 0.125 mM EDTA, 0.1 μM leupeptin, 0.1 μM pepstatin, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride), and sonicated for 30 s (three bursts of 10 s) on ice. After sonication, the cell suspension was centrifuged at 14,000 × *g* for 20 min at 4 °C, and the pellet was resuspended in buffer A. Protein concentrations of the supernatant and pellet fractions were measured by Bradford assay (Pierce). Equal concentrations of protein (100 μg), unless otherwise stated, were loaded onto SDS-PAGE gels for subsequent analyses.

Immunocytochemistry—Immunofluorescence studies were performed on rat cardiac muscle H9c2 cells grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum on glass coverslips in 6-well tissue culture (NUNC) plates for 72 h at 37 °C. Cells were fixed in 3.7% formalin in PBS, pH 7.4, extracted in absolute acetone at −20 °C, and rehydrated in PBS + 0.1% BSA before incubation with affinity-purified primary antibodies (1:100 dilution anti-goat RII and 1:250 dilution AKAP100) for 1 h at room temperature. Control experiments with antibody to signal sequence receptor (1:50 dilution, kindly provided by Dr. T. A. Rapoport, Max-Delbruck center for Molecular Medicine, Germany) and preimmune sera were performed under identical conditions. Cells were washed with excess PBS + 0.1% BSA (3 times) before incubation with secondary antibodies (1:100) for 1 h at room temperature. RII was detected with fluorescein isothiocyanate-conjugated donkey anti-goat IgG, and AKAP100 was detected with Texas Red donkey anti-rabbit IgG. Unbound secondary antibodies were removed by washing with excess PBS + 0.1% BSA (three times). Coverslips were mounted with Vectashield™ mounting medium. Staining patterns were observed on a Leitz Fluovert FU confocal photomicroscope under oil immersion using a 63/1.4 OEL PL APO lens.

Purification of the RII-AKAP100 Complex by cAMP-Agarose—RII-AKAP complexes were isolated from rat skeletal muscle cell extracts as previously described (36). Unbound protein was removed from the cAMP-agarose by washing with hypotonic buffer plus 1 M NaCl and four additional washes with hypotonic buffer. Finally, specifically bound proteins were eluted from the affinity resin by incubation at room temperature with hypotonic buffer containing 75 mM cAMP.

Other Methods—Antibodies against AKAP100 peptide and recombinant AKAP100 were produced by Bethyl Laboratories, Inc. (Montgomery, TX). Oligonucleotide sequencing primers were purchased from the Center for Gene Research and Biotechnology, Oregon Science University (Corvallis, OR).

RESULTS

Cloning of AKAP100—Clones encoding AKAPs were isolated from a human hippocampal cDNA expression library by the RII overlay method using RIIα as a probe. One positive clone was isolated from ~250,000 recombinants. This clone was called hhBD-1 (1888 bp) and contained a partial open reading frame of 599 amino acids (Fig. 1*A*). When Northern blots were screened with a hhBD-1 probe, the only mRNA species detected in human tissues was 8 kilobases, confirming that hhBD-1 was a partial fragment of the full-length cDNA. The human hippocampal cDNA library was rescreened with a 1400-bp *Hin*dIII-*Bam*HI fragment excised from the 5′-end of hhBD-1, but this did not yield clones with any additional information. However, a random and oligo(dT)-primed human fetal brain cDNA library (provided by Dr. W. Michael Gallatin, ICOS Corp., Seattle, WA) was screened with the same probe, and 12 positive clones were identified from ~500,000 recombinants. DNA from seven of the positive clones was sequenced and indicated that one clone, called hFB 7–1 (1314 bp), overlapped with hhBD-1 and yielded 822 bp of additional 5′-sequence. A composite sequence of 2595 is presented in Fig. 1*A* and contains an open reading frame encoding a 655-amino acid protein with a predicted M_r 78,172. However, the protein migrates on SDS-polyacrylamide gels with an apparent molecular mass of 100 kDa (see below); thus, in accordance with the nomenclature proposed for RII anchoring proteins by Hirsch and colleagues (39), we have named the protein AKAP100.

Comparison of the AKAP100 sequence to the DNA and protein data base did not identify any overall similarity to other known proteins. Computer-aided analysis of the AKAP100 sequence identified a putative amphipathic α-helix located between residues 392 and 408 (Fig. 1*B*). This region is compared with the RII-binding regions of other AKAPs (Fig. 1*C*). Both of these findings are consistent with the notion that residues 392–408 of AKAP100 form the RII-binding domain.

The Tissue Distribution of AKAP100 mRNA—To establish the tissue expression pattern of AKAP100, filters of immobilized poly(A)$^+$ RNA from several human tissues were screened

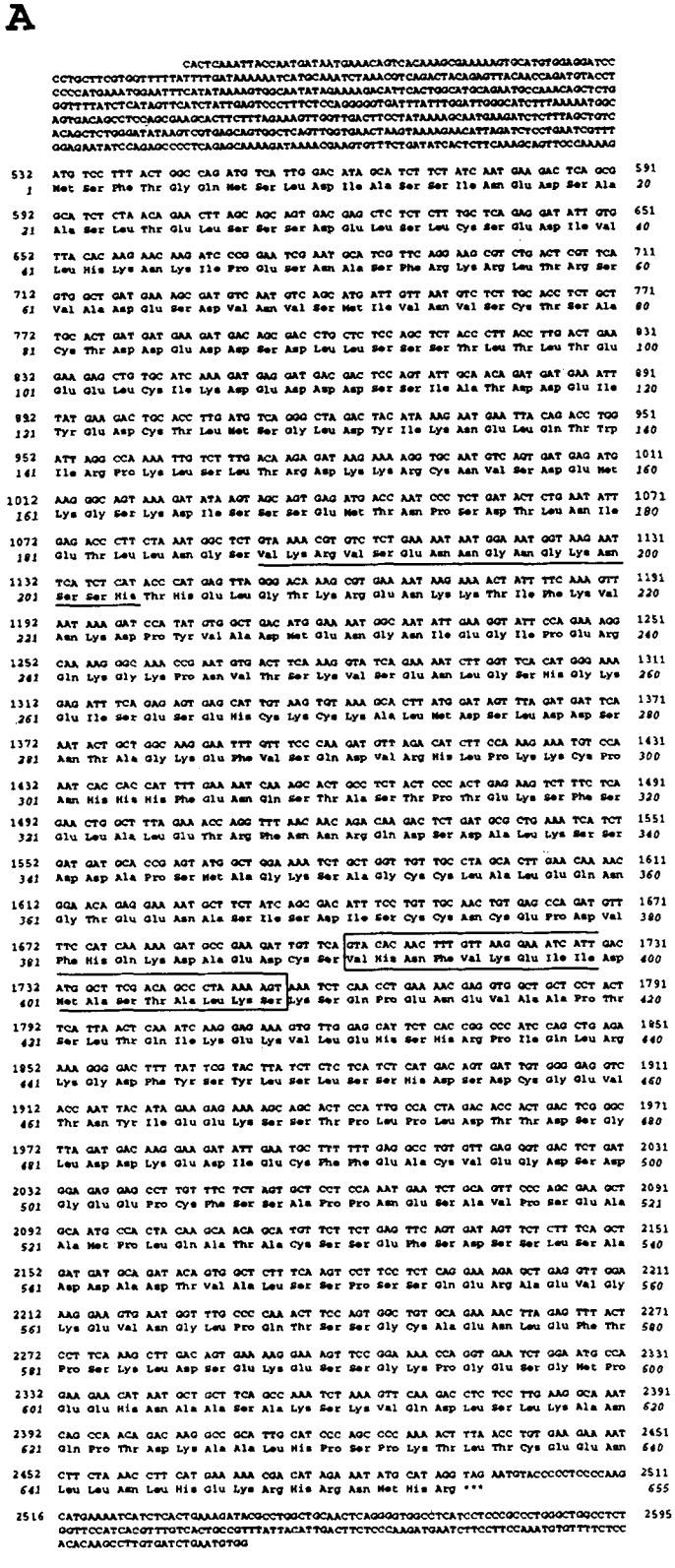

FIG. 1. **Sequence of AKAP100.** *A*, the nucleotide sequence (*upper line*) and deduced amino acid sequence (*lower line*) of a cDNA encoding the protein kinase A anchor protein AKAP100. The *boxed region* indicates the putative RII-binding region, and a sequence used to raise peptide antisera is *underlined*. *B*, sequence homology between AKAP100 (residues 396–411) and the RII-binding regions of three other AKAPs, MAP2, AKAP150, and Ht31. *Boxed regions* show amino acid identity, and conservative changes (*) are indicated. *C*, helical wheel representation of AKAP100 (residues 392–408) drawn as a α-helix of 3.6 amino acids/turn. The *shaded* area indicates hydrophobic residues, and the *open* area indicates hydrophilic residues. Amino acids are indicated in the one-letter code. The *arrow* indicates the direction of the helix.

with a radiolabeled 1400-bp *Hin*dIII-*Bam*HI fragment excised from hhBD-1. A single mRNA species of 8 kilobases was detected in certain human tissues and a variety of brain regions (Fig. 2, *A* and *C*). The AKAP100 mRNA was most highly expressed in the heart and skeletal muscle (Fig. 2*A*). When the filters were probed with a β-actin probe, equal levels of mes-

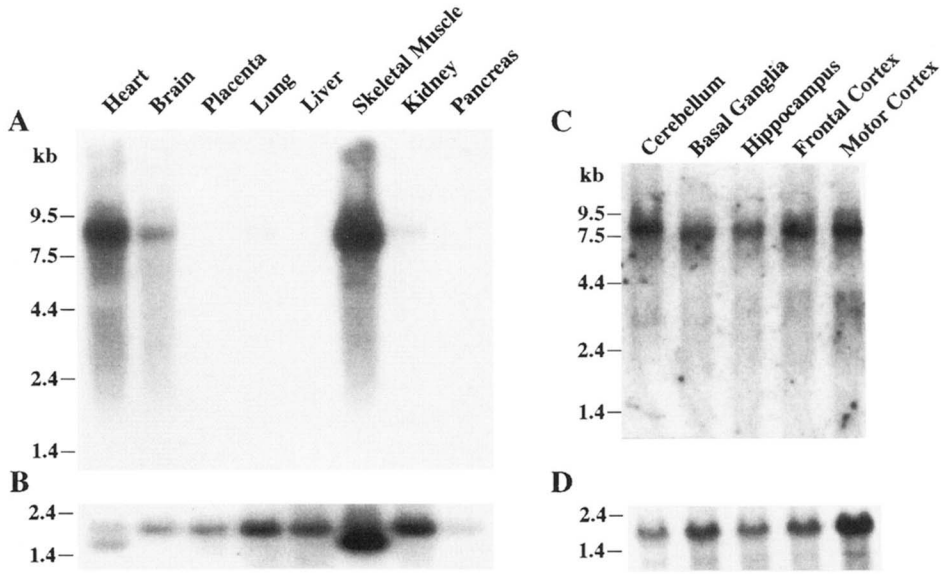

FIG. 2. **The tissue distribution of the AKAP100 mRNA.** 2 μg of poly(A)$^+$ RNA from human tissues (human MTN, Clontech) (*A*) and selected brain regions (5 μg) (*C*) on nitrocellulose were probed with a ^{32}P-radiolabeled 1400-bp *Hin*dIII-*Bam*HI fragment excised from the 5′-end of hhBD-1 as described under "Experimental Procedures." Human tissues (human MTN, Clontech) (*B*) and selected brain regions (*D*) were probed with ^{32}P-radiolabeled β-actin. Hybridizing mRNA species were detected by autoradiography. The tissue source of each RNA is indicated *above* each *lane*. *kb*, kilobases.

sage were detected in each lane (Fig. 2, *B* and *D*).

Expression and Characterization of AKAP100—To confirm that AKAP100 was an RII binding protein, the full-length cDNA was expressed in *E. coli* using the kfc fusion system (34) as described under "Experimental Procedures." A 115-kDa protein was detected by SDS-PAGE in bacterial extracts of induced cells and was purified to homogeneity by affinity chromatography on calmodulin-Sepharose (Fig. 3*A*). The expression of the recombinant fusion protein was monitored by Western blot (Fig. 3*A*) using anti-peptide antisera raised against residues 188–203 of AKAP100 (Val-Lys-Arg-Val-Ser-Glu-Asn-Asn-Gly-Asn-Gly-Lys-Asn-Ser-Ser-His). The recombinant protein bound ^{32}P-RII as assessed by a direct overlay (Fig. 3*C*). Solid-phase RII binding was blocked when overlay blots were incubated with the anchoring inhibitor Ht31-(493–515) peptide (Fig. 3*D*). This peptide has been previously shown to block RII-AKAP interaction (24, 36). Nonspecific binding to a band of 80 kDa was detected upon prolonged exposure of the control blot (Fig. 3*D*).

The binding affinity of AKAP100 was assessed by quantitative overlays using RIIα protein at a specific activity of 2.1–1.5 $\times$ 10^5 cpm/pmol. The binding to immobilized AKAP100 and Ht31 over a range of 0.01–10 nmol/100 μl was detected by autoradiography and was measured by densitometry. Both proteins bound RII with high affinity, with half-maximal binding values calculated at 10 μM for AKAP100 and 2.5 μM for Ht31 (Fig. 4). Collectively, these data are consistent with the notion that the AKAP100 cDNA encodes a high affinity RII binding protein.

The Subcellular Location of AKAP100—The tissue distribution of AKAP100 mRNA suggested that the protein may be predominately expressed in cardiac and skeletal muscle tissues. To test this hypothesis, affinity-purified polyclonal AKAP100 antisera, raised against purified recombinant AKAP100, was used to probe protein extracts from a variety of muscle cell lines including the rat cardiac muscle (H9c2) and rat skeletal muscle (L6P) cell lines. A single immunoreactive protein of 100 kDa was detected by Western blot for both H9c2 (Fig. 5, *lane 2*) and L6P (see below) cells. There was no immunoreactivity when identical blots were probed with preimmune serum (Fig. 5, *lane 3*). Numerous RII binding proteins ranging

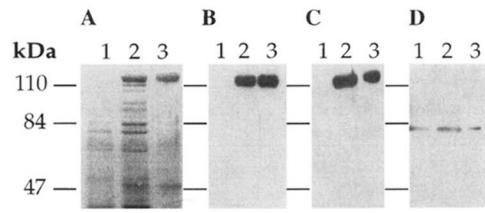

FIG. 3. **Recombinant AKAP100 binds RIIα.** The entire coding region of AKAP100 was expressed using the pET11dkfc plasmid. Expression of recombinant protein was induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside to growing bacterial cultures. Bacterial extracts (100 μg) or purified protein were separated by electrophoresis on 10% (w/v) SDS-polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes. Blots were stained with Coomassie Blue (*A*) and analyzed by autoradiography (*B–D*). *B*, AKAP100 was detected by Western blot with anti-peptide antibodies. RII binding proteins were detected by a solid-phase binding assay (17) using ^{32}P-radiolabeled RIIα as a probe in absence (*C*) or presence (*D*) of 1 μM anchoring inhibitor Ht31-(493–515) peptide. Sample sources, indicated *above* each *lane*, are uninduced bacterial lysate (*lane 1*), isopropyl-1-thio-β-D-galactopyranoside-induced bacterial lysate (*lane 2*), and affinity-purified protein (*lane 3*). Molecular mass markers are indicated on each *panel*.

in size from 250 to 40 kDa were detected in both cell lines by direct overlay (data not shown).

The subcellular location of AKAP100 was examined using indirect immunofluorescence techniques (Fig. 6). In quiescent H9c2 cells (Fig. 6*A*), AKAP100 staining was restricted to the perinuclear regions (Fig. 6*C*) and exhibited a similar staining pattern to the sarcoplasmic reticulum marker protein, signal sequence receptor (Fig. 6*B*). Conversely, the staining pattern of the Golgi marker protein, mannosidase II, was distinct from AKAP100 (data not shown). Double immunofluorescence staining for AKAP100 (Fig. 6*C*) and RII (Fig. 6*D*) suggested that both proteins had overlapping cellular distributions and were concentrated in the same cellular compartment. This observation was confirmed by confocal microscopy showing that AKAP100 and RII have overlapping staining patterns in 0.1-micron-thick focal sections of H9c2 cells (Fig. 6, *C* and *D*). Control experiments confirmed that no staining was observed with preimmune serum (Fig. 6, *E* and *F*) or secondary antibody alone (data not shown). These experiments are consistent with

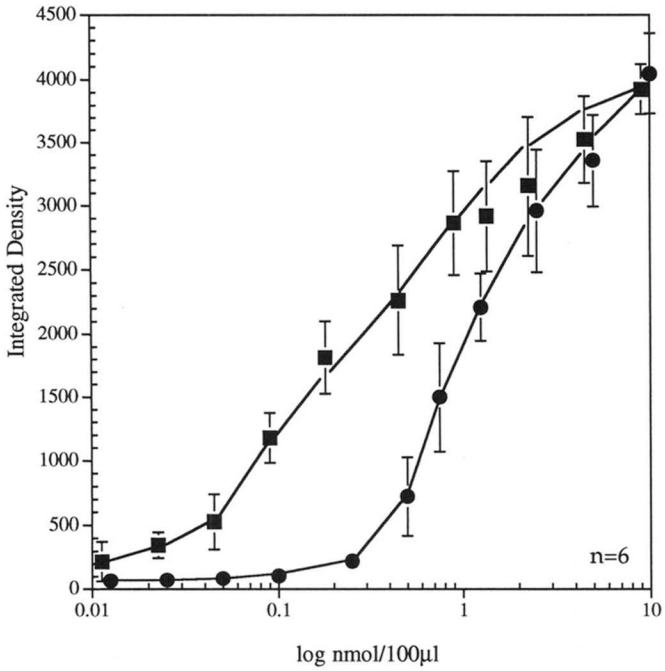

FIG. 4. **Quantitation of AKAP binding to RIIα.** The binding of ^{32}P-radiolabeled RIIα to AKAP100 and a fragment of the human thyroid anchoring protein Ht31 was measured by a quantitative overlay procedure. Aliquots of purified anchoring proteins, ranging from 0.01 to 10 nmol/100 µl were immobilized onto nitrocellulose filters. Individual filters were probed with excess ^{32}P-radiolabeled RIIα (specific activity ranging from 2.1 to 1.5 × 10^5 cpm/nmol). Unbound ^{32}P-radiolabeled RIIα was washed off with Tris-buffered saline (pH 7.4). Detection of binding was by autoradiography. Quantitation of binding over the range of AKAP concentrations was measured by densitometry of the autoradiographs. Signals were normalized for the specific activities of each RIIα probe. Binding curves for AKAP100 (○) and Ht31 (■) are presented from six experiments, and the standard deviation is indicated.

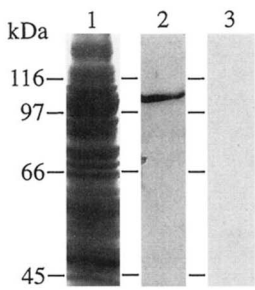

FIG. 5. **AKAP100 is present in an H9c2 cell lysate.** A solubilized extract (250 µg) of H9c2 cells was stained with Coomassie Blue (*lane 1*) or probed for AKAP100 (*lane 2*) using purified antibodies. Detection was by enhanced chemiluminescence. Preimmune sera is shown in *lane 3*. Molecular mass markers are indicated.

the localization of AKAP100 and a significant proportion of RII to the sarcoplasmic reticulum of H9c2 cells. An indistinguishable staining pattern for AKAP100, RII, and marker proteins was obtained when these experiments were repeated in L6P cells (data not shown).

Purification of the RII-AKAP100 Complex—To examine whether AKAP100 was associated with RII in cell lysates, we attempted to purify the RII-AKAP100 complex using affinity chromatography on cAMP-agarose. L6P cells were used for these experiments because they grow more rapidly and, thereby, provide more starting material. Western blot analysis of solubilized L6P cell lysates indicated that the lysates contained RII that was purified by affinity chromatography on cAMP-agarose (Fig. 7*A*). Identical blots probed with anti-AKAP100 antibodies indicated that a proportion of the AKAP100 present in the lysate copurified with RII (Fig. 7*B*). These results provide evidence that RII-AKAP100 complexes exist and can be purified from cell lysates.

DISCUSSION

In this report, we describe the cloning and characterization of a novel A-kinase anchor protein, AKAP100, which is specifically localized to the sarcoplasmic reticulum. AKAP100 is selectively expressed in certain tissues, and high levels of the 8-kilobase message are detected in various brain regions and cardiac and skeletal muscle. This finding is supported by cloning studies that isolated the original cDNA fragment from a human hippocampal library. The calculated molecular weight of AKAP100 is 78,172, although the protein migrates with a mobility of M_r 100,000 on SDS-polyacrylamide gels. Apparently, anomalous migration on SDS-PAGE gels is a characteristic of several AKAPs. For example, the bovine neural-specific anchoring protein, AKAP75, has a calculated molecular weight of 47,085 but migrates with an apparent mobility of 75 kDa on SDS-PAGE gels (24). This has led Hircsh and colleagues (39) to suggest that the abundance of acidic residues in the amino-terminal portion of the protein alters its migration pattern. Consistent with this hypothesis, AKAP100 also contains stretches of acidic side chains between residues 80 and 110 of the protein.

A growing body of evidence suggests that AKAPs contain a conserved RII-binding site responsible for interaction with PKA. Recently, we have shown that RII binding proceeds through sites in the extreme amino terminus of RII, and isoleucines 3 and 5 on each RII protomer are required for interaction with an amphipathic helix on the surface of the AKAP (22). Site-directed mutagenesis studies have demonstrated that an intact α-helical structure is required for RII-AKAP interaction (23), and synthetic peptides encompassing the amphipathic helix regions of three AKAPs (Ht31, AKAP79, and AKAP95) block RII-AKAP interaction *in vitro* (19, 24, 36). On the basis of these observations, it seems likely that residues 392–408 may form the RII-binding site on AKAP100. This region exhibits a high probability of amphipathic α-helix formation and shares 30–40% sequence identity with the RII-binding regions of other AKAPs (Fig. 1*B*); deletion constructs lacking residues 392–410 are unable to bind RII by the overlay assay.[2] Moreover, the anchoring inhibitor peptide, Ht31-(393–415), effectively competes with AKAP100 for RII binding in direct overlays.

It would also appear that AKAP100 binds RIIα with high affinity, as half-maximal binding to the immobilized anchoring protein was measured in the micromolar range. Although it is technically infeasible to measure precise binding constants due to aggregation of AKAP100, it would appear that the intracellular levels of AKAP100 and RII (assessed by Western blotting) are within a concentration range sufficient to permit complex formation *in vivo*. This notion is supported by our evidence that AKAP100 is copurified with RII from L6P cell lysates by affinity chromatography on cAMP-agarose. Since recombinant AKAP100 does not display any intrinsic cAMP binding affinity, it is therefore likely that the anchoring protein was purified as complex with RII. In addition, both RII and AKAP100 were detected in the flow-through from the cAMP-agarose (Fig. 7). There are two potential explanations for this observation: the amount of RII in the cell lysate was in excess of the capacity of the cAMP-agarose preventing retention of all RII complexed with AKAP and an undetermined proportion of AKAP100 was not associated with RII. This observation is consistent with

[2] B. M. Little and J. D. Scott, unpublished observation.

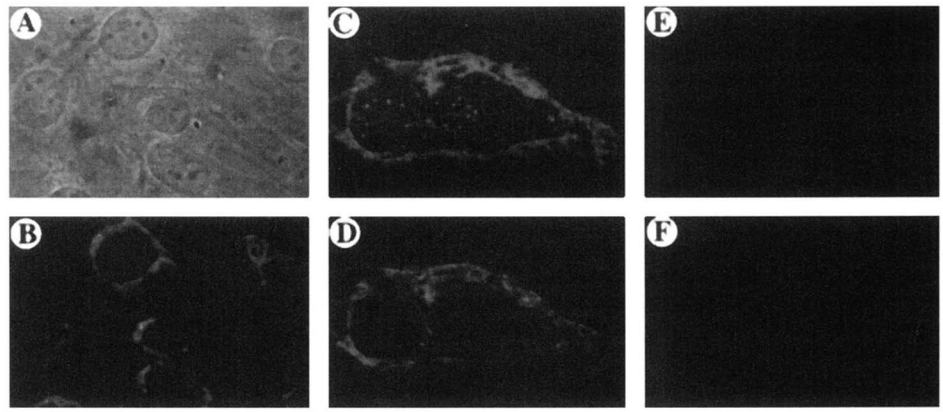

FIG. 6. **Immunocytochemical analysis of rat cardiac muscle cell line (H9c2).** H9c2 cells were formalin-fixed (*A*) and incubated with anti-sequence-specific receptor antibodies (*B*), anti-AKAP100 antibodies (*C*), anti-RII antibodies (*D*), or preimmune serum for AKAP100 (*E*) and for RII (*F*) under the conditions described under "Experimental Procedures." Fluorescein isothiocyanate-conjugated anti-rabbit secondary antiserum was used in *panels B*, *C*, and *E*). Texas red conjugated anti-goat secondary antiserum was used in *panels D* and *F*.

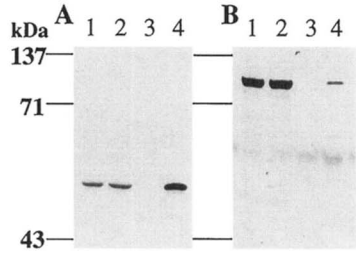

FIG. 7. **AKAP100 copurifies with RII on cAMP-agarose.** Detection of proteins on Western blots is shown for fractions isolated from cAMP-agarose affinity chromatography. Separate filters were probed for either RIIα (*A*) or AKAP100 (*B*) using purified antibodies and enhanced chemiluminescence detection. A solubilized extract of L6P cells (*lane 1*) was incubated with cAMP-agarose. Unbound protein (*lane 2*) was separated from the affinity matrix by centrifugation, and the pellet containing protein bound to cAMP-agarose was washed extensively in washing buffer. After a final wash (*lane 3*), proteins remaining bound to the affinity matrix were eluted in 75 mM cAMP (*lane 4*) and boiled in sample buffer. Fractions (100 μg, *lanes 1* and *2*; 10 μg, *lane 4*) were separated on 7.5% SDS-polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes. Molecular mass markers are indicated.

similar findings for AKAP79 and AKAP95 (24, 36), which indicate that a proportion of the RII-binding sites on anchoring proteins are unoccupied. Furthermore, our findings show that only a fraction of RII purified is associated with AKAP100 (Fig. 7*B*, *lane 4*). This is because the cAMP-agarose purification protocol isolates the total R subunit pool, which includes soluble RI, soluble RII, and RII associated with other AKAPs present in the L6P cell extract.

Previous studies have proposed that the type II PKA is associated with native sarcoplasmic reticulum vesicles (40). This finding is supported by our immunocytochemical data showing that AKAP100 and a significant proportion of the cellular RII pool appear to be localized at the sarcoplasmic reticulum. In light of both observations, we suggest that AKAP100 functions to adapt PKA for a role in the phosphorylation of proteins in or surrounding the sarcoplasmic reticulum. Although the precise identity of these target substrates remains to be determined, anchoring of kinases close to ion channels is an attractive hypothesis. Levitan and others (13, 40–44) have proposed that targeting of kinases and phosphatases could permit the precise regulation of ion channel phosphorylation status. This view is supported by evidence suggesting that anchoring of PKA by AKAPs is required for modulation of glutamate receptor ion channels. Intracellular perfusion of cultured hippocampal neurons with anchoring inhibitor peptides derived from the conserved kinase-binding domain of AKAP79 or Ht31 prevented PKA-mediated regulation of the AMPA-kainate currents (25). Therefore, the AKAP100-PKA complex might interact directly with ion channels or may be targeted to structural proteins within the sarcoplasmic reticulum. For example, the ryanodine receptor, which forms the intracellular Ca^{2+} release channel of the sarcoplasmic and endoplasmic reticulum, is activated by PKA (45), influencing Ca^{2+} mobilization within the cell (46, 47). Other reports have suggested PKA activity is closely associated with a reconstituted calcium-activated potassium channel (41) and a sarcoplasmic reticulum-associated chloride channel (40). Moreover, recent studies suggest that voltage-dependent potentiation of the L-type Ca^{2+}-gated channels in skeletal muscle requires anchored PKA. Catterall and colleagues (48) have shown that perfusion of the Ht31 anchoring inhibitor peptide prevents cAMP-responsive potentiation of the channel. In addition, AKAP100 may function to target other enzymes involved in signal transduction to the sarcoplasmic reticulum, as we have recently demonstrated that AKAP79 forms a ternary complex with PKA and the phosphatase 2B, calcineurin (28). These results suggest that both kinase and phosphatase are targeted to subcellular sites by association with a common anchor protein to regulate the phosphorylation state of key substrates.

In conclusion, these studies suggest that AKAP100 is a novel protein that binds RIIα with high affinity. The anchoring protein is selectively expressed in certain human tissues and has been localized to the sarcoplasmic reticulum in rat cardiac H9c2 cells. Current studies are focusing on identifying other proteins that interact with AKAP100 to establish those molecules responsible for targeting the entire complex to the sarcoplasmic reticulum.

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