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Cloning and Characterization of AKAP 95, a Nuclear Protein That Associates with the Regulatory Subunit of Type II cAMP-dependent Protein Kinase*

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The subcellular location of the type II cAMP-dependent protein kinase is dictated by the interaction of the regulatory subunit (RII) with A-kinase anchor proteins (AKAPs). Using an interaction cloning strategy with RIIα as a probe, we have isolated cDNAs encoding a novel 761-amino acid protein (named AKAP 95) that contains both RII- and DNA-binding domains. Deletion analysis and peptide studies revealed that the RII-binding domain of AKAP 95 is located between residues 642 and 659 and includes a predicted amphipathic helix. Zinc overlay and DNA binding studies suggest that the DNA-binding domain is composed of two CC/HH-type zinc fingers between residues 464 and 486 and residues 553 and 576. The AKAP was detected in a nuclear matrix fraction, and immunofluorescence using purified anti-AKAP 95 antibodies revealed a distinct nuclear staining in a variety of cell types. Direct overlay of fluorescein isothiocyanate-labeled RIIα onto fixed rat embryo fibroblasts showed that high-affinity binding sites for RII exist in the nucleus and that these sites are blocked by an anchoring inhibitor peptide. Furthermore, AKAP 95 was detected in preparations of RII that were purified from cellular extracts using cAMP-agarose. The results suggest that AKAP 95 could play a role in targeting type II cAMP-dependent protein kinase for cAMP-responsive nuclear events.

The action of many hormones and neurotransmitters requires the generation of cAMP as intracellular second messenger (Sutherland, 1972). Upon synthesis by adenylate cyclase, cAMP diffuses or is transported from the inner face of the plasma membrane to its site of action, where it activates the cAMP-dependent protein kinase (PKA).[1] Four molecules of cAMP bind each dormant PKA holoenzyme, activating the kinase by release of two catalytic (C) subunits from the regulatory (R) subunit-cAMP complex (Krebs and Beavo, 1979). Phosphorylation of target substrates by PKA potentiates the hormonal response by altering the biological activity of key enzymes and structural proteins. An apparent paradox is that PKA is a multifunctional kinase with broad substrate specificity, but triggers discrete physiological responses, even in the same cell (reviewed by Harper *et al.* (1985) and Scott (1991)). For example, phosphorylation of membrane-bound neurotransmitter receptor channels modulates the flow of ions into the cell (Wang *et al.*, 1991; Greengard *et al.*, 1991), while phosphorylation of nuclear transcription factors (*e.g.* cAMP response element-binding protein) alters the activity of certain genes (Meyer and Habener, 1993). One hypothesis to account for the selectivity of PKA action is that individual effectors activate particular pools of the kinase that are compartmentalized at intracellular sites close to preferred substrates (Harper *et al.*, 1985; Scott and Carr, 1992).

Compartmentalization of PKA is dictated by the R subunit (Rubin *et al.*, 1979). Two classes of R subunit exist (RI and RII), which form the type I and II PKA holoenzymes, respectively (Hofmann *et al.*, 1975; Corbin *et al.*, 1975). Type II PKA is present in all cells, whereas the tissue distribution of type I PKA is more restricted (Rubin *et al.*, 1972; Corbin *et al.*, 1978). The subcellular distributions of PKA isoforms also appear to be distinct. The RI isoforms (RIα and RIβ) are reported to be primarily cytoplasmic, while certain tissues contain up to 75% of RII isoforms (RIIα or RIIβ) in particulate form associated with the plasma membrane, cytoskeletal components, secretory granules, or nuclei (Corbin *et al.*, 1975; Leiser *et al.*, 1986; Salvatori *et al.*, 1990; Nigg *et al.*, 1985a, 1985b; Joachim and Schwoch, 1990). Type II PKA localization is directed by association of RII and specific A-kinase anchor proteins (AKAPs) (Lohmann *et al.*, 1984; Lieser *et al.*, 1986; Bregman *et al.*, 1989; Carr *et al.*, 1992a). Previous reports have shown that RII dimerization is required for anchoring protein interaction (Scott *et al.*, 1990) and that only the first 50 amino acids on RII are required for AKAP binding (Luo *et al.*, 1990), while studies with purified anchoring proteins and peptides have shown that acidic amphipathic helices form the high-affinity RII-binding domains (Carr *et al.*, 1991, 1992a, 1992b). In addition to binding RII, each anchoring protein contains regions that localize the AKAP (and presumably type II PKA) to specific subcellular structures. The intracellular targeting domain of MAP 2 has been defined as a COOH-terminal octadecapeptide repeat that localizes the protein to microtubules (Joly *et al.*, 1989), and it has recently been reported that the neuronal anchoring protein AKAP 75 contains two noncontiguous regions that localize AKAP 75 to the cytoskeleton (Glantz *et al.*, 1993).

In this report, we describe the cloning and characterization of a new AKAP (called AKAP 95) that contains two zinc finger

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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) U01914.

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; C, catalytic; R, regulatory; AKAP, A-kinase anchor protein; bp, base pair(s); HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; FITC, fluorescein isothiocyanate.

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domains and binds DNA. In all cell types examined, AKAP 95 is localized in the nucleus, where it appears to be associated with the nuclear matrix. The findings provide the first evidence of nuclear anchoring sites for the regulatory subunit of type II PKA.

MATERIALS AND METHODS

Cloning of AKAP cDNAs—A rat pituitary (GH_4C_1) cDNA λZap library (kindly provided by Dr. Thom Segerson, Vollum Institute, Portland, OR) was screened using a modified RII overlay procedure (Lohmann *et al.*, 1984). Plaques were blotted in duplicate to isopropyl-1-thio-β-D-galactopyranoside-soaked filters as described (Sambrook *et al.*, 1989). After blocking for 2 h at 25 °C in 5% milk and 0.1% bovine serum albumin in Tris-buffered saline, the filters were incubated with 0.2 μg/ml recombinant murine RIIα at 25 °C for 2 h. Following three washes in Tris-buffered saline, the filters were incubated with anti-RII P13 peptide antibodies (1:1000), washed, and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. Positive plaques were visualized by enhanced chemiluminescence (ECL, Amersham Corp.). Fifteen positive clones were identified from ~500,000 recombinants in the initial screening. Secondary and tertiary screens were performed by direct overlay with ^{32}P-labeled RIIα as described (Lohmann *et al.*, 1984). For DNA screening, the GH_4C_1 cDNA library and a random-primed rat olfactory bulb cDNA library were screened using a 620-bp *XhoI*/*EcoRI* fragment that was excised from the 5′-end of one of the GH_4C_1 clones and nick-translated as described (Sambrook *et al.*, 1989). The labeled 620-bp fragment was also used to probe a mouse mRNA Northern blot (multiple tissue Northern blot, Promega) at 42 °C in 50% formamide. All sequencing reactions were performed on both strands of DNA using 19–22-mer oligonucleotides (Operon Technologies, Inc.) and the dideoxy chain termination method of Sanger *et al.* (1977). Compressions were resolved by including 5-deaza-GTP in reactions with either Sequenase (at 37 °C; U. S. Biochemical Corp.) or *Taq* DNA polymerase (at 70 °C).

Expression in Escherichia coli—A 935-bp fragment of cDNA encompassing both zinc fingers and the putative amphipathic helix RII-binding domain of AKAP 95 was amplified by polymerase chain reaction. Primers were designed to create an *NcoI* site at the 5′-end and a *BglII* site at the 3′-end of the polymerase chain reaction product. After digestion with both restriction enzymes, the insert was ligated into the bacterial expression vector pET11d (Novagen), and expression of recombinant protein was as described by Studier *et al.* (1990) in *E. coli* pLysS cells. Crude bacterial lysates were fractionated by centrifugation at 10,000 × *g*, and recombinant protein was recovered from the pellet fraction by resuspension in 6 M urea, 2 mM dithiothreitol, and 50 mM Tris-HCl (pH 6.5). After centrifugation to remove insoluble material, recombinant protein was reconstituted by gradual dialysis at 4 °C against degassed 50 mM Tris-HCl (pH 7.8) and 5 mM $ZnSO_4$. The preparation was concentrated by ultrafiltration (Amicon, Inc.) and loaded onto a 7.8 × 300-mm gel filtration HPLC column (Protein-Pak 300SW, Waters) at 500 p.s.i. Fractions containing RII binding activity (as assessed by RII overlay) were pooled and concentrated by ultrafiltration. For preparation of soluble fusion protein, the 935-base pair cDNA fragment was cloned into pET-kfc, and recombinant fusion protein was purified from the soluble fraction of induced pLysS cells using calmodulin-agarose as described (Stofko-Hahn *et al.*, 1992).

RII and Zinc Overlays—Proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose. AKAPs were detected using recombinant murine RIIα that was either phosphorylated by the C subunit of PKA using [^{32}P]ATP and used in modified Western blots as described previously (Lohmann *et al.*, 1984) or overlaid directly on the blot and detected using affinity-purified rabbit anti-RII and the ECL Western assay. In control experiments, RII probe was preincubated with 0.4 μM AKAP 95-(642–659)-peptide (EVAAEVLAEVITAAVKAV) or anchoring inhibitor Ht 31-(493–515)-peptide (Carr *et al.*, 1992a). For zinc overlays, proteins separated by SDS-PAGE were transferred to nitrocellulose and immediately immersed in binding buffer (100 mM Tris-HCl (pH 7.4) and 50 mM NaCl) at 25 °C for 60 min. $^{65}ZnCl$ (Dupont NEN) was added at 1 μCi/ml, and after incubation for 90 min, the blots were washed extensively in binding buffer and exposed to x-ray film for 18 h.

Western Blots—Proteins were separated by SDS-PAGE as described (Laemmli, 1970) and transferred to PVDF membrane (Immobilon, Millipore Corp.) using a semidry blotter. Rabbit antibodies to the HPLC-purified AKAP 95 recombinant fragment were affinity-purified using homogeneous preparations of recombinant AKAP-kfc fusion protein coupled to Affi-Gel 15 (Bio-Rad) and used at 1:1000 dilution. Anti-RII antibodies (raised in rabbits) were affinity-purified on columns containing recombinant RIIα-Affi-Gel and used at 1:2000 dilution. The purified rat nuclear matrix preparation was kindly provided by Dr. Ronald Berezney (State University of New York, Buffalo, NY).

DNA Binding—For Southwestern blots, proteins were separated by SDS-PAGE and transferred to Immobilon. After blocking in binding buffer (20 mM Tris-HCl, 40 mM KCl, 0.1 mM EDTA, 0.4 mM 2-mercaptoethanol, and 50 μM $ZnSO_4$) containing 0.5% nonfat dry milk for 45 min, blots were incubated in fresh binding buffer with 10^5 cpm/ml nick-translated salmon sperm DNA for 18 h. After three 30-min washes in binding buffer, the blots were exposed to x-ray film for 2 h. For DNA-cellulose experiments, recombinant protein was phosphorylated by the C subunit using [^{32}P]ATP and purified on a desalting column (Excellulose GF-5, Pierce Chemical Co.). The labeled protein (57,000 cpm) was incubated with 0.1 g of double-stranded DNA-cellulose (Sigma) in 0.4 ml of binding buffer for 20 min. The cellulose was washed with 5 column volumes of binding buffer (flow-through fraction) followed by sequential washes with binding buffer containing 0.4 and 0.8 M NaCl prior to elution. The elution buffer consisted of binding buffer (prepared without zinc) containing 8 mM 1,10-phenanthroline. The amount of labeled protein (measured in counts/minute) in the fractions was determined by scintillation counting.

Immunocytochemistry—Horizontal cryostat sections of mouse brain were fixed, and the cellular distribution of AKAP 95 was determined using affinity-purified anti-AKAP 95 antibodies (1:100 dilution). Immune complexes were detected by the diaminobenzidine staining method as described (Nilaver and Kozlowski, 1989). Immunofluorescence studies were performed on rat embryo fibroblasts (REF-52) grown on glass coverslips and either fixed in 3.7% Formalin in phosphate-buffered saline followed by extraction in absolute acetone at −20 °C or fixed and extracted directly in absolute methanol at −20 °C. Cells were rehydrated in phosphate-buffered saline and 0.1% bovine serum albumin and then stained with affinity-purified primary antibodies (1:100 dilution). Following incubation at 37 °C for 60 min and staining for primary antibodies with biotinylated anti-rabbit antiserum, AKAP 95 distribution was visualized by staining with Texas Red-conjugated streptavidin, and DNA was visualized by Hoechst staining. The cells were mounted and photographed as described elsewhere (Lamb *et al.*, 1991). For DNase treatments, REF-52 cells grown on coverslips were treated with DNase I and ammonium sulfate essentially as described (Nickerson *et al.*, 1992). Anti-serum response factor antiserum (Gauthier-Rouviere *et al.*, 1991) was used at 1:100 dilution and visualized by staining with FITC-conjugated anti-rabbit antiserum.

For fluorescent *in situ* overlay studies, cells fixed in Formalin were incubated for 60 min at 37 °C with 12 μg/ml FITC-conjugated recombinant RIIα in the absence and presence of 0.4 μM anchoring inhibitor Ht 31-(493–515)-peptide and washed extensively with phosphate-buffered saline and 0.1% bovine serum albumin. Cells were observed and photographed on a Zeiss Axiophot photomicroscope using a 40 × planapochromat (1.4 numerical aperture) oil immersion lens.

cAMP-Agarose Purification—Mouse lymphoma YAC-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% newborn calf serum (300 ml) for 72 h and harvested by centrifugation at 3000 × *g*. After two washes in phosphate-buffered saline, 1.5 ml of packed cells were resuspended in 3 ml of hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 0.5 mM dithiothreitol, 1 mM benzamidine, 10 μM isobutylmethylxanthine). This cell suspension was incubated at 4 °C for 2 h followed by addition of 0.1% Nonidet P-40 and homogenization with an ice-cold Dounce apparatus and type A pestle. The cell lysate was centrifuged at 15,000 × *g* for 15 min at 4 °C, and the supernatant was added to cAMP-agarose (Sigma) equilibrated in hypotonic buffer. After mixing at 4 °C for 18 h, the cAMP-agarose pellet was washed five times with 3 ml (total of 37.5 column volumes) of hypotonic buffer. The flow-through fraction and final wash were saved for Western blot analysis. cAMP-binding proteins were eluted, boiled in SDS-PAGE sample buffer for 10 min, and analyzed by Western blotting as described above.

RESULTS

Cloning of AKAP 95—Clones encoding AKAPs were isolated from a GH_4C_1 cDNA expression library by a modification of the RII overlay method of Lohmann *et al.* (1984) using RIIα as a probe. One clone (called GH4/16 (3408 bp)) contained a partial open reading frame of 691 amino acids. When Northern blots were screened with a GH4/16 cDNA probe, the predominant mRNA species detected in a variety of mouse tissues was ~4.3 kilobases (data not shown), confirming that GH4/16 was a par-

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A.

B.

AKAP 95 (642-659)	EVAAEVLAEVITAAVKAV
Ht 31 (495-513)	EEAASRIVDAVIEQVKAA
MAP 2 (89-105)	EVSA-RIVQVVTAEAVAV

C.

FIG. 1. **Sequence of AKAP 95.** *A*, the nucleotide sequence (*upper line*) and deduced amino acid sequence (*lower line*) of a cDNA encoding the A-kinase anchoring protein AKAP 95. The *boxed* region indicates the putative RII-binding domain, and zinc finger motifs are *underlined*. *B*, sequence homology between AKAP 95 (residues 642–659) and the RII-binding regions of two other AKAPs, Ht 31 and MAP 2. *Boxed* regions show amino acid identity. *C*, helical wheel representation of AKAP 95 (residues 642–659) drawn as an 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 one-letter code. The *arrow* indicates the direction of the helix.

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tial fragment of the full-length cDNA. The GH_4C_1 library was rescreened with a 620-bp *XhoI/EcoRI* fragment excised from the 5′-end of GH4/16, but this did not yield clones with additional information. However, a random-primed rat olfactory bulb cDNA library was screened with the same probe, and 30 positive clones were detected from ~250,000 recombinants. The sequence of one clone (called OlF 4 (2715 bp)) overlapped with GH4/16 and yielded 687 bp of additional 5′-nucleotide sequence. A composite sequence of 4072 bp is presented in Fig. 1*A* and contains an open reading frame encoding a 761-amino acid protein with a predicted M_r of 84,720. However, the protein from a variety of murine tissues migrates on SDS-polyacrylamide gels with an apparent molecular mass of 95 kDa (see below); thus, in accordance with the nomenclature of Hirsch *et al.* (1992), we have named the protein AKAP 95.

RII Binding—Comparison of the AKAP 95 sequence to DNA and protein data bases did not identify any overall similarity to other known proteins. However, analysis of the AKAP 95 sequence revealed some important structural features. Residues 642–659 of the protein were homologous to the RII-binding domains of two other AKAPs, MAP 2 and Ht 31 (Fig. 1*B*). Computer-generated analysis of protein secondary structure suggested that this region of AKAP 95 had a high probability of forming an amphipathic α-helix, and when this sequence was drawn as a helical wheel (Fig. 1*C*), the hydrophilic and hydrophobic residues were aligned on opposite faces.

To confirm that this region contained RII binding activity, a DNA fragment encoding COOH-terminal residues 452–761 of AKAP 95 was expressed in *E. coli* as described under "Materials and Methods." A 45-kDa protein was detected by SDS-PAGE in bacterial extracts of induced cells and was partially purified by cell fractionation followed by gel filtration chromatography (Fig. 2*A*). The recombinant protein fragment bound ^{32}P-labeled RIIα as assessed by a direct overlay method (Fig. 2*B*). Solid-phase RII binding was inhibited when overlay blots were incubated in the presence of either a synthetic peptide corresponding to residues 642–659 of AKAP 95 (Fig. 2*C*) or anchoring inhibitor Ht 31-(493–515)-peptide (data not shown), which has previously been shown to block RII/AKAP interaction (Carr *et al.*, 1992a).

DNA Binding—In addition to an RII-binding domain, the AKAP 95 cDNA also encoded two zinc finger motifs located between residues 464 and 486 and residues 553 and 576 (Fig. 1*A*). Zinc binding to the 45-kDa fragment of AKAP 95 (which includes both zinc fingers) was demonstrated by an overlay procedure using ^{65}Zn as a probe (Fig. 2*D*). Partial purification of the recombinant AKAP fragment was achieved by gel filtration HPLC, but surprisingly, the protein was excluded to the void volume of the column and eluted with an apparent molecular mass in excess of 300 kDa (Fig. 3*A*). This was unusual as the RII binding activity in the eluted fractions was associated with a single band that migrated with an apparent molecular mass of 45 kDa on SDS-polyacrylamide gels (Fig. 3*A*, *inset*). Because the AKAP fragment contained zinc fingers of the CC/HH variety often found in DNA-binding proteins (Kaptein, 1991) and was associated with the insoluble fraction of bacterial lysates, we reasoned that the high apparent molecular mass might be due to nonspecific interaction with bacterial DNA. This hypothesis was supported by the observation that the AKAP preparation contained high molecular mass material that positively stained with ethidium bromide on agarose gels (data not shown).

To facilitate purification of the polypeptide in a form that was both soluble and free of bound DNA, we cloned the cDNA fragment encoding the 45-kDa polypeptide into the bacterial expression vector pET-kfc (Stofko-Hahn *et al.*, 1992). Induction of bacteria containing this construct resulted in expression of a 58-kDa AKAP 95/calmodulin-binding fusion protein (AKAP-kfc), a proportion of which was soluble and was purified to homogeneity on calmodulin-agarose. Immobilized AKAP-kfc bound radiolabeled salmon sperm DNA on Southwestern blots (Fig. 3*B*, *lane 2*), whereas kfc fusion proteins made with fragments of either RIIα or the human thyroid AKAP Ht 31 did not bind the DNA (*lanes 4* and *5*). A nuclear matrix preparation known to contain DNA-binding proteins (Hakes and Berezney, 1991a) and a purified preparation of the cAMP response element-binding protein served as positive controls in these experiments (Fig. 3*B*, *lanes 1* and *3*). The AKAP-kfc fusion protein also contained a consensus substrate site for PKA and was phosphorylated by the recombinant C subunit. Radiolabeled AKAP-kfc bound double-stranded DNA-cellulose in the presence of up to 0.8 M NaCl, but was eluted by 8 mM phenanthroline, a zinc-chelating agent (Fig. 3*C*). These results indicate that AKAP 95 binds DNA *in vitro* in a zinc-dependent manner.

Cellular and Subcellular Distribution of AKAP 95—Antiserum against the HPLC-purified 45-kDa AKAP fragment was raised in rabbits. Affinity-purified AKAP 95 antibodies detected a protein band of 95 kDa on Western blots of rat GH_4C_1 and mouse 3T3 cell extracts (Fig. 4*A*, *first* and *third lanes*). A 95-kDa band was also identified by Western analysis of cellular extracts from rat fibroblasts (REF-52), rat granulosa, rat Sertoli cells, rat liver, mouse lymphoma cells (EL-4 and YAC-1), mouse kidney, and mouse brain (data not shown), suggesting a ubiquitous expression pattern for AKAP 95.

AKAP 95 was also immunologically detected on Western blots of a highly purified rat liver nuclear matrix preparation (Fig. 4*A*, *second lane*). This finding was supported as RII-binding proteins of 95 kDa were also detected in the nuclear matrix when identical filters were probed with RIIα by the overlay procedure (Fig. 4*B*, *first* through *third lanes*). Furthermore, DNA-binding proteins in this molecular mass range were detected in the same nuclear matrix preparation by Southwestern blotting (Fig. 3*B*, *lane 1*).

To examine the cellular and subcellular distribution of AKAP 95 *in situ*, we conducted immunohistochemical and immunofluorescence experiments using affinity-purified AKAP 95 antiserum. Initial studies of frozen brain sections showed a distinct nuclear staining pattern for AKAP 95 throughout murine brain that was particularly apparent in cells within the choroid plexus (Fig. 5*A*) and was not observed with nonimmune serum (Fig. 5*B*).

The nuclear localization of AKAP 95 was examined further using indirect immunofluorescence techniques on cultured cells. In quiescent REF-52 fibroblasts, AKAP 95 staining was restricted to nuclear structures, but was excluded from nucleoli (Fig. 6*A*) and appeared to be colocalized with DNA (Fig. 6*B*). Furthermore, AKAP 95 staining was maintained in nuclei after DNase treatment and high salt extraction (Fig. 6*D*). These extraction conditions resulted in the loss of DNA staining (Fig. 6*E*) and an associated transcription factor, serum response factor (Fig. 6, *C* and *F*). These findings are consistent with our Western blot results and suggest that AKAP 95 is tightly associated with the nuclear matrix. A nuclear staining pattern has been observed for AKAP 95 in all mammalian tissues and cell lines we have studied to date, including human HS68 fibroblasts, monkey CV1 kidney cells, mouse hippocampal neurons, and rat L6 myoblast and GH4 lymphoma cells (data not shown). No significant nuclear staining was detected in control experiments using secondary antiserum alone, nonimmune serum, or anti-AKAP 95 antibodies that were preabsorbed on the immobilized recombinant AKAP 95 fragment (data not shown).

Distribution of RII-binding Sites—Direct cytofluorescent

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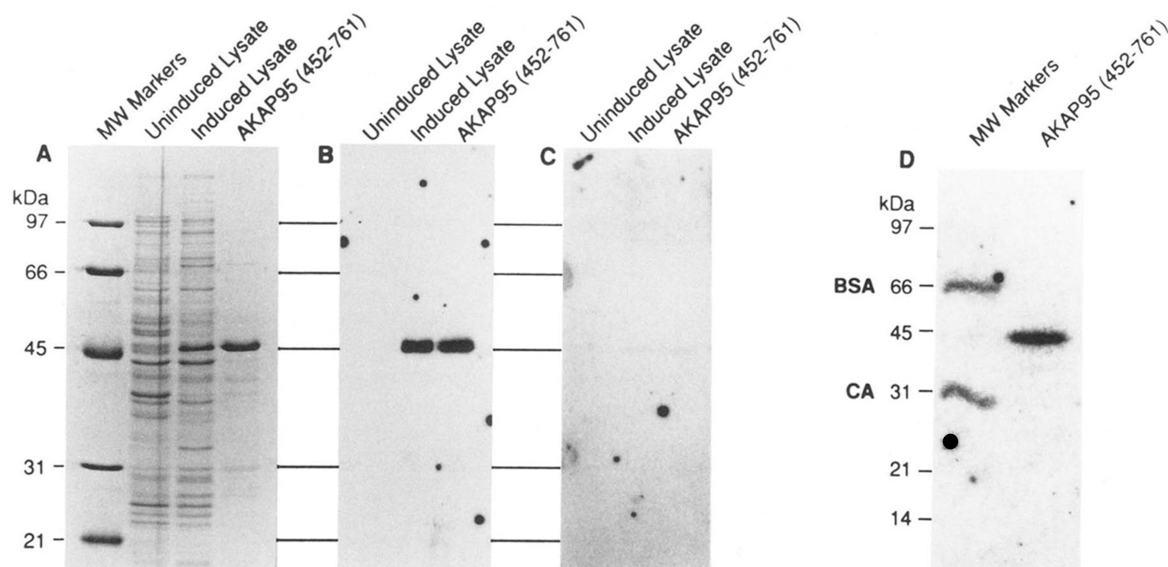

FIG. 2. **Recombinant AKAP 95 fragment binds RII and zinc.** A fragment of the AKAP cDNA (encoding residues 452–761 of the protein) was expressed using the pET11d plasmid. Expression of the recombinant protein was induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside to growing bacterial cultures. Bacterial extracts (100 μg) or purified protein (5 μg) was separated by electrophoresis on 10% (w/v) SDS-polyacrylamide gels and transferred to PVDF membrane. Blots were stained with Coomassie Brilliant Blue (*A*) and analyzed by autoradiography (*B–D*). RII-binding proteins were detected by a solid-phase binding assay (1) using ^{32}P-radiolabeled RIIα as a probe in the absence (*B*) or presence (*C*) of 0.4 μM AKAP 95-(642–659)-peptide. Zinc binding was also detected by a solid-phase binding assay (*D*) using ^{65}Zn as a probe. Sample sources are indicated above each lane. Bovine serum albumin (*BSA*) and carbonic anhydrase (*CA*) were used as internal controls for zinc-binding blots. Molecular mass markers are indicated on each panel.

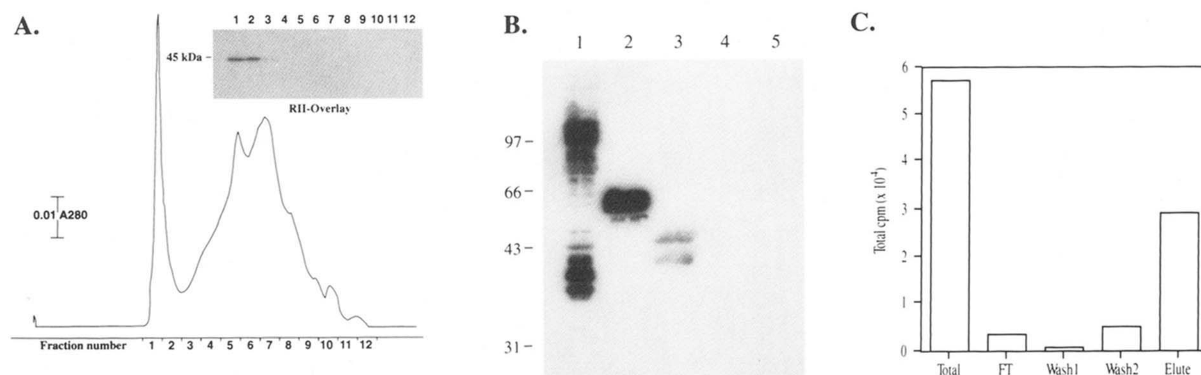

FIG. 3. **Purified recombinant AKAP 95 fragment binds DNA.** *A*, purification of AKAP 95-(452–761) fragment. Crude bacterial extracts (50 μg) from induced cells expressing the AKAP 95 fragment were separated on a Protein-Pak 300SW gel filtration column. The *inset* shows detection of RII-binding proteins in fractions eluted from the column by the RII overlay method as described under "Materials and Methods." The migration position of the AKAP 95 fragment is indicated. *Lane numbers* correspond to column fractions. *B*, solid-phase binding of the AKAP-kfc recombinant fusion protein to salmon sperm DNA. Protein samples (1–5 μg) were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to PVDF membrane. DNA-binding proteins were detected by the Southwestern blot procedure with radiolabeled salmon sperm DNA as a probe. Protein samples are rat nuclear matrix extract (*lane 1*), AKAP-kfc (*lane 2*), cAMP response element-binding protein (*lane 3*), RIIα-kfc (*lane 4*), and Ht 31-kfc (*lane 5*). *C*, zinc-dependent binding of AKAP-kfc to DNA-cellulose. Radiolabeled AKAP-kfc (57,000 cpm; *Total*) was absorbed onto double-stranded DNA-cellulose as described under "Materials and Methods." Sequential washes with binding buffer (flow-through fraction (*FT*)), 0.4 M NaCl (*Wash1*), and 0.8 M NaCl (*Wash2*) were followed by elution with 8 mM 1,10-phenanthroline (*Elute*), a zinc-chelating agent. Detection of the radiolabeled AKAP-kfc fragment was by liquid scintillation counting.

techniques using FITC-labeled RIIα as a probe were employed to determine the distribution of RII-binding sites in quiescent REF-52 fibroblasts. RII-binding sites were detected throughout the cells, including the Golgi apparatus, mitochondria, and the nucleus (Fig. 7*A*). The nuclear distribution of RII-binding sites excluded nucleoli (Fig. 7*A*) and was similar to the pattern of AKAP 95 staining detected by indirect immunofluorescence (Fig. 6*A*). No significant staining was observed in control experiments using fluorescent RII probe preincubated with anchoring inhibitor Ht 31-(493–515)-peptide (Fig. 7*B*) or using RII probe that had previously been boiled in SDS (data not shown). The results demonstrate the presence of nuclear RII-binding sites in quiescent cells and suggest that these sites could be provided by AKAP 95.

Copurification of RII and AKAP 95—To examine whether AKAP 95 was associated with RII in cell lysates, we attempted to purify the RII·AKAP 95 complex using affinity chromatography on cAMP-agarose. YAC-1 lymphoma cells were chosen for these experiments because they can be grown in suspension, where they divide rapidly, thus facilitating acquisition of sufficient starting material. Western blot analysis of solubilized YAC-1 lysates indicated that the cells contained RII, which was purified by affinity chromatography on cAMP-agarose (Fig. 8*A*). Identical blots probed with anti-AKAP 95 antibodies indicated that a proportion of the AKAP 95 present in the lysate copurified with RII (Fig. 8*B*). The results provide evidence that RII·AKAP 95 complexes exist and can be purified from cell lysates.

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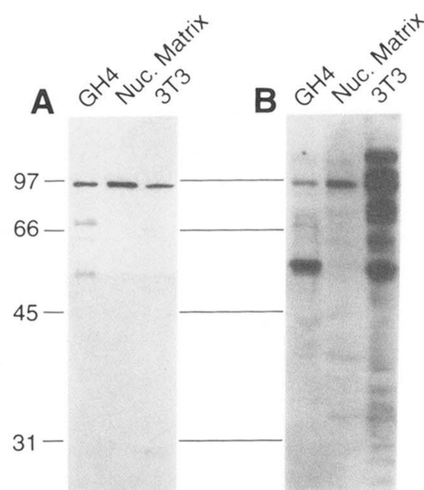

FIG. 4. **Detection of AKAP 95 in nuclear matrix fraction.** Protein extracts from rat pituitary GH_4C_1 (*GH4*) cells and NIH-3T3 (*3T3*) cells and a purified rat liver nuclear matrix fraction (*Nuc. Matrix*) were separated by 10% SDS-PAGE and transferred to PVDF membrane. Blots were subjected to either Western blot analysis using anti-AKAP 95 antiserum (*A*) or analysis of RII binding by overlay with RIIα (*B*). Molecular mass markers (in kilodaltons) are indicated on each panel.

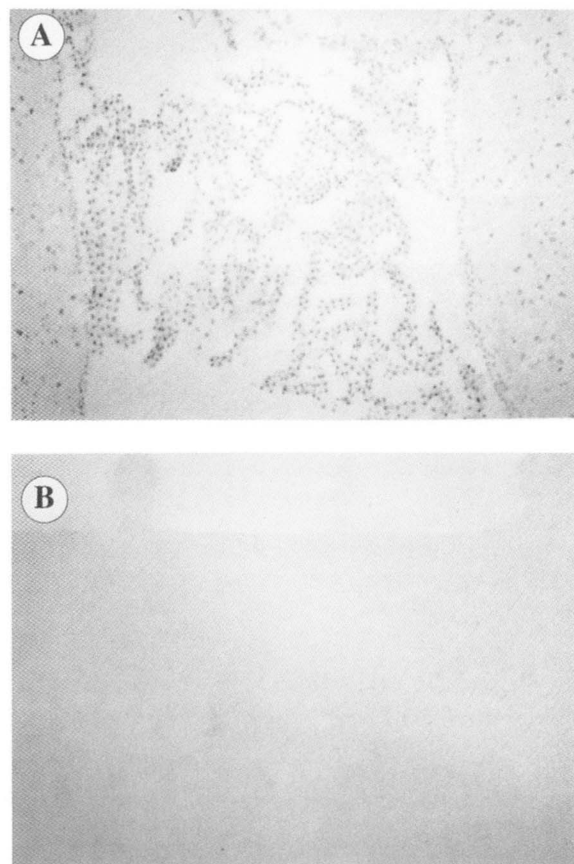

FIG. 5. **Immunological localization of AKAP 95.** Horizontal sections of mouse brain were fixed and incubated with either rabbit anti-AKAP 95 antibodies (*A*) or nonimmune rabbit serum (*B*). Immune complexes were detected using diaminobenzidine staining. Cells of the choroid plexus are shown at a magnification × 100.

DISCUSSION

In this report, we describe the cloning of cDNA encoding a novel A-kinase anchor protein (called AKAP 95) that binds RIIα *in vitro*. Studies on other AKAPs have revealed that their RII-binding domains are composed of amphipathic helix regions (Carr *et al.*, 1991). Peptides encompassing the putative amphipathic helix region in one of these proteins, the human thyroid AKAP Ht 31, bind RIIα or the type II PKA with nanomolar affinity (Carr *et al.*, 1992a). Residues 642–659 of AKAP 95 appear to represent the RII-binding domain because this sequence is predicted to form an amphipathic helix and is homologous to the binding regions of two AKAPs, Ht 31 and MAP 2. Furthermore, a truncated form of AKAP 95 that includes the putative amphipathic helix binds RII in overlay assays, and this binding is blocked by a synthetic peptide corresponding to residues 642–659 of AKAP 95. These results support our previous observation that AKAPs contain a conserved region of secondary structure responsible for RII binding (Carr *et al.*, 1991).

In addition to an RII-binding domain, we have proposed that each AKAP contains regions responsible for targeting to specific subcellular structures (Coghlan *et al.*, 1993). The AKAP 95 sequence contains two zinc finger motifs that conform closely to the consensus sequence of Tyr/Phe-X-Cys-X_{2-4}-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His (X represents any amino acid), which is representative of the transcription factor IIIA-like zinc fingers (Berg, 1990). This motif is found in a large number of eukaryotic proteins, many of which have been demonstrated to interact directly with DNA (Jacobs, 1992; Desjarlais and Berg, 1992). The ability of AKAP 95 to bind DNA was first indicated by the observation that a recombinant protein fragment (including both zinc fingers) expressed in *E. coli* copurified with bacterial DNA. Nonspecific DNA binding activity was confirmed by Southwestern blotting and affinity chromatography on DNA-cellulose. AKAP 95 appears to bind DNA in a sequence-independent manner; several attempts to select specific DNA-binding site preferences from libraries of random-sequence oligonucleotides were unsuccessful.[2] While these data would imply that AKAP 95 is unlikely to be a transcription factor, it could play a role in promoting the binding of such factors to DNA.

An apparent lack of DNA-binding site specificity is characteristic of nuclear matrix proteins, which maintain the three-dimensional architecture of the nucleus (van Driel *et al.*, 1991; Hakes and Berezney, 1991b). Immunological detection of AKAP 95 in the insoluble nuclear matrix fraction suggests that it is the predominant, if not only, RII-anchoring protein in the matrix. Moreover, the bright nuclear staining pattern observed that remains after DNase digestion and high salt extraction of other nuclear proteins is further evidence that AKAP 95 remains associated with nuclear structures and is entirely consistent with the properties of a nuclear matrix protein (Staufenbiel and Deppert, 1984; Fey *et al.*, 1986). The resistance of AKAP 95 to these harsh extraction conditions indicates that the protein may be tethered to the matrix scaffold through association with other matrix components. A primary function of the nuclear matrix is the organization of chromatin loops of discrete structural and functional units (van Driel *et al.*, 1991). It has previously been shown that the PKA holoenzyme is associated with transcriptionally active chromatin and that the R subunits are bound to the nuclear matrix in rat liver (Sikorska *et al.*, 1988). It is tempting to speculate that AKAP 95 might be involved in the interaction of type II PKA with chromatin loops, and we are currently investigating this possibility. AKAP 95-directed targeting of type II PKA near functional transcription units is an intriguing prospect as it would also optimally position the kinase for phosphorylation of transcription factors such as cAMP response element-binding protein (Mellon *et al.*, 1989).

[2] V. M. Coghlan and J. D. Scott, unpublished data.

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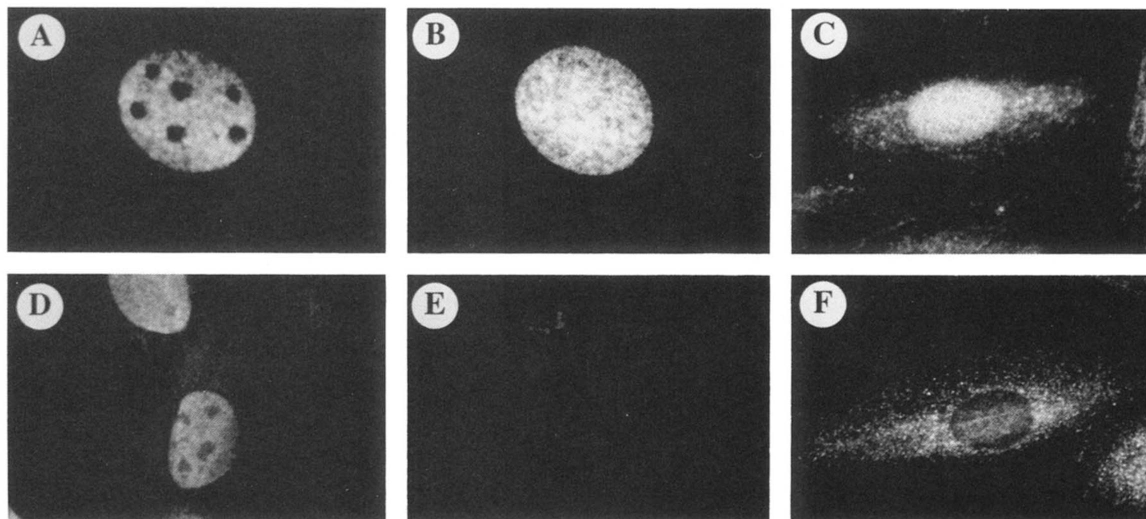

FIG. 6. **Immunofluorescence detection of AKAP 95.** REF-52 cells were Formalin-fixed and incubated either with anti-AKAP 95 antibodies (*A* and *D*) and Hoechst stain (*B* and *E*) or with antibodies against the transcription factor serum response factor (*C* and *F*). FITC-conjugated anti-rabbit secondary antiserum was used for staining. Cells in *A–C* were fixed under the standard conditions described under "Materials and Methods," whereas cells in *D–F* were treated with DNase I and extracted with ammonium sulfate to isolate nuclear matrices.

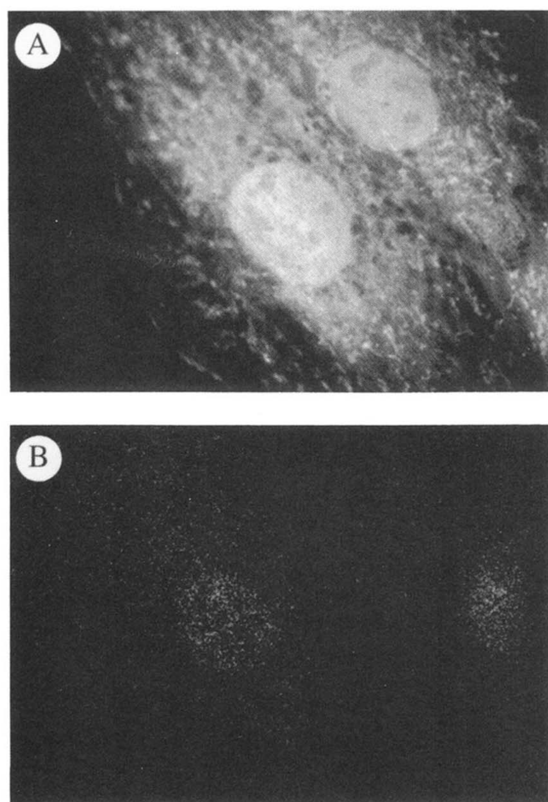

FIG. 7. **Detection of RII-binding sites by *in situ* overlay.** RII-binding sites were detected *in situ* by direct overlay of FITC-labeled RIIα (12 μg/ml) onto Formalin-fixed REF-52 fibroblasts. Incubations were at 37 °C for 60 min in the absence (*A*) or presence (*B*) of 0.4 μM anchoring inhibitor Ht 31-(493–515)-peptide.

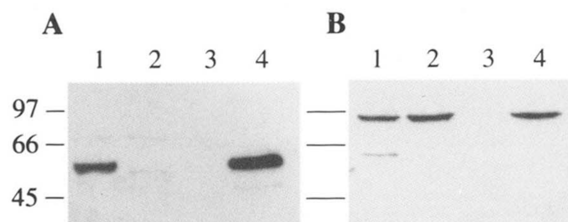

FIG. 8. **AKAP 95 copurifies with RII on cAMP-agarose.** Immunochemiluminescent detection of proteins on Western blots is shown for fractions isolated from cAMP-agarose affinity chromatography. Separate filters were probed for either RIIα (*A*) or AKAP 95 (*B*) using purified antibodies, horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence detection. A solubilized extract of YAC-1 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 with binding buffer. After a final wash (*lane 3*), proteins remaining bound to the affinity matrix were eluted (*lane 4*) and boiled in SDS sample buffer. Fractions were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membrane.

Although we have established that AKAP 95 is a nuclear protein, the occurrence of R subunits in the nucleus remains controversial. Indirect immunofluorescence with subunit-specific antiserum has detected RII in the nuclei of medullary neurons (Cumming *et al.*, 1981), Madin-Darby bovine kidney cells (Nigg *et al.*, 1985a, 1985b), and H411E hepatoma cells (DeCamilli *et al.*, 1990). In addition, R subunits have been detected in the nuclei of hepatoma cells using fluorescein isothiocyanate-labeled C subunit as a probe (Byus and Fletcher, 1982; Fletcher *et al.*, 1986), and immunocolloidal gold electron microscopy shows that RII is associated with the perinuclear membranes of these same cell types (Kuettel *et al.*, 1985). In contrast, microinjection of fluorescently tagged type I PKA subunits into living cells suggests that the labeled RI subunits do not enter the nucleus, even upon dissociation of the holoenzyme with 8-bromo-cAMP (Meinkoth *et al.*, 1990). However, these microinjection studies were performed using the RI subunit, which has been shown to be exclusively soluble (Corbin *et al.*, 1975), while only the RII subunit has been shown to be associated with subcellular particles (Corbin *et al.*, 1977). Tsien and co-workers used fluorescence resonance energy transfer to detect the dissociation of double-labeled type I and II PKA holoenzymes (called $FlCRhR^{I}$ and $FlCRhR^{II}$) and showed that the FITC-labeled C subunit translocated into the nucleus upon elevation of cAMP, while the rhodamine-labeled R subunit remained cytoplasmic (Adams *et al.*, 1991; Bacskai *et al.*, 1993). Although this is compelling evidence for nuclear exclusion of fluorescent R subunits in stimulated cells, it is possible that the rhodamine label interferes with nuclear targeting. It is also possible that free RII probe would first saturate anchoring sites in the cytoplasm before nuclear staining would become apparent. This is a reasonable hypothesis given that individual cell types express 10–15 different AKAPs that

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appear to bind RII with nanomolar affinity (Carr and Scott, 1992; Carr *et al.*, 1992a). To circumvent some of these limitations, we utilized a direct *in situ* overlay technique using fluorescein isothiocyanate-labeled RIIα as a probe. The results demonstrate the presence of high-affinity RIIα-binding sites throughout the cell, including the nucleus. We presume that the staining represents unoccupied sites on various AKAPs since control studies performed in the presence of an anchoring inhibitor peptide blocked fluorescent RII binding. Whether the binding sites identified contribute to physiologically important interactions remains an unanswered question. Nevertheless, our finding that AKAP 95 copurified from cell extracts with RII suggests that this interaction can occur *in vivo*. Undoubtedly, the challenge remains to determine the consequences of this interaction and the physiological role AKAP 95 may play in the regulation of cAMP-responsive nuclear events.

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