

Cloning and Characterization of a Novel A-kinase Anchoring Protein

AKAP 220, ASSOCIATION WITH TESTICULAR PEROXISOMES*

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Compartmentalization of the type II cyclic AMP-dependent kinase (PKA) is achieved through association of the regulatory subunit (RII) with A-kinase anchoring proteins (AKAPs). Using an interaction cloning strategy with RII α as a probe, we have isolated cDNAs encoding a novel 1129-amino acid protein that contains both a PKA binding region and a peroxisome targeting motif. Northern analysis detected mRNAs of 9.7 and 7.3 kb in several rat tissues with the highest levels present in the brain and testis. Western analysis and RII overlay experiments showed that the protein is approximately 220 kDa and was, therefore, named AKAP 220. Immunoprecipitation of AKAP 220 from rat testis extracts resulted in co-purification of the type II PKA holoenzyme. The specific activity of PKA increased 458-fold from 7.2 pmol/min/mg in the cell lysate to 3.3 nmol/min/mg in the immunoprecipitate. Immunohistochemical analysis of rat testicular TM4 cells showed that AKAP 220 and a proportion of RII were co-localized in microbodies that appear to be a subset of peroxisomes. Collectively, these results suggest that AKAP 220 may play a role in targeting type II PKA for cAMP-responsive peroxisomal events.

Intracellular responses of many hormones are mediated by signal transduction pathways that alter the phosphorylation state of key regulatory proteins (1). Protein phosphorylation is a reversible process involving two classes of signaling enzymes: protein kinases, which catalyze the phosphotransfer reaction, and phosphoprotein phosphatases that catalyze dephosphorylation (1, 2). The activity of both enzyme classes is tightly regulated and responds to fluctuations in diffusible second messengers such as Ca²⁺, phospholipid, and cAMP (1). The predominant effect of cAMP is to activate a cAMP-dependent protein kinase (PKA)¹ that modifies serine and threonine residues on substrate proteins (3). Since PKA has a broad substrate specificity and is present in relatively high intracellular

concentrations (4), it is critical to restrict the activity of the enzyme and its substrate availability. One way to accomplish this is by localizing PKA to specific cellular compartments (5). Compartmentalization of the kinase seems to represent a regulatory mechanism that could increase the selectivity and intensity of a cAMP-mediated hormonal response. This is achieved, in part, through the association of the kinase with a family of A-kinase anchoring proteins (AKAPs) (6).

In recent years, numerous AKAPs have been identified that target PKA to the plasma membrane, cytoskeleton, endoplasmic reticulum, Golgi, mitochondria, and nuclear matrix (7–15). These proteins represent a growing family of signaling molecules that contain a conserved PKA binding motif and function to localize the kinase to particular subcellular sites (16). In this report, we describe the cloning and characterization of another AKAP, called AKAP 220. This protein associates with PKA in testis where it may target the kinase to peroxisomes.

EXPERIMENTAL PROCEDURES

Cloning of AKAP 220 cDNAs—A 1933-bp cDNA was obtained by screening a rat pituitary (GH₄C₁) [λ Zap II, cDNA expression library by a direct overlay method with ³²P-labeled RII α as a probe as described previously (17). Full-length cDNAs were obtained from a rat olfactory bulb λ Zap II cDNA library after screening with a [α -³²P]dCTP random primed 198-bp *EcoRI*-*NcoI* fragment excised from the 5' end of the original clone. Nucleotide sequencing was performed on both strands by the dideoxy chain termination method of Sanger (18) or on an automated sequencer (ABI).

Northern Analysis—A rat multitissue Northern blot of immobilized poly(A)⁺ mRNAs (Clontech) was probed with a ³²P-radiolabeled 936-bp fragment of the original AKAP 220 cDNA. The blot was prehybridized in 400 mM sodium phosphate (pH 6.6), containing 1 mM EDTA, 5% SDS, 1 mg/ml bovine serum albumin, 50% formamide for 6 h at 42 °C. The radiolabeled probe was added to the same buffer and hybridized overnight at 42 °C. Nonhybridized probe was removed by washing the blot in 2 \times SSC, 0.05% SDS for 45 min at room temperature followed by washes in 0.1 \times SSC, 0.1% SDS for 40 min at 50 °C. Signals were detected by autoradiography. In control experiments, the blot was probed with a ³²P-radiolabeled β -actin cDNA fragment using similar conditions.

Bacterial Expression of AKAP 220—A 1104-bp fragment encompassing the coding region of the original AKAP 220 cDNA was amplified by polymerase chain reaction. Primers were designed to create a *NdeI* site (CAGCGGATCCACATATGGGGTTTAGAAGGAGATTTC) at the 5' end and a *BamHI* site (CGCGCTCGAGGATCCTACATGTAATCA) 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, pET 16b (Novagen). Expression of the recombinant His-tag fusion protein was as described by Studier (19) in *Escherichia coli* BL21 (DE3 pLysS). The recombinant protein was induced at an OD of 0.3 with 1 mM isopropyl- β -D-thiogalactopyranoside. After 2 h, cells were pelleted at 6,000 \times g and lysed by sonication 3 \times 10 s in buffer. The crude cell lysate was fractionated by centrifugation at 10,000 \times g and recovered from the particulate fraction by solubilization in 20 mM Tris-HCl (pH 7.9), containing 6 M urea, 5 mM imidazole, 0.5 mM NaCl. The protein was renatured by sequential dialysis at 4 °C against 40 mM MOPS (pH 7.9), 0.1 mM EDTA, and 0.5 mM dithiothreitol with decreas-

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¹ The abbreviations used are: PKA, protein kinase A; AKAP, A-kinase anchoring protein; bp, base pair(s); MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

ing concentrations of urea (0–4 M). Purification of the His-tag protein was achieved on a His-bind 228 metal chelation resin as described previously (20).

RII Overlays—Proteins were separated by SDS-PAGE (21) and electrotransferred to PVDF membranes (Immobilon, Millipore Corp.). Overlay assays were conducted as described (22) with control experiments performed by preincubating blots with 0.5 μ M anchoring inhibitor peptide, Ht-31 (493–515) (23). Semiquantitative overlays were performed using a slotblot apparatus as described previously (20). All overlays were performed in solutions containing approximately 250,000 cpm of 32 P-radiolabeled RII α and binding was detected by autoradiography. Films were digitally scanned and analyzed by densitometry using the National Institutes of Health Image Software (version 1.55).

Western Blots—Western blots were performed by standard procedures using affinity-purified rabbit polyclonal antibodies against the recombinant AKAP 220 fragment (produced by Bethyl Laboratories, Inc., Montgomery, TX) and rat anti-peptide antibodies against the PKA catalytic subunit (kindly provided by Dr. S. L. Pelech, Kinetek Corp. Vancouver, British Columbia). Enhanced chemiluminescence detection of antigens (DuPont) was achieved with horseradish peroxidase-conjugated secondary antibodies (Amersham Corp.).

Preparation of Tissue and Cell Extracts—Rat tissues were obtained from euthanized animals. The tissue was quick-frozen in liquid nitrogen and pulverized with a mortar and pestle and suspended in a hypotonic buffer consisting of 10 mM HEPES (pH 7.9), containing 1.5 mM MgCl₂, 10 mM KCl, 1 mM 4-(2-aminoethyl)benzenesulfonfyl fluoride, 2 μ g/ml pepstatin/leupeptin, 1 mM benzamide, 10 μ M isobutylmethylxanthine, and 0.5% Nondiet-P 40. After incubation in the hypotonic buffer for 1 h at 4 °C, the tissue or cell preparations were lysed by Dounce homogenization (20 strokes). The detergent extracted supernatant fraction was collected after centrifuging at 15,000 rpm for 30 min at 4 °C. Protein concentrations were determined by a modified Lowry assay (24).

Purification of the AKAP220 Complex—PKA-AKAP complexes were purified from rat testis extracts by immunoprecipitation as described (25). Cell lysates were incubated with affinity-purified antibody (15 μ g) in hypotonic buffer (see above) overnight at 4 °C. Following this step, the antibody/lysate mixture was incubated with protein A-Sepharose (10%, v/v) for 2 h at 4 °C. After washing with high salt (hypotonic buffer/750 mM NaCl) and three washes with hypotonic buffer proteins were eluted with 0.5 mM of the Ht 31 anchoring inhibitor peptide, 75 mM cAMP or SDS-PAGE buffer. Control experiments were performed with 150 μ g of preimmune sera.

Protein Kinase A Assay—Protein kinase A activity was measured in immunoprecipitate fractions as described previously (26). C subunit activity was defined as the difference in 32 P incorporated into kemptide (LRRASLG) after incubation for 10 min at 30 °C in the presence and absence of the specific kinase inhibitor, PKI, 5–24 peptide (TTYADFI-ASGRTGRRNAIHD). Excess cAMP (1 mM) was present in all fractions.

Immunohistochemistry—Immunofluorescence studies were performed on rat Sertoli TM4 cells grown on glass coverslips. Cells were fixed with a picric acid, 2% (v/v) paraformaldehyde solution, and permeabilized with 0.2% Triton X-100. Cells were rehydrated in PBS, 0.1% bovine serum albumin and incubated with primary antibodies for 1 h at 30 °C. Affinity-purified AKAP 220 antibody was used at a dilution of 1:100, affinity-purified goat-anti-RII antibody at 1:1000, and anti-70-kDa peroxisomal protein at 1:100 (kindly provided by Dr. Suresh Subramani, University of California, San Diego). Immune complexes were detected by incubating the cells with secondary antibodies labeled with either fluorescein isothiocyanate or Texas Red for 1 h at 30 °C. Control experiments were performed with preimmune sera at a concentration of 5 μ g/100 μ l or with affinity-purified AKAP 220 antibody pre-absorbed with excess (50 μ g/100 μ l) of AKAP 220 protein. The coverslips were washed with PBS, 0.1% bovine serum albumin to remove excess antibodies. Coverslips were mounted with Vectashield 228 mounting media. The cells were examined and photographed on either a Nikon confocal photomicroscope using a 63 \times planapochromat oil immersion lens or a Leitz Fluovert FU photomicroscope with a 63/1.4 OEL PL APO lens.

RESULTS

Cloning of AKAP 220—Clones encoding RII-binding proteins were isolated from a GH₄C₁ cDNA expression library as described previously (14) using 32 P-radiolabeled RII α as a probe. Fifteen positive clones were identified from an initial screening of \sim 500,000 recombinants. One clone, called GH₄-12, was 1933 bp in length and contained a continuous open reading frame of 368 amino acids (Fig. 1A). The size of the transcript was deter-

mined by probing a Northern blot of various rat RNAs with a 32 P-radiolabeled cDNA fragment of the original clone. The mRNA for GH₄-12 showed two predominant message sizes of 9.7 and 7.3 kb in rat heart, liver, lung, kidney, and testis, whereas a third message of 5.5 kb was detected in brain (Fig. 2). These mRNA sizes confirmed that the GH₄-12 clone encompassed a partial fragment of the full-length message. Therefore, a 198-bp *EcoRI-NcoI* fragment excised from the 5' end of the GH₄-12 insert was used to screen a rat olfactory bulb cDNA library for more complete transcripts of the message. Three overlapping clones were obtained that extended the sequence to 9733 bp. The entire nucleotide sequence has been placed in GenBank™. The contiguous sequence of GH₄-12, presented in Fig. 1A, contains an open reading frame of 3,387 bp that encodes for a 1,129 amino acid protein. Fig. 1A represents the entire open reading frame, although there is an additional 5406 bp of 5'-untranslated region.

The AKAP 220 nucleotide and protein sequence were compared with the Genbank™ data base, and there was no overall homology to known protein sequences. However, the last three amino acids of the protein, Cys-Arg-Leu, fulfill the criteria for a COOH-terminal peroxisomal (microbody) targeting signal (Fig. 1A). In addition, residues 905–918 are likely to represent an RII binding site as this region exhibits high probability for forming an amphipathic α -helix (Fig. 1B) and shows limited homology to the RII binding regions of other AKAPs (Fig. 1C).

Expression and Characterization of AKAP 220—A fragment of the AKAP 220 cDNA encoding the COOH-terminal 368 amino acids of the protein was subcloned into the pET16b His-tag expression vector then expressed in *E. coli*. A 58-kDa protein was detected in bacterial extracts from cells induced with isopropyl- β -D-thiogalactopyrnoside. The recombinant protein was purified by affinity chromatography on His-binding resin (Fig. 3A). This protein was specifically recognized by polyclonal antibodies raised against AKAP 220 (Fig. 3B) and bound 32 P-labeled RII α as assessed by the overlay method (Fig. 3C). Control experiments demonstrated that RII binding was inhibited by preincubating the blot with 0.5 μ M anchoring inhibitor peptide, Ht 31 (493–515) (Fig. 3D). The RII-binding affinity of the AKAP 220 fragment was measured by a semiquantitative overlay procedure and compared with a 55-kDa fragment derived from the human thyroid AKAP, Ht 31 (27). The half-maximal binding values were calculated to be 20 and 25 ng for AKAP 220 and Ht 31, respectively (Fig. 4). These findings suggest that the RII binding region of AKAP 220 is located in the COOH-terminal third of the molecule and it binds RII with an affinity similar to Ht 31.

Tissue Distribution of AKAP 220—Affinity-purified antibodies against the 58-kDa fragment were used to screen rat tissue extracts for expression of the native protein. Two predominant bands detected in rat testis (Fig. 5B) corresponded to RII-binding bands (Fig. 5A). In order to assess the size of these immunoreactive proteins, molecular weight measurements were performed on SDS-PAGE gels of different acrylamide concentrations (5–7.5%). The average molecular mass of the larger immunoreactive protein was calculated to be 220 kDa (data not shown). The lower band was 180 kDa and may well represent a proteolytic fragment of the larger band. Therefore, we have named the protein AKAP 220. Furthermore, the prominent expression of AKAP 220 in rat testis provided us with a suitable tissue source for further studies to determine whether the anchoring protein bound PKA in extracts and testis derived cell-lines.

Purification of PKA-AKAP 220 Complex—To examine whether AKAP 220 was associated with PKA in testis, affinity-purified antibodies were used to immunoprecipitate the

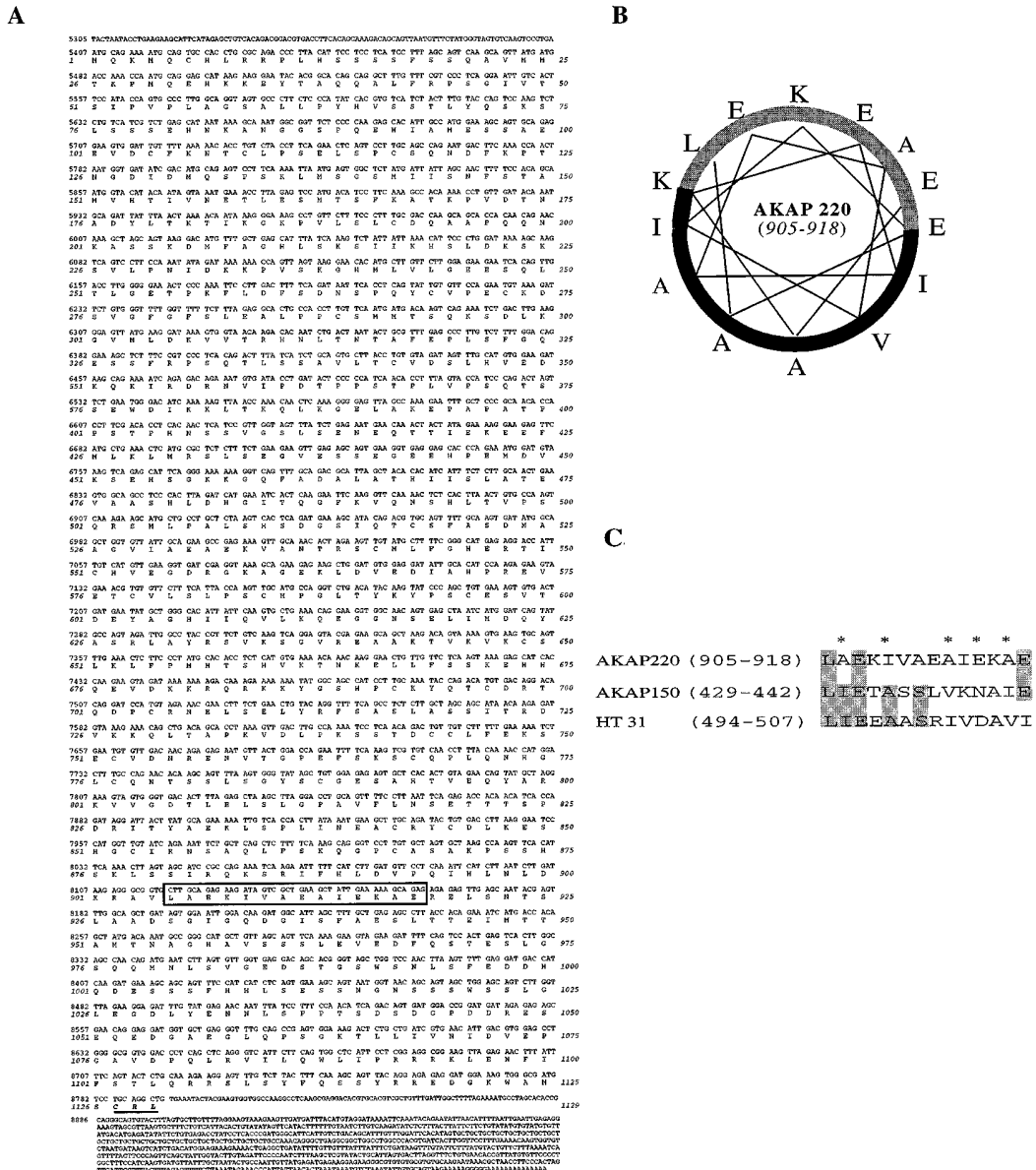


Fig. 1. Sequence of AKAP 220. **A**, the nucleotide sequence and the deduced amino acid sequence of the cDNA encoding the A-kinase anchoring protein, AKAP 220. The boxed area indicates the putative RII binding region, while the peroxisome-targeting sequence is underlined and in italics. **B**, helical wheel representation of AKAP 220 (residues 905–918) drawn as an α -helix of 3.6 amino acids/turn. The shaded area indicates the hydrophilic residues, and the black area indicates the hydrophobic residues. **C**, sequence homology between AKAP 220 (residues 905–918) and the RII binding regions of two other AKAPs, AKAP 150 and Ht 31. The shaded area indicates amino acid identity and conserved amino acids are indicated (*).

AKAP220 complex (Fig. 6A). AKAP 220 was detected in the immunoprecipitate by Western blot (Fig. 6B). Complementary studies were initiated to detect the PKA subunits in the immunoprecipitate. The specific activity of the C subunit increased 458-fold from 7.2 ± 2.1 pmol/min/mg ($n = 3$) in the testis lysate to 3.3 ± 0.15 nmol/min/mg ($n = 3$) in the immunoprecipitate (Fig. 6C). In contrast, minimal PKA activity, 0.026 nmol/min/mg ± 0.07 ($n = 3$), was detected in control samples immunoprecipitated with preimmune serum (Fig. 6C). Since 99.3% of C subunit activity was inhibited by the PKI 5–24 peptide, which is a specific inhibitor of the C subunit, we believe that the majority of the kinase activity co-precipitated with AKAP 220 was PKA. RII was also detected in the immunoprecipitates and reciprocal experiments detected AKAP 220 in the eluate from a cAMP-agarose affinity column (data not shown). In addition, incubation with the anchoring inhibitor peptide Ht 31 (493–515) released the C subunit of PKA from the immunopre-

cipitates (Fig. 6D). Since the Ht 31 peptide specifically disrupts RII-AKAP interaction these experiments definitively show that the type II PKA holoenzyme associates with AKAP 220 in rat testis.

Subcellular Distribution of AKAP 220—The subcellular distribution of AKAP 220 was evaluated by immunohistochemistry in the rat testis cell lines (TM3 and TM4). Immunofluorescence detection of AKAP 220 showed an unusual punctuate cytoplasmic staining pattern in both cell lines, which was more distinct in TM4 cells (Fig. 7B). Confocal analysis showed that AKAP 220 (Fig. 7B) and RII (Fig. 7A) exhibited distinct but overlapping staining patterns. Double imaging experiments suggested that a proportion of the RII signal occupied the same focal plane as the anchoring protein. Control experiments using preimmune serum (Fig. 7D) or antibodies preabsorbed with AKAP 220 protein (Fig. 7E) were negative. These findings support our biochemical studies on the isolation of an

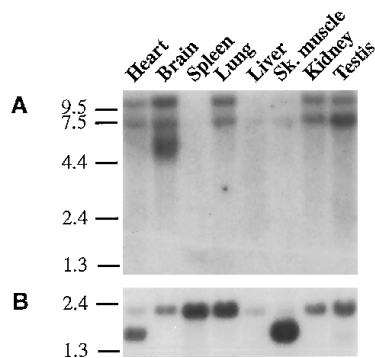


FIG. 2. **The tissue distribution of the AKAP 220 mRNA.** *A*, 2 μ g of poly(A)⁺ RNA from rat tissues (rat MTN, Clontech) were probed with a ³²P-radiolabeled 936-bp fragment excised from the 3' end of GH₄-12 as described under "Experimental Procedures." *B*, the same filter was probed with ³²P-radiolabeled β -actin. Hybridizing mRNA species were detected by autoradiography. The tissue source of each RNA is indicated above each lane. Kilobase markers are indicated beside each panel.

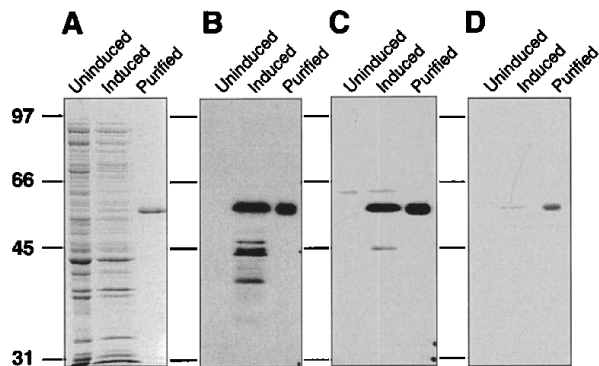


FIG. 3. **Recombinant AKAP 220 fragment specifically binds RII α .** A fragment of the AKAP 220 cDNA (encoding residues 761-1129 of the protein) was expressed using the pET16b bacterial expression vector. Bacterial extracts, induced or uninduced (100 μ g), or purified protein (10 μ g) were separated by electrophoresis on 10% (w/v) SDS-PAGE and electrotransferred to PVDF membranes. *A*, gels were stained with Coomassie Blue dye. *B*, the recombinant AKAP 220 fragment was detected by Western blot with affinity-purified antibodies. RII binding proteins were detected by a solid-phase binding assay (22) using ³²P-radiolabeled RII α as a probe in the absence (*C*) or presence (*D*) of 1 μ M anchoring inhibitor peptide, Ht 31 (493-515). Sample sources are indicated above each lane, and the molecular weight markers are indicated beside each panel.

AKAP-PKA complex. Although the AKAP 220 staining pattern in TM4 cells was distinct and reproducible, it was not immediately evident where the anchoring protein was localized. Furthermore, the AKAP 220 signal did not overlap with the staining patterns for the Golgi or endoplasmic reticulum marker proteins, Mannosidase II and signal sequence receptor (data not shown). However, the punctuate cytoplasmic staining pattern was reminiscent of "endosomal-like" structure. This, combined with the identification of a peroxisomal targeting motif on AKAP 220, provided the impetus for additional immunofluorescence studies with a peroxisomal marker, "the 70-kDa peroxisomal protein" (28). Staining with the peroxisomal marker protein produced a punctate cytoplasmic pattern that was similar to AKAP 220 (Fig. 7C). Therefore, we propose that the anchoring protein may associate with a subset of peroxisomes in TM4 cells.

DISCUSSION

Anchoring of PKA at specific subcellular sites is thought to be a regulatory mechanism that permits the kinase access to certain substrates. Recently, it has become evident that AKAPs

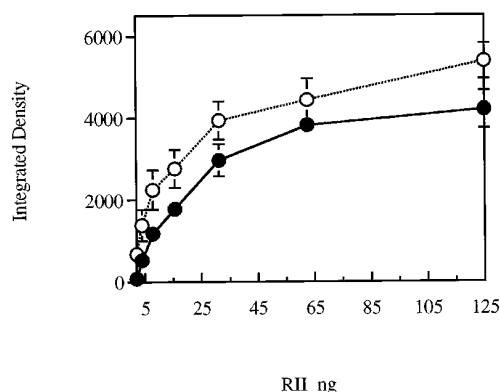


FIG. 4. **Estimation of the AKAP220 binding affinity for RII α .** Binding of ³²P-radiolabeled RII α to the COOH-terminal fragment of AKAP 220 (residues 761-1129) and a corresponding fragment of the human thyroid anchoring protein Ht 31 was measured by a semiquantitative overlay procedure. Aliquots of the purified protein ranging from 5 to 125 ng were immobilized onto nitrocellulose filters using a slot-blot apparatus. Individual filters were probed with excess ³²P-radiolabeled RII α (specific activity: 2.1–1.5 \times 10⁵ cpm/nmol). Detection of immobilized RII α was by autoradiography. Quantitation of binding over the range of protein concentrations was determined by densitometry of the radiographs. Signals were normalized for the specific activities of each RII α probe. Binding curves for AKAP 220 (○) and Ht 31 (●) are presented from three experiments; the standard deviation of the integrated density is indicated.

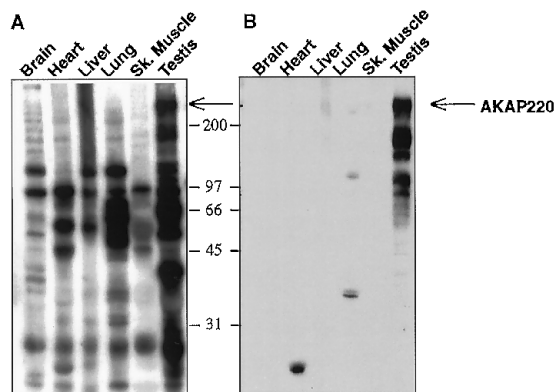


FIG. 5. **AKAP 220 is enriched in rat testis.** Detergent-solubilized extracts of several rat tissues (100 μ g of protein/lane) were separated on 7.5% SDS-PAGE and electrotransferred to PVDF. *A*, the solid-phase overlay method was used to detect RII binding proteins in these tissues. The blots were probed with ³²P-radiolabeled RII α , and the RII-binding proteins were detected by autoradiography. *B*, Western blot analysis was performed on identical filters as described under "Experimental Procedures." The migration of an immunoreactive protein that corresponds to AKAP220 is indicated by an arrow. Tissue sources are indicated above each lane, and molecular weight markers are indicated beside each panel.

are a growing family of functionally related proteins which serve to localize the type II regulatory subunit (RII) of PKA to certain subcellular locations (29, 30). Since there is little or no conservation in primary structure between AKAPs, the principle criterion used to identify these proteins is their ability to bind RII. Two predominant techniques are used: a solid-phase RII overlay assay and screening of bacterial expression libraries with RII as a probe (17). Overlay techniques have shown that most cell types express 5–10 AKAPs (23, 31), whereas other studies have demonstrated that hormonal stimulation or developmental signals promote the induction of certain anchoring proteins (15, 32). Likewise, RII expression cloning techniques have been used to isolate several cDNAs and 8 AKAP sequences have been published to date (13–15, 22, 23, 33, 34). One important property that has emerged from these cloning

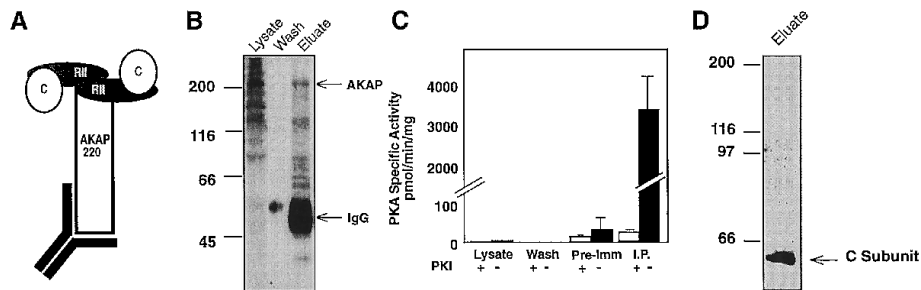


FIG. 6. AKAP 220 associates with type II PKA in rat testis. *A*, immunoprecipitation of the AKAP 220/PKA complex from rat testis was performed with 15 μ g of affinity-purified AKAP 220 antibody as described under "Experimental Methods." Fractions collected from the protein A-Sepharose column were separated by electrophoresis on a 7.5% SDS-PAGE gel and electrotransferred to PVDF. *B*, AKAP 220 was detected by Western blot. Sample sources are indicated above each lane. *C*, PKA activity was measured in all fractions by a filter paper assay using kemptide as a substrate. *D*, the catalytic subunit of PKA was specifically eluted from the immunoprecipitate with 0.5 mM anchoring inhibitor peptide, Ht 31 (493–515), and was detected by Western blotting.

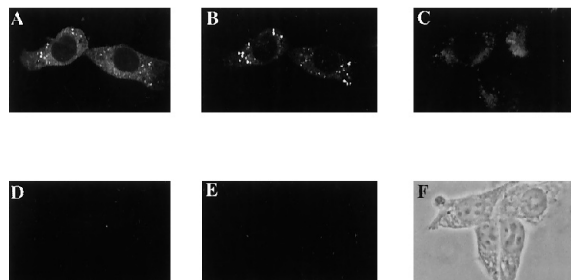


FIG. 7. Immunocytochemical analysis of rat testicular cells (TM4). TM4 cells were fixed with picric acid/paraformaldehyde and incubated with anti-RII antibodies (*A*), anti-AKAP 220 antibodies (*B*), anti-peroxisome 70-kDa protein antibodies (*C*), preimmune sera (*D*), or anti-AKAP 220 antibody preabsorbed with 10 μ g of purified AKAP 220 protein (*E*). *F*, a phase micrograph of TM4 cell is shown. Immune complexes were detected with fluorescein isothiocyanate-conjugated anti-rabbit secondary antiserum (*A*, *C*, *D* and *E*) and Texas red-conjugated anti-goat secondary antiserum (*B*). Double label immunofluorescence of RII (*A*) and AKAP 220 (*B*) were analyzed in the same focal plane.

studies is that all AKAPs isolated by interaction cloning retain the ability to bind RII under the denaturing conditions used in the overlay assay.

The putative RII binding site of AKAP 220 is located in the COOH-terminal third of the protein and probably includes residues 905–918. This sequence shares similarity with the RII binding regions of AKAP 150, Ht 31, and MAP 2 (14, 32, 34, 35). In addition, a recombinant fragment, including this region exhibits a similar affinity for RII α as the human thyroid anchoring protein, Ht 31 (27). Residues 905–918 are predicted to adopt a helical confirmation and exhibit a partitioning of hydrophobic and hydrophilic side chains when plotted on a helical wheel. All AKAPs seem to possess an amphipathic helix region that forms the principle site of contact with the RII dimer. Mutagenesis studies demonstrate that disruption of secondary structure by the insertion of prolines abolishes the interaction with RII (22, 27). Other evidence is that a peptide derived from residues 393–415 of Ht 31, which mimics the amphipathic helix, binds RII with nanomolar affinity to block the AKAP interaction (23). Since this peptide blocks RII-AKAP 220 interactions, it suggests that AKAP 220 binds the RII dimer in a manner similar to other AKAPs. AKAP 220 is, therefore, a prototypic AKAP based on its ability to bind RII.

Another hallmark of AKAPs is their anomalous migration on SDS-PAGE gels. In fact, AKAP 75, AKAP 95, and MAP 2 all display apparent molecular weights which are approximately double their calculated molecular mass (14, 36, 37). AKAP 220 follows this pattern as the calculated molecular weight from the cDNA sequence is 124,427, whereas the native protein migrates with a mobility of approximately 220 kDa on SDS-

polyacrylamide gels. Potential explanations for this discrepancy in apparent molecular weight may be related to the numerous acidic residues in the amino-terminal portion of the protein or by phosphorylation or other post-translation modifications.

Although most AKAPs share some common RII binding characteristics, each anchoring protein is targeted to specific subcellular sites (6). In recent years AKAPs have been identified that tether PKA to specific subcellular sites (13–15, 22, 23, 33–36). It appears that AKAP 220 may be targeted to peroxisomes through the last three residues of the protein, Cys-Arg-Leu. This sequence conforms to a peroxisomal targeting signal 1 motif (38–40). Work originally performed on the COOH terminus of the firefly luciferase has demonstrated that the sequence Ser-Lys-Leu is necessary and sufficient for peroxisomal targeting (39). Analysis of mammalian peroxisomal targeting motifs have determined that Leu is the only invariant residue in the triplet, whereas Ser can be replaced by Cys or Ala at the first position and Arg can be substituted for Lys at position two. Therefore, the Cys-Arg-Leu triplet in AKAP 220 is likely to facilitate peroxisomal localization of AKAP 220 and may represent its targeting domain (38–40). Our immunocytochemical analysis supports this association of AKAP 220 with testicular peroxisomes. Future studies are planned to establish whether the Cys-Arg-Leu triplet is sufficient for the peroxisomal targeting of AKAP 220 and whether the anchoring protein is an integral component of the peroxisomal matrix.

Peroxisomes are small organelles present in all cell types that function prominently in cellular lipid metabolism, such as the β -oxidation of fatty acids. These organelles have been associated with cAMP-responsive events such as androgen biosynthesis (41). In particular, there appears to be a direct correlation between testicular peroxisome volume and testosterone secretion (42, 43). However, the role of targeted PKA in peroxisomes is currently unknown. At least two classes of peroxisomes exist in most cells, these can be distinguished by catalase staining methods (44). Our double label immunofluorescence experiments, using the 70-kDa peroxisomal marker, show that AKAP 220 overlaps with only a portion of peroxisomes. At this time we do not know which subset of peroxisomes co-localize with AKAP 220; however, the association of AKAP 220 with rat testicular peroxisomes may provide a potential role for targeted PKA regulation of steroid biosynthesis.

AKAP 220 may also serve as an adaptor protein that co-localizes PKA with other signaling molecules. Until recently, AKAPs were thought to function exclusively in the localization of PKA. However, this view has been modified to include recent findings that demonstrate that AKAP 79 also binds the calcium/calmodulin-dependent phosphatase 2B, calcineurin (25). Likewise, another AKAP, MAP 2, has been shown to co-purify

with subcellular fractions that contain type 2A phosphatase (45). Co-localization of kinases and phosphatases may account for the exquisite modulation of certain phosphorylation events that are necessary for maintaining cellular homeostasis (46). In addition, proteins with the peroxisomal targeting signal have been shown to function as carrier proteins that piggy-back other molecules into peroxisomes (47). Therefore, AKAP 220 has the potential to regulate peroxisomal functions through multiple mechanisms. Future studies are under way to define the role of AKAP 220 and associated proteins in testicular signaling events.

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