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Identification of Sperm-specific Proteins That Interact with A-kinase Anchoring Proteins in a Manner Similar to the Type II Regulatory Subunit of PKA*

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The cAMP-dependent protein kinase (PKA) is targeted to specific subcellular compartments through its interaction with A-kinase anchoring proteins (AKAPs). AKAPs contain an amphipathic helix domain that binds to the type II regulatory subunit of PKA (RII). Synthetic peptides containing this amphipathic helix domain bind to RII with high affinity and competitively inhibit the binding of PKA with AKAPs. Addition of these anchoring inhibitor peptides to spermatozoa inhibits motility (Vijayaraghavan, S., Goueli, S. A., Davey, M. P., and Carr, D. W. (1997) *J. Biol. Chem.* 272, 4747–4752). However, inhibition of the PKA catalytic activity does not mimic these peptides, suggesting that the peptides are disrupting the interaction of AKAP(s) with proteins other than PKA. Using the yeast two-hybrid system, we have now identified two sperm-specific human proteins that interact with the amphipathic helix region of AKAP110. These proteins, ropporin (a protein previously shown to interact with the Rho signaling pathway) and AKAP-associated sperm protein, are 39% identical to each other and share a strong sequence similarity with the conserved domain on the N terminus of RII that is involved in dimerization and AKAP binding. Mutation of conserved residues in ropporin or RII prevents binding to AKAP110. These data suggest that sperm contains several proteins that bind to AKAPs in a manner similar to RII and imply that AKAPs may have additional and perhaps unique functions in spermatozoa.

PKA[1] is a ubiquitous, multifunctional kinase involved in the regulation of a diverse array of cellular events. The PKA holoenzyme consists of four subunits, two catalytic and two regulatory. The regulatory subunits form dimers through an interaction at the N terminus whereas the C terminus contains two tandem repeat sequences, which form the cAMP binding sites. Binding of cAMP to the regulatory subunits promotes the dissociation and activation of the catalytic subunits. A second function of the regulatory subunits is to target or anchor PKA to specific subcellular locations within the cell.

A major advance in signal transduction research in recent years is the understanding that the actions of many signaling molecules are spatially restricted and coordinated through cell- and function-specific targeting of these enzymes and their substrates (1). PKA is anchored to specific cellular compartments through the interaction of the regulatory subunit with a family of proteins referred to as A-kinase anchoring proteins (AKAPs) (reviewed in Refs. 2–4). Numerous AKAPs have been cloned and biochemically characterized. Several AKAPs have been shown to simultaneously bind to PKA and other signal transduction molecules such as calmodulin, protein phosphatase 1 (PP1), calcineurin (PP2B), and protein kinase C (5–9). This has led to a model in which AKAPs act as scaffolding molecules that coordinate the actions of several kinases and phosphatases all located within one cellular compartment.

The structural feature of AKAPs that promotes interaction with PKA has been known for some time (10). AKAPs contain an amphipathic helix region and bind to the type II regulatory subunit of PKA via the hydrophobic face. The identification of this binding domain has facilitated the design of reagents that have been used to determine experimentally the physiological consequences of the interaction of PKA with AKAPs. Synthetic peptides encompassing the amphipathic helix binding domain are potent competitive inhibitors of PKA·AKAP interaction (11) and therefore are referred to as anchoring inhibitor peptides (AIPs). Addition of AIPs to a variety of somatic cells inhibits PKA modulation of cellular events. For example, microinjection of AIPs into hippocampal neurons causes a time-dependent decrease in AMPA/kainate-responsive currents whereas control peptides had no effect on channel activity, suggesting that PKA anchoring is required for PKA modulation of the AMPA/kainate channels (12). Since this initial finding, several laboratories have used AIPs to demonstrate that anchoring is required for PKA modulation of L-type Ca^{2+} channels (13), calcium-activated potassium channels (14), insulin secretion from pancreatic beta cells (15), and phosphatidylinositide turnover in myometrial cells (16, 17). In all of the above examples, the action of AIPs is mimicked by the addition of reagents that inhibit the catalytic activity of PKA. These data support a model where AKAPs interact with the PKA regulatory subunit

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF231410 and AF239723.

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAPs, A-kinase anchoring proteins; RII, type II regulatory subunit of PKA; AIPs, anchoring inhibitor peptides; ASP, AKAP-associated sperm protein; FSII, fibrosheathin II; SP17, sperm protein 17; PKI, PKA inhibitor peptide; GST, glutathione *S*-transferase; RACE, rapid amplification of cDNA ends; kb, kilobase(s); PCR, polymerase chain reaction.

to anchor or target the catalytic subunit to relevant physiological substrates.

Several sperm AKAPs have been identified and characterized (18–27). The most prominent AKAP detected by RII overlay assay of bovine, human, mouse, and monkey spermatozoa is AKAP110 (20, 21, 27). Northern analysis suggests that AKAP110 is expressed only in spermatozoa, and immunofluorescence studies detecting AKAP110 in the flagella suggest that this protein may be involved in regulating motility. Addition of S-Ht31 (stearated-Ht31 is a cell permeable AIP) to bovine caudal epididymal spermatozoa inhibits motility in a time- and concentration-dependent manner (26). A control peptide, S-Ht31-P, identical to S-Ht31 except for an isoleucine to proline substitution that prevents amphipathic helix formation, had no effect on motility. Surprisingly, inhibition of PKA catalytic activity by addition of H-89 or S-PKI had little effect on basal motility or motility stimulated by agents previously thought to work via PKA activation (26). These data suggest that proteins interacting with sperm AKAPs regulate motility in a manner that is independent of PKA catalytic activity. These results have been confirmed by a different approach. McKnight and colleagues (28), using mutant mice lacking RIIα, have shown that the catalytic subunit is no longer located along the flagellum but instead is concentrated in the cytoplasmic droplet, yet the spermatozoa are motile and the mice are fertile. These data suggest that neither RIIα nor RIIα-dependent localization of PKA catalytic subunit is necessary to support motility. Thus, it appears that AIPs may be exerting an effect on spermatozoa that is independent of AKAP·RIIα interaction.

One hypothesis consistent with the above data is that sperm AKAPs are interacting with proteins other than RII via the amphipathic helix domain. This would explain how AIPs could be regulating motility in a manner independent of both the regulatory and catalytic subunits of PKA. Using the yeast two-hybrid system to screen a human testis library, we have now identified several sperm-specific proteins that bind to fragments of AKAP110 containing the amphipathic helix domain. Binding studies demonstrate that these proteins bind to AKAP110 in a manner homologous with RII. Based on these data, we propose a model for the function of AKAPs in spermatozoa that is very different than the model for AKAPs in somatic cells.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The yeast strain EGY48(p2op-lacZ) containing constructs of pLexA human AKAP110, truncated hAKAP110-(1–350), and human ropporin was transformed with a pB42AD-fused human testis cDNA library following protocols for the Matchmaker LexA Two-Hybrid System (CLONTECH). Approximately 2×10^8 transformants were screened with each construct on 150-mm plates containing 5×10^4 clones with SD/Gal/Raf/-His/-Trp/-Ura/-Leu/+Xgal + BU salts. Blue colonies, positive for β-galactosidase activity, were re-streaked on SD/-His/-Trp/-Ura plates, then replated on SD/Gal/Raf/-His/-Trp/-Ura/-Leu/+X-gal + BU salts to re-test for activation of both the lacZ and LEU2 reporter genes. Double positives were then plated on master plates containing SD/-His/-Trp/-Ura and used for yeast PCR and sequence analysis.

Purification of Recombinant Proteins—Ropporin and mutant ropporin (L18A) were expressed as pET30a N-terminal His_6-tagged fusion proteins in *Escherichia coli* (BL21(DE3)) and were purified by fast protein liquid chromatography using Hi-Trap chelating Sepharose columns (Amersham Pharmacia Biotech). One liter of LB broth + 30 μg/ml kanamycin was inoculated and grown to mid-logarithm phase before adding 1 mM isopropyl-1-thio-β-D-galactopyranoside to induce protein expression at 37 °C for 2 h. The cells were then pelleted by centrifugation at 2000 × *g* for 20 min, sonicated in buffer A on ice (20 mM HEPES, 500 mM NaCl, 20 mM imidazole, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, pH 7.4), and clarified by centrifugation at 15,000 × *g* for 20 min at 4 °C, and the resultant supernatant was passed through a 0.45-μm filter. The supernatant was then applied to the Ni^{2+}-charged Hi-Trap chelating column. Bound proteins were eluted with a stepwise gradient of imidazole (0–0.5 M) in buffer A. Fractions containing purified protein were identified by Coomassie Blue staining of 10% SDS-polyacrylamide gel electrophoresis gels. pET11d-RIIα and mutant RIIα (L14A) were expressed in *E. coli* (BL21(DE3)) and purified by cAMP-agarose affinity column as previously described (10).

Plasmids—Plasmids for expression of recombinant hAKAP110, hAKAP110-(1–350), mRIIα, h-ropporin, hASP, hSP17, and hFSII in a yeast two-hybrid system, as histidine-tagged or GST fusion proteins in *E. coli* were prepared as follows. The pET30a hAKAP110 full-length construct and its truncated form hAKAP110-(1–350) (27) were digested with *Eco*RI and *Xho*I, gel-purified, and ligated into pLexA, pB42AD (CLONTECH, Palo Alto, CA), and pGEX5x-1 (Amersham Pharmacia Biotech, Piscataway, NJ) vectors cut with the same restriction enzymes. PCR was performed on pET11d RIIα with forward primer 5′-CCGGAATTCATGAGCCACATCCAGATCCCG-3′ and reverse primer 5′-CCGCTCGAGCACACTGAGAAGGCTCCAAGATTC-3′, respectively, containing an *Eco*RI and *Xho*I restriction site. The PCR product was digested with *Eco*RI/*Xho*I, gel-purified, and ligated into pLexA and pB42AD. Full-length PCR products of ropporin, SP17, and FSII were obtained by 5′-RACE of a Marathon Ready Human Testis cDNA library (CLONTECH). Ropporin forward primer 5′-GGATTCATGGCTCAGACAGATAAGCCAACATG-3′ and reverse primer 5′-CCCTCGAGAATTGTGCTGTTACTCCAGCCAAACC-3′, SP17 forward primer 5′-AGATCCATGTCGATCCATTCTCCAACACCCA-3′ and reverse primer 5′-ATTTGCGGCCGCTGGAGGTAAAACCAGTGTCCTCACTTG-3′, FSII forward primer 5′-AGATCCATGTCGATCCATTCTCCAACACCCAC-3′ and reverse primer 5′-ATTTGCGGCCGCTGGAGGTAAAACCAGTCTCCTCACTTG-3′. Ropporin had *Eco*RI/*Xho*I restriction sites added to the primers, SP17 and FSII primers had *Bam*HI/*Not*I restriction sites added. Ropporin was digested and ligated into pET30a, pLexA, and pB42AD; SP17 and FSII were subcloned into pLexA. ASP was obtained using the yeast two-hybrid system and Human Testis Matchmaker LexA cDNA (CLONTECH), *Eco*RI/*Xho*I-digested, and ligated into pLexA and pET30a.

Northern Blot Analysis of Human ASP—A Mouse Multiple Tissue Northern blot (CLONTECH), containing 2 μg of poly(A)$^+$ RNA per lane, was screened using the full-length ASP fragment generated in the plasmids section. The probe was ^{32}P-labeled using the High Prime DNA labeling kit (Roche Molecular Biochemicals). Hybridization of the probe was carried out at 42 °C for 18 h in Ullrich's buffer/2% SDS. The blot was washed at room temperature 2× in SSC, 2× in SSC/2% SDS at 65 °C and finally in 0.1% SSC at room temperature. An exposure of the blot was then made on x-ray film for 72 h.

In Vitro Binding Assay of PKA Regulatory Subunit (RIIα) and Ropporin to AKAP110—E. coli transformed with pGEX-5X-1 plasmid encoding human AKAP110-(1–350) or AKAP110-P (containing a proline substitution for leucine 131) were grown to mid-logarithm phase at 37 °C in 1 liter of LB medium. They were cultured for an additional 2 h at 37 °C in the presence of 0.2 mM isopropyl-β-D-thiogalactopyranoside to induce synthesis of the fusion protein. Crude extracts were prepared by sonicating the bacteria in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride. 2% Tween 20 (v/v) was added to the extract and rotated at room temperature for 1 h. The extract was centrifuged at 15,000 × *g* for 20 min before passing the supernatant through a 0.45-μm filter. The supernatant was incubated for 30 min at room temperature with 3 ml of glutathione-Sepharose before extensive washing in phosphate-buffered saline to remove nonspecifically bound proteins.

Prior to the addition of purified proteins, 200 μl of the GST-hAKAP110-(1–350) was incubated with 750 μl of Blotto and 10 mM dithiothreitol for 30 min at room temperature. 1 μg of RIIα, RIIα (L14A), 3 μg of ropporin and 4 μg of ropporin (L18A) were added to separate tubes containing the Blotto/AKAP110 mix and rotated at room temperature for 2 h. After washing extensively with phosphate-buffered saline and 10 mM dithiothreitol, proteins were eluted by boiling in SDS gel-loading buffer and separated by 10% SDS-polyacrylamide gel electrophoresis. Regulatory subunit RIIα was detected by Western blotting using rabbit antisera against RIIα (6825) and secondary anti-rabbit horseradish peroxidase conjugate (Sigma Chemical Co., St. Louis, MO). Ropporin was detected by conjugated horseradish peroxidase-Anti-S-Protein (Novagen, Madison, WI).

PCR and Sequencing—PCR was done directly on diethyl pyrocarbonate-H_2O suspensions of yeast cell positives using vector-specific primers flanking the multiple cloning site of the pB42AD vector (29, 30). The resulting PCR products were gel-purified, and sequence analysis was performed using the Big-Dye Terminator Sequencing Kit (Applied Bio-

systems, Foster City, CA) and the same vector specific primers. The Y-Der yeast DNA extraction kit (Pierce, Rockford, IL) was used to recover plasmid DNA from positives that wouldn't amplify using the above method. Analysis of sequence data, sequence comparison, and alignments was performed using the MacVector ClustalW program (Oxford Molecular Group) and the BLAST program (31) provided by the NCBI server at the National Library of Medicine/National Institutes of Health.

Rapid Amplification of 5′-cDNA Ends (5′-RACE)—The 5′-RACE was performed using a Marathon cDNA amplification kit and Human Testis Marathon-Ready cDNA (CLONTECH) as described with the accompanying procedures. The primers used to obtain full-length PCR products were previously stated in the plasmids section.

Site-directed Mutagenesis—Plasmid constructs pET30a mouse ropporin, pET11d mouse RIIα, and pGEX5x-1 containing hAKAP110-(1–350) were used as PCR templates for the QuikChange 1-day site-directed mutagenesis method (Stratagene, La Jolla, CA). Aligned leucines (see Fig. 4*A* below) at positions 18 and 14 of ropporin and RIIα, respectively, were mutated to alanines using the following primers: Ropporin (L18A), forward primer 5′-TGCCGGAATTGGCAAAGCAGTTTAC-3′, reverse primer 5′-GTAAACTGCTTTGCCAATTCCGGCA-3′; RIIα (L14A), forward primer 5′-TCACGGAGCTGGCACAGGGCTACA-3′, reverse primer 5′-TGTAGCCCTGTGCCAGCTCCGTGA-3′. Leucine 131 within the amphipathic helix region of hAKAP110 was mutated to proline using the following primers: forward primer 5′-ATGCTAACCGCCCAACGAATCTAG-3′, reverse primer 5′-CTAGATTCGTTGGGCGGTTAGCAT-3′.

The resulting mutated DNA was transformed into *E. coli* Super Competent JM109 cells (Promega, Madison, WI) and grown on antibiotic-resistant LB Agar plates. The mutations were verified by sequence analysis using vector-specific primers.

Immunocytochemistry—Adult male mice were sacrificed by CO_2 asphyxiation, and a sperm suspension was obtained by mincing the cauda epididymides in buffered saline (145 mM NaCl and 5 mM Hepes, pH 7.4). Sperm were then fixed 30 min in 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4), attached to coverslips, permeabilized in absolute acetone for 10 min at −20 °C and air-dried. For immunostaining, cells were first incubated 1 h in blocking buffer of Tris-saline (TN = 150 mM NaCl, 25 mM Tris-HCl, pH 8.0, and 0.05% Tween 20) containing 2.5% bovine serum albumin and 5% goat serum and then successively incubated in primary and secondary antibodies also diluted in blocking solution. Between all incubation steps, coverslips were washed three times in TN containing 1% goat serum. Primary antibodies, prepared in rabbits, included affinity-purified IgG to AKAP110 and ropporin. Control samples substituted identical levels of affinity-purified non-immune rabbit IgG for immune IgG. Cy3-conjugated affinity-purified secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

RESULTS

Yeast Two-hybrid Screening of Human Testis cDNA Library—AKAP110 is a sperm-specific AKAP that binds to the type II regulatory subunit of PKA via an amphipathic helix-binding motif located at amino acid position 124–143 (27). To determine if other proteins also bind to AKAP110 via the amphipathic helix binding domain, a human testis cDNA library was screened using an N-terminal fragment of AKAP110-(1–350) as bait in a yeast two-hybrid procedure. Positives were then co-transformed in the two-hybrid system with another fragment of AKAP110-(349–660) that does not contain an amphipathic helix domain. Three positives were identified that bound to the amphipathic helix-containing fragment (1–350) but not to the other fragment (349–660) (Fig. 1). The pB42AD plasmids were recovered from these clones, and the cDNA inserts (1.4, 1.3, and 1.1 kb in size) were sequenced.

Sequence Analysis of AKAP110 Binding Proteins—Sequence analysis demonstrated that the 1.4-kb insert encodes for the human type II regulatory subunit of the cAMP-dependent protein kinase, RIIα (GenBank™ accession number NM004147). The first base of the insert lines up with base 142 of the deposited sequence, which lists the coding region as 190–1404. Thus, the 1.4-kb insert contains the full-length coding region for PKA RIIα protein plus an additional 16 amino acids from the 5′-untranslated region. Although this additional region would normally not be translated, there are no stop codons within this segment, and thus these additional amino acids functioned as a bridge between the pB42AD fusion partner and RIIα.

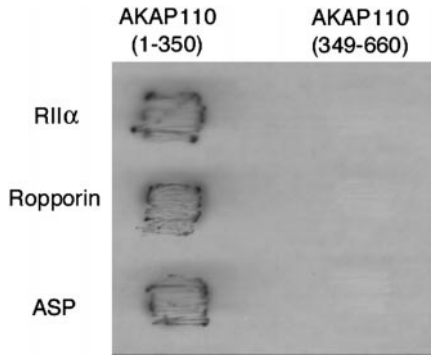

FIG. 1. **Fragments of AKAP110 containing the amphipathic helix binds RII, ropporin, and ASP.** A human testis library was screened using AKAP110-(1–350) as bait. Positives included RIIα, ropporin, and AKAP-associated sperm protein (*ASP*). These positives were then co-transformed with a fragment of AKAP110-(349–660) that does not contain the amphipathic helix. The transformants were plated on a selective medium and subjected to β-galactosidase assay.

The sequence obtained from the 1.3-kb insert is 94% identical to the amino acid sequence of the murine protein ropporin (GenBank™ accession number AF178531), which we previously isolated as a binding partner of a Rho effector, rhophilin (32). The human ropporin (h-ropporin) sequence has been submitted to the GenBank™ data base (accession number AF231410). Ropporin is a sperm-specific protein localized in the principal piece and the end piece of sperm flagella (32). Ropporin forms homodimers and binds to rhophilin, and both proteins have been shown to co-precipitate *in vitro* with Rho (32). Once again, in addition to sequence that was homologous to the coding region for m-ropporin, the pB42AD insert contained sequence upstream (165 bases) of the ropporin start site, adding 55 amino acids of 5′-untranslated repeat as a bridge between the pB42AD fusion protein and the h-ropporin.

The sequence obtained from the 1.1-kb insert is a novel protein. It is 39% identical to h-ropporin but does not match any other protein in the GenBank™ data base and will hereafter be referred to as AKAP-associated sperm protein or ASP. ASP also contains a 5′-untranslated repeat bridge (57 bases, 19 amino acids) between the vector and the start site. This sequence has been submitted to the GenBank™ data base (accession number AF239723). The optimal alignment (MacVector ClustalW Alignment Program) of m-ropporin, h-ropporin, and ASP is shown in Fig. 2. ASP is highly homologous with the N-terminal 80 residues of ropporin and only moderately homologous with the rest of the molecule, suggesting the N terminus contains a conserved domain that may have an important function in spermatozoa.

Tissue Distribution of ASP mRNA—We have previously shown that m-ropporin is detected only in the testis and then only in the most inner part of the seminiferous tubules region, suggesting this protein is expressed in developing spermatozoa (32). To determine the tissue distribution of ASP, Northern blots containing 2 μg of poly(A)$^+$ RNA from eight different adult mouse tissues were probed with ^{32}P-labeled ASP cDNA. A single message was detected only in the testis (Fig. 3), suggesting this protein is testis-specific and possibly sperm-specific. Using a linear regression analysis of a plot of the $\log_{10}$ *versus* the R_F of the molecular weight markers, the ASP mRNA was calculated to be ~1.05 kb. This was bigger than expected based on the insert size (850 bases) from the yeast vector. To

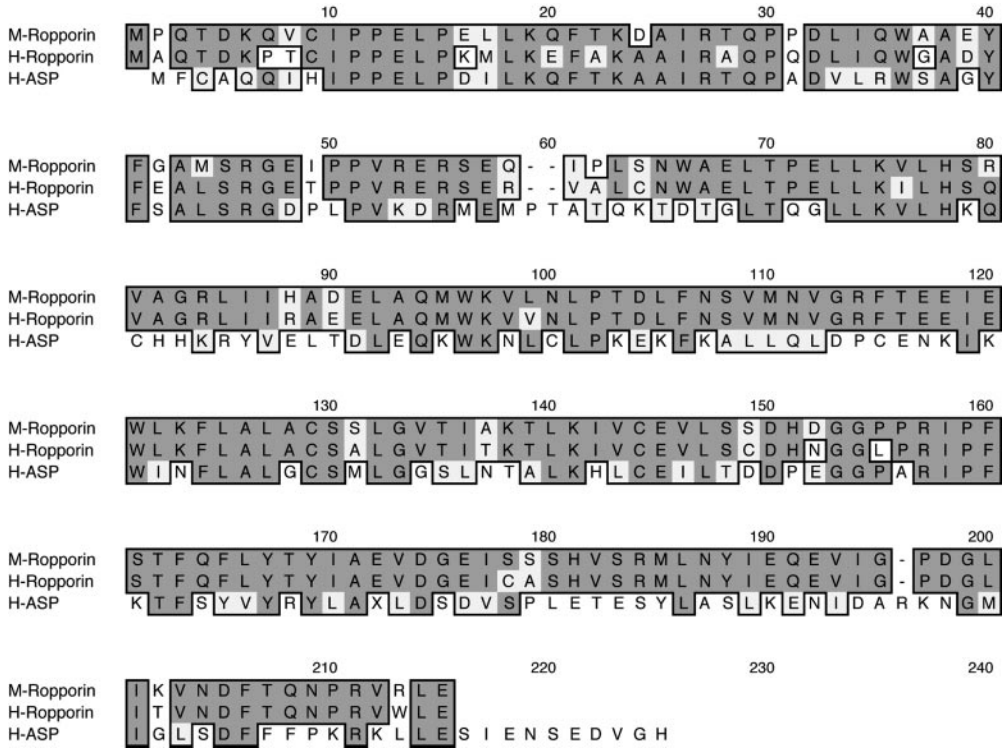

FIG. 2. **Amino acid sequence homology of mouse ropporin, human ropporin, and AKAP-associated sperm protein (*ASP*).** Identical amino acids shared by more than one protein are *shaded dark gray*. Functionally similar amino acids are *shaded light gray*.

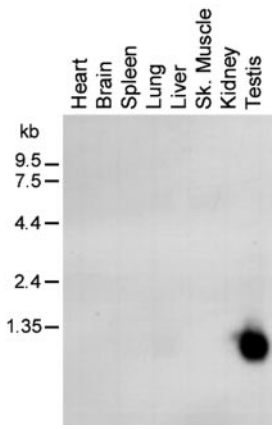

FIG. 3. **Northern blot analysis of tissue distribution of ASP mRNA expression.** A Northern blot containing 2 μg of poly(A)$^+$ RNA per lane was probed with ^{32}P-labeled ASP cDNA and detected by autoradiogram. The tissue source of the RNA is indicated at the *top* of each lane, and the positions of the molecular size markers (bp $\times$ 10^{-3}) are shown on the *left*.

determine if the insert represented full-length cDNA for ASP, 5′-RACE was performed using Human Testis Marathon-Ready cDNA (CLONTECH) as template. An additional 144 bases were identified using this technique, bringing the total number of bases to 994 or approximately the same size as the calculated mRNA. Although this new region contains an open reading frame continuous with the rest of the protein, it does not contain an alternate start site. At the time of submission of this manuscript, a BLAST search of the human genome data base does not detect either human ropporin or ASP.

Alignment of Sperm Proteins with RII—As mentioned above, the N-terminal regions of ropporin and ASP are the most conserved. The sequence in this region is also similar to the N terminus of the type II regulatory subunit of PKA and two other sperm-specific proteins, SP-17 (GenBank^{TM} accession number Q15506) (33, 34) and fibrousheathin II (FSII) (GenBank^{TM} accession number NM_012189).[2] Optimal alignment (MacVector ClustalW alignment program) of RIIα and RIIβ (residues 1–46 or 45, respectively, of both human and mouse) with the corresponding regions of ropporin, ASP, SP17, and FSII is shown in Fig. 4*A*. The *dark gray shading* indicates sequence identity, and the *light gray shading* indicates sequence similarity. Although these sperm proteins share high sequence similarity with the N-terminal region of RII, they have little or no homology with other regions of RII such as the nucleotide binding domains. The only other protein identifiable by sequence homology, sharing the characteristics of having an AKAP binding domain but not a cyclic-nucleotide binding domain, is a hypothetical protein from *Caenorhabditis elegans* F39H12.3. The location of this protein in *C. elegans* is still undetermined.

The N-terminal 44 amino acids of RII contain the domains responsible for homodimerization and binding to AKAPs (Fig. 4*B*). The sequence identity of h-RIIβ, h-ropporin, hASP, hSP17, hFSII, and hRIα with hRIIα (1–44) is 70, 30, 32, 45, 34, and 18%, respectively. It is interesting to note that all of these newly identified AKAP-binding proteins have a higher sequence homology with RII than RI does with RII (RI is not even picked up in the position-specific iterated BLAST search), suggesting that all of these proteins may bind AKAPs with a higher affinity than RI. The positions that are most conserved within this region are those that have been identified as being key residues involved in either homodimerization or AKAP binding (35–37). For example, Li and Ruben (36) showed that substitution of Ala for Leu-13 or Phe-36 in RIIβ generates monomeric RIIβ that cannot bind to AKAPs. Both of these residues are 100% conserved in all of the above proteins.

Functional Comparison of RIIα with Ropporin—In vitro binding assays were performed to determine if ropporin interacts with AKAPs in a manner functionally homologous with

[2] A. Mandal, M. J. Wolkowicz, S. Naaby-Hansen, and J. C. Herr, unpublished data.

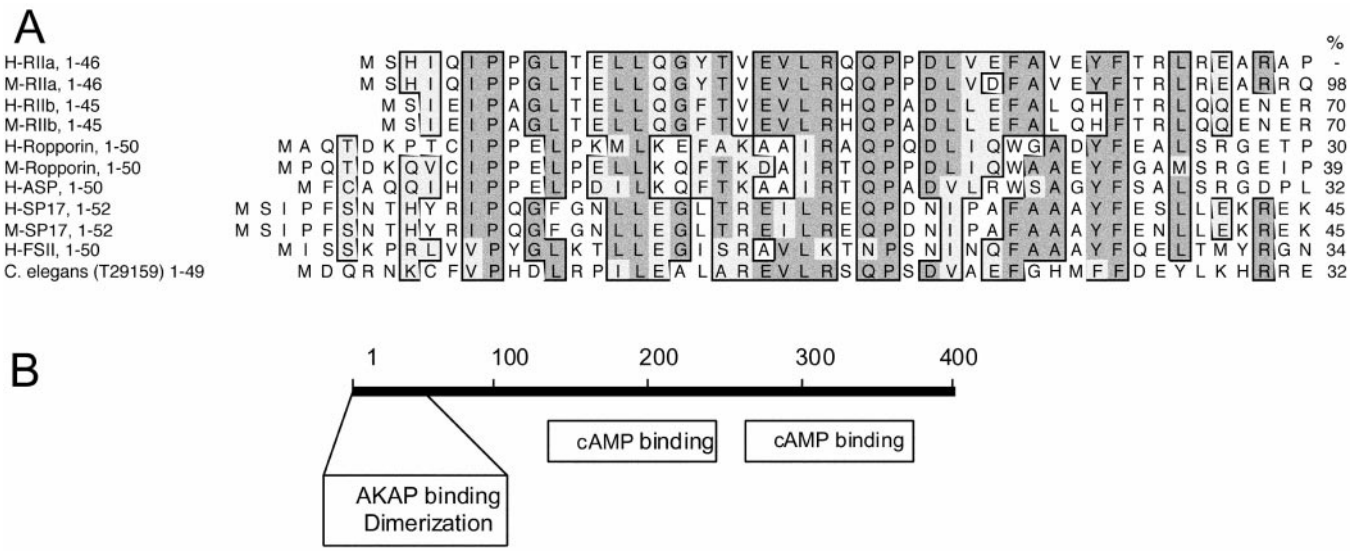

FIG. 4. **Sequence alignment of the AKAP binding domain of RII with ropporin, ASP, SP17, FSII, and an unknown *C. elegans* protein.** *A*, the N terminus of RIIα, RIIβ, ropporin, ASP, SP17, FSII, and a *C. elegans* protein were aligned using the MacVector ClustalW software program. Identical amino acids shared by more than five proteins are *shaded dark gray*. Functionally similar amino acids are *shaded light gray*. The percent sequence identity of each protein compared with human RIIα is shown in the *right-hand column*. *B*, the location of the AKAP binding, dimerization, and cAMP binding domains of RIIα are shown.

RIIα. As mentioned above, mutation of Leu-13 (Leu-13 in RIIβ is equivalent to Leu-14 in RIIα) to Ala inhibits RII binding to AKAPs (36). To confirm these results, a pull-down assay using GST-AKAP110 was performed. Wild type RIIα bound to AKAP110, but the RIIα alanine mutant did not, even in the presence of excess mutant protein (Fig. 5*A*). A comparable mutant was made for ropporin, substituting Ala for Leu-18 (see alignment in Fig. 4*A*). As with RIIα, only the wild type ropporin bound to AKAP110 (Fig. 5*B*). One characteristic of RIIα·AKAP interaction is that disruption of the secondary structure of the amphipathic helix region, by insertion of a proline residue, disrupts binding. Incubation of either RIIα or ropporin (wild type or Ala mutant) with AKAP110-P (proline substituted for leucine at position 130) did not produce any detectable interaction.

Colocalization of AKAP110 and Ropporin in Mouse Spermatozoa—Cauda epididymal mouse spermatozoa immunostained with antibodies to AKAP110 exhibited specific staining of both the principal piece segment and of the dorsal margin of the acrosomal segment (Fig. 6, *A* and *B*); specific staining was not detectable in other sperm segments. Cauda epididymal spermatozoa immunostained with anti-ropporin exhibited specific staining of the principal piece segment and as well as of the cytoplasmic droplet located at the distal end of the midpiece (Fig. 6, *C* and *D*). Control samples exposed to non-immune rabbit IgG exhibited no detectable fluorescence (not shown). These data indicate that AKAP110 and ropporin are both located in the principal piece. In addition, each protein appears to uniquely occupy another compartment, AKAP110 in the acrosome and ropporin in the cytoplasmic droplet and midpiece.

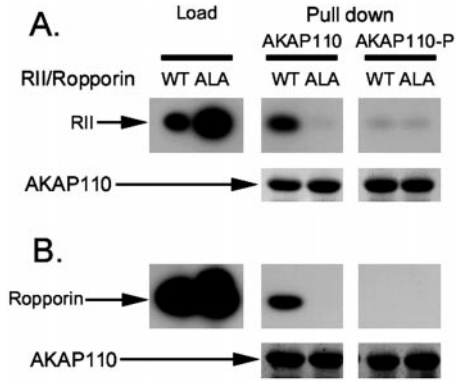

FIG. 5. ***In vitro* binding of wild type and mutant RIIα and ropporin to immobilized wild type and mutant AKAP110.** An equal amount of AKAP110-(1–350) or AKAP110-P (1–350 with a proline for leucine substitution at position 131) was conjugated to glutathione beads (*AKAP110* with *arrows* indicates Coomassie Blue stain of AKAP110 or AKAP110-P immobilized on each column). *A*, cell lysates containing wild type RIIα or mutant RIIα (*ALA*, indicating a substitution of alanine for leucine 14) or ropporin (*B*) (*WT* or alanine for leucine 18) were incubated with the AKAP110 or AKAP110-P beads and then washed. Bound proteins were precipitated and subjected to Western blot analysis of total lysates (*Load*, *left panel*), AKAP110 pull-down (*middle panel*), or AKAP110-P pull-down (*right panel*). The positions of RII and ropporin are shown by *arrows*.

DISCUSSION

In somatic cells, experimental data suggest that AKAPs anchor or target PKA to key physiological substrates. Disruption of PKA·AKAP interaction in a cell results in the loss of cAMP/PKA modulation of specific events. For example, oxytocin stimulates uterine smooth muscle contraction by increasing phosphatidylinositide turnover, which in turn promotes the release of intracellular calcium. Addition of cAMP to myometrial cells inhibits the action of oxytocin by stimulating PKA activity. However, cAMP does not inhibit oxytocin if the myometrial cells have been pretreated with anchoring inhibitor peptides (AIPs) or with PKA inhibitors such as H-89 (17). These data support a model in which AKAPs interact with the PKA regulatory subunit to anchor the catalytic subunit to a location within the cells where it is available to phosphorylate the appropriate substrate if and when it becomes activated.

Although AIPs are potent inhibitors of bovine sperm motility (26), several lines of evidence suggest that the function of AKAPs in sperm is different from somatic cells. First, PKA inhibitors such as H-89 or S-PKI have little or no effect on bovine sperm motility (26), and second, altering the subcellular location of the PKA catalytic subunit using targeted gene disruption of the type II regulatory subunit does not significantly effect mouse sperm motility (28). These data support our hypothesis that sperm AKAPs are interacting with proteins other than PKA. To be consistent with the above data, this new

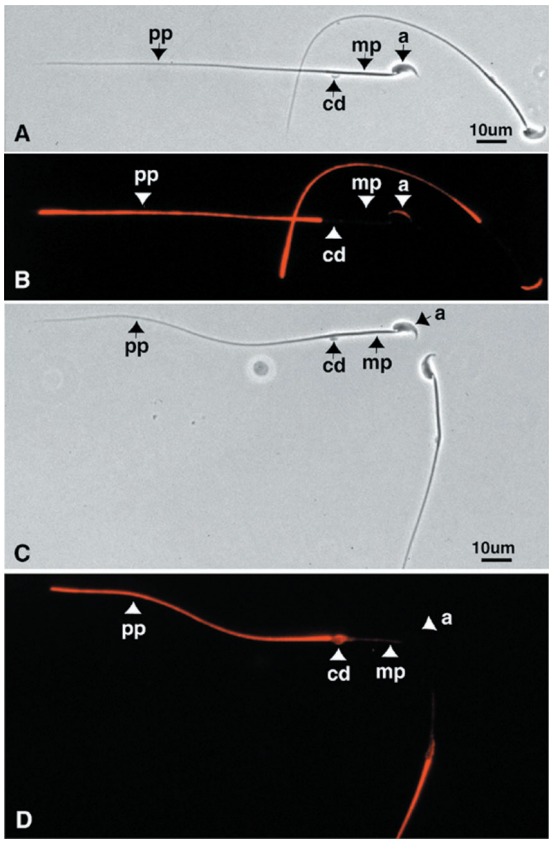

FIG. 6. **Colocalization of AKAP110 and ropporin in sperm flagellum.** Matched phase contrast (*A* and *C*) and fluorescence images (*B* and *D*) of cauda epididymal spermatozoa immunostained with anti-AKAP110 (*A* and *B*) and anti-ropporin (*C* and *D*). Both antibodies give positive staining of the principal piece segment of the flagellum (*pp*). Positive staining of the dorsal surface of the acrosome (*a*) is detected with anti-AKAP110, and positive staining of the cytoplasmic droplet (*cd*) is noted with the anti-ropporin antibody. *mp*, midpiece segment. All photographs were taken at the same magnification, and the *bar* in *A* and *C* represents 10 μm.

interaction would have to involve the amphipathic helix domain of the AKAPs and therefore be subject to disruption by the addition of AIPs. To determine if sperm AKAPs interacted with proteins other than RII, a human testis library was screened using a fragment of AKAP110 that contained the amphipathic helix domain. Three proteins were identified that bound to this fragment, but not other fragments, of AKAP110. One protein was the regulatory subunit of PKA, RIIα. A second protein was the human homolog of mouse ropporin (32), and the third was a novel protein with 39% sequence similarity to ropporin. We have named the novel protein AKAP-associated sperm protein or ASP.

Both ropporin and ASP share strong sequence similarity with the AKAP binding domain of RIIα, suggesting that, like RIIα, they interact with the amphipathic helix region of AKAPs. Using BLAST position-specific iterated searches, two other sperm proteins, SP17 and fibrosheathin II, have also been identified as having sequence similarity with RII in this region, suggesting spermatozoa contain at least four proteins capable of binding the amphipathic helix region of AKAPs. These other proteins do not contain consensus motifs that would indicate they bind to either cyclic nucleotides or the catalytic subunit of PKA. The amino acids that are most conserved between RII and these other four proteins are the same amino acids that have been identified as being important for RII dimerization and RII·AKAP interaction (35, 36). Site-directed substitution of alanine for leucine at position 14 in RIIα or the equivalent position in ropporin disrupted the interaction of these proteins with AKAP110. Likewise, proline substitution at position 132 within the amphipathic helix region of AKAP110 disrupted interaction with both RIIα and ropporin. These data suggest that ropporin and the other molecules are functional homologs of RIIα in their ability to bind to AKAPs. Thus, it appears that the classic model of AKAP function in somatic cells is fundamentally different in spermatozoa.

The main function of somatic cell AKAPs is to anchor or target the PKA catalytic subunit through an interaction with the regulatory subunit. No other somatic cell proteins have yet been identified that would compete with RII for binding to AKAPs. In contrast, sperm AKAPs can bind RII, but their ability to regulate sperm functions such as motility and the acrosome reaction appears to be independent of the location of the PKA catalytic subunit. In addition, all of the sperm RII homologs could potentially compete with RII, or each other, for binding to sperm AKAPs. These data suggest that sperm AKAPs may have additional and perhaps unique functions compared with somatic cells. Although a more exhaustive study will need to be performed, present data suggest that spermatozoa are the only cells that contain proteins, other than RII, that interact with the amphipathic helix domain of AKAPs. The fact that sperm expresses at least four of these proteins indicates they have evolved a unique and important use for this interaction. It is possible that the different AKAP-binding proteins might act on different pathways. Several AKAP-binding proteins (RIIα, ropporin, SP17, and FSII) are located in the flagellum, suggesting any or all of them may be involved in regulating motility. Both AKAP110 and SP17 have been detected in the acrosomal region of the head, implying these proteins may be involved in regulating head associated functions such as the acrosome reaction or sperm/egg binding.

One possible role of sperm AKAPs might be to function as a scaffolding protein for the Rho-GTPase pathway. AKAP110 and ropporin co-localize in the sperm flagellum. Ropporin was originally identified in a yeast two-hybrid screen of a mouse testis library using rhophilin as bait (32). Rhophilin serves as an adaptor protein capable of binding both ropporin and Rho simultaneously. Rho is a small GTPase that functions as a molecular switch, regulating various cellular processes such as cell adhesion, gene expression and cytokinesis, smooth muscle contraction, and motility (38). Rho has also been shown to regulate protein phosphatases through activation of Rho-kinase (39). Regulation of myosin phosphatase by Rho and Rho-kinase controls smooth muscle contraction and actin/myosin interaction in non-muscle cells (40). We hypothesize a similar pathway may exist in sperm. Evidence supporting this hypothesis includes: 1) inhibition of Rho by ADP ribosylation using C3 exoenzyme inhibits bovine sperm motility (41); and 2) addition of AIPs to spermatozoa produces a 2-fold increase in PP1γ2 activity and inhibits motility.[3] PP1γ2, a unique sperm serine/threonine phosphatase, is a key biochemical factor regulating motility (42, 43). Several AKAPs have been shown to interact with phosphatases (44), including AKAP220 (9), which is expressed in spermatozoa (25) and shares a 33% sequence similarity with AKAKP110. These observations suggest that Rho may regulate sperm motility via a mechanism similar to the one controlling smooth muscle contraction and that AKAP110 or other sperm AKAPs may function as scaffolding molecules controlling the location of Rho, rhophilin, ropporin, and possibly Rho-kinase and PP1. Further experiments will be needed to confirm the precise role of sperm AKAPs and the Rho-GTPase pathway in regulating sperm motility.

[3] S. Vijayaraghavan and D. W. Carr, unpublished results.

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Identification of Sperm-specific Proteins That Interact with A-kinase Anchoring Proteins in a Manner Similar to the Type II Regulatory Subunit of PKA

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