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Localization of a Novel Human A-Kinase-Anchoring Protein, hAKAP220, during Spermatogenesis

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Using a combination of protein kinase A type II overlay screening, rapid amplification of cDNA ends, and database searches, a contig of 9923 bp was assembled and characterized in which the open reading frame encoded a 1901-amino-acid A-kinase-anchoring protein (AKAP) with an apparent SDS–PAGE mobility of 220 kDa, named human AKAP220 (hAKAP220). The hAKAP220 amino acid sequence revealed high similarity to rat AKAP220 in the 1167 C-terminal residues, but contained 727 residues in the N-terminus not present in the reported rat AKAP220 sequence. The hAKAP220 mRNA was expressed at high levels in human testis and in isolated human pachytene spermatocytes and round spermatids. The hAKAP220 protein was present in human male germ cells and mature sperm. Immunofluorescent labeling with specific antibodies indicated that hAKAP220 was localized in the cytoplasm of premeiotic pachytene spermatocytes and in the centrosome of developing postmeiotic germ cells, while a midpiece/centrosome localization was found in elongating spermatocytes and mature sperm. The hAKAP220 protein together with a fraction of PKA types I and II and protein phosphatase I was resistant to detergent extraction of sperm tails, suggesting an association with cytoskeletal structures. In contrast, S-AKAP84/D-AKAP1, which is also present in the midpiece, was extracted under the same conditions. Anti-hAKAP220 antisera coimmunoprecipitated both type I and type II regulatory subunits of PKA in human testis lysates, indicating that hAKAP220 interacts with both classes of R subunits, either through separate or through a common binding motif(s).

Key Words: **cAMP-dependent protein kinase; A-kinase-anchoring protein; centrosome; sperm; midpiece.**

INTRODUCTION

Physiological effects of a large number of hormones and neurotransmitters are mediated by protein kinases and protein phosphatases via tightly controlled phosphorylation and dephosphorylation events. Key cellular processes, including cell growth and differentiation, metabolism, and sperm motility, are regulated by cyclic AMP (cAMP)[2] (Scott, 1991; Francis and Corbin, 1994). The primary target for cAMP is cAMP-dependent protein kinase (PKA), a tetrameric enzyme containing a regulatory (R) subunit dimer and two catalytic (C) subunits (Beebe and Corbin, 1986). Four genes encoding human R subunits (RIα, RIβ, RIIα, and RIIβ) and three genes encoding human C subunits (Cα, Cβ, and Cγ) have been identified (Taskèn *et al.*, 1994). Binding of four cAMP molecules, two to each R subunit, activates the PKA holoenzyme by release of catalytically active monomeric C subunits.

PKA has broad substrate specificity. Despite this, PKA has the ability to selectively phosphorylate individual substrates in response to discrete hormonal stimuli. Increasing evidence suggests that anchoring of the R subunit of PKA to A-kinase-anchoring proteins (AKAPs) targets PKA in close proximity to relevant substrates and conveys specificity in the cAMP/PKA signaling pathway (Scott, 1991; Rubin, 1994). Numerous different AKAPs have been identified from various tissues and species (Colledge and Scott, 1999).

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[2] Abbreviations used: cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; AKAP, A-kinase-anchoring protein; C, catalytic, and R, regulatory subunit of PKA; FSC1, fibrous sheath component 1; SOB1, sperm–oocyte binding protein 1; PBS, phosphate-buffered saline.

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Activation of PKA by cAMP elicits initiation and maintenance of flagellar movement in mature spermatozoa (Tash and Means, 1982; Brokaw, 1987). Although the underlying mechanisms are still unknown, sperm have been shown to contain a distinct adenylyl cyclase (Gordeladze and Hansson, 1981; Buck *et al.*, 1999), PKA type I and II isozymes (Landmark *et al.*, 1993), and phosphodiesterases (Salanova *et al.*, 1999), implying that sperm have the machinery to generate and execute cAMP effects. Also, protein phosphatase 1 $\gamma 2$ (PP1$\gamma 2$) is present in sperm and has been shown to influence sperm motility (Smith *et al.*, 1996), indicating that dephosphorylation events may also be important for sperm function. Furthermore, anchoring of PKA through AKAPs to distinct intracellular sites in sperm is believed to be essential for regulating sperm motility since disruption of the AKAP–PKA interaction results in motility arrest (Vijayaraghavan *et al.*, 1997). Several AKAPs have been identified in sperm, notably in the flagellum (Carrera *et al.*, 1994; Vijayaraghavan *et al.*, 1999; Miki and Eddy 1998), midpiece (Lin *et al.*, 1995), and acrosomal region (Vijayaraghavan *et al.*, 1999). Thus, anchoring of PKA may be essential for sperm function and consequently for fertility.

In the present study, we describe the cloning and characterization of several overlapping human cDNA clones encoding a 1901-amino-acid protein, hAKAP220, that binds PKA RI and RII subunits. hAKAP220 has an apparent molecular weight of 220 kDa and shares high homology with the C-terminus of rat AKAP220, but extends 727 amino acids farther into the N-terminus. The hAKAP220 protein is expressed throughout spermatogenesis. Immunostaining of hAKAP220 dynamically redistributes from a granular cytoplasmic staining in premeiotic germ cells to a centrosomal localization in postmeiotic cells and to the midpiece/centrosome area in mature sperm. There, hAKAP220 anchors both RI and RII and partially cofractionates with PP1. The data argue toward the establishment of a functional signaling unit in the midpiece of spermatozoa during spermatogenesis.

MATERIALS AND METHODS

Screening and isolation of cDNA clones. A total of 10^6 recombinant clones from a human Jurkat cell cDNA library in Lambda ZAP Express *Eco*RI (Stratagene, La Jolla, CA; Cat. No. 938200) were screened using an overlay technique with a ^{32}P-labeled recombinant RIIα protein (see Bregman *et al.*, 1989). The cDNA was retrieved from Lambda ZAP into the plasmid pBK-CMV by helper phage infection as outlined by the manufacturer, and the insert-containing plasmid was used for sequencing.

5′-Rapid amplification of cDNA ends (RACE). Human testis RACE-Ready cDNA was purchased from Clontech Laboratories, Inc. (Palo Alto, CA; Cat. No. 7414-1). Primer RACE1 (5′-GCCTGATCACAGTGGGAAAATGGAG-3′, nucleotides +2747 to +2771), anchor-specific primer (AP1; Clontech), and the Clontech Advantage PCR kit (Clontech Cat. No. K1905-1) were used in a PCR with 30 cycles of denaturation at 94°C for 30 s and with annealing and extension at 68°C for 4 min. Three parallel, independent PCRs were performed, the 2945-bp PCR products were subcloned, and three independent clones were sequenced.

PCR on human testis cDNA. Human testis RACE-Ready cDNA (Clontech; Cat. No. 7414-1) was used in a PCR with PCR primers 5′-GGCTGTCACGTTTTCCCCTTCTTTTCACAATCA-3′, nucleotides +2214 to +2246, and 5′-TCTTCCCTGAGTGC-TCCGACTTCACATCCA-3′, nucleotides +3554 to +3583, or 5′-CTGTCACGTTTTCCCCTTCTTTTCACAATCAAG-3′, nucleotides +2216 to +2248, and 5′-CTTCTTCCCTGAGT-GCTCCGACTTCACATC-3′, nucleotides +3556 to +3585, using the Clontech Advantage PCR kit. Thirty cycles were performed with denaturation at 94°C for 30 s and annealing and extension at 68°C for 4 min. Amplification products were subcloned and sequenced.

Sequencing of cDNA clones. Plasmids from the overlay screening and 5′-RACE and PCR products containing AKAP220 cDNA fragments were sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) using cycle sequencing protocols (ThermoSequenase kit, Cat. No. US79760; Amersham Life Science Inc., Cleveland, OH 44128), ^{33}P-labeled dideoxynucleotides (Cat. No. AH9539; Amersham), and a combination of vector- and insert-specific primers. Nucleotide and amino acid sequence data were analyzed using the GCG program package (program manual for the Wisconsin package, version 8, September 1994; Genetics Computer Group, Madison, WI 53711).

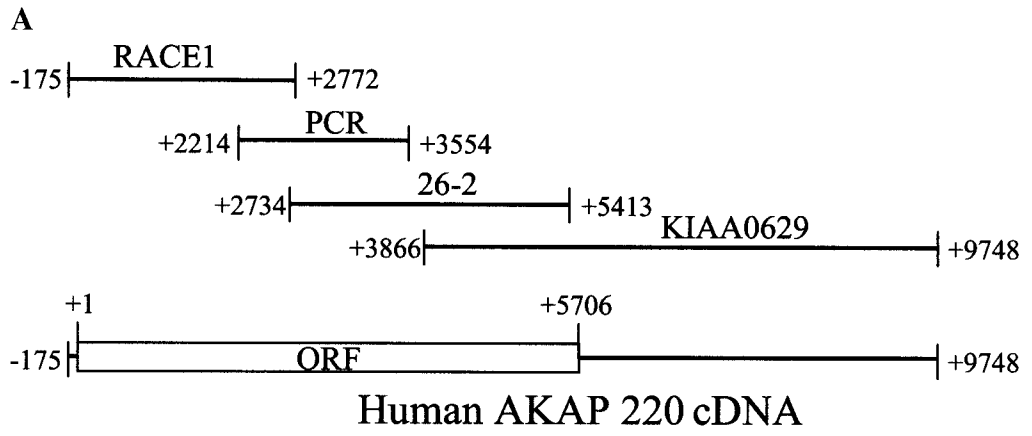
Preparation of tissues. Human testis and liver tissues were obtained from transplantation donors. Tissues were decapsulated and homogenized in homogenization buffer (20 mM potassium phosphate, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 50 mM benzamidine) by the use of an Ultra Thurrax homogenizer (Janke and Kunkel GmgH and Co., Staufen, Germany) and used for Western blot analysis, RII overlay, and immunoprecipitation.

Fractionation of germ cells from human testis. Germ cells were isolated by combined treatment with trypsin and DNase (Grootegoed *et al.*, 1977), followed by separation using a centrifugal elutriation method essentially as described elsewhere (Blanchard *et al.*, 1991). Purity of the cell fractions was evaluated by analysis of DNA content by flow cytometry and phase contrast microscopy.

Sperm fractionation. Mature motile sperm from human donors were purified using the swim-up procedure (Wikland *et al.*, 1987). Removal of sperm heads was performed by sonicating sperm for 3 min in PBS and subsequent sedimentation of heads at 1500*g* for 5 min. For Triton X-100 extraction, sperm or sperm tails were extracted 30 min with 1% Triton X-100 essentially as described elsewhere (San Agustin *et al.*, 1998).

Northern analysis. Total RNA from human germ cell fractions was extracted by the guanidine isothiocyanate/CsCl method as previously described (Knutsen *et al.*, 1996). Northern analysis was performed using 10–20 μg RNA/lane. Ethidium bromide staining of the gel verified the loading in each lane. Filters with human germ cell fractions and tissue blots purchased from Clontech (human multiple-tissue blot, Cat. Nos. 7759-1h and 7759-1H) were probed with ^{32}P-labeled 2676-bp AKAP220 cDNA (clone 26-2) from the overlay screening. In addition, the multiple-tissue blots were probed with a ^{32}P-labeled probe for chicken β-actin.

Antibodies. Two anti-hAKAP220 antisera, designated αAKAP220 [1112-1127] and αAKAP220 [159-179], were made by immunizing rabbits with hemocyanin-coupled synthetic peptides (antigen EP 980098, NH_2-SSEWDIKKLTKKLKGEC-$CONH_2$, and EP 980911, NH_2-FLHQKHQLETTDEDDDDTNQS-$CONH_2$; Eurogentec, Seraing, Belgium) corresponding to amino acids 1112 to



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1    MATFRNNHMKTKASVRKSFSEDVFQSVKSLLQSQKELCSVTAEDCLQQDEHANLTEVTFLGFNEETDAAHIQDLA 75
76   AVSLELPDILNSLHFCSLNENEIICMKNINKPLDISSDPLNQSHPSGMLCVMRVSPTSPRLRIDFIFSLLSKYAT 150
151  GIRYTLDTFLHQKHQLETTDEDDDDTNQSVSSIEDDFVTAFEHLEEEETSKPYNDGMNITVLRSQCDAASQTVTG 225
226  HHLETHDLKILISSGQQKSLAKPSTSSVNVLGHKELPSVKTSVTTSISEPWTQRSFYRSSNASDKDSDLQKTFFS 300
301  SSPAYSSESECSSPSPVIFLDEEGYQKSLKAKLELPKIPVMKDDIEDSDSEVSEFFDSFDQFDELEQTLETCLFN 375
376  KDPVIGKSSQRKGHKHGKSCMNPQKFKFDRPALPANVRKPTPRKPESPYGNLCDAPDSPRPVKASREDSGLFSPI 450
451  RSSAFSPLGGCTPAECFCQTDIGGDRIHENHDSVYYTYEDYAKSISCEVLGSVLRTHHTNTLSNINSIKHGENKT 525
526  VTFKHGNLDQKNKSKNKSLMIKDSIQKFAADLVEKSFGSAFKDLQKGVSSCTNALYHLAIKLTSSVLQMAFDELR 600
601  RQRAFSLKERAISGLANFLVSEALSNALKDLQYVKKQIFTNTVARFAADLAEELVFEGIMEVCQFSYPQTPASPQ 675
676  CGSFDFEDKVVKLYAKDLSESVIQEAFIELSQVDVTFTTKAAVSVSTDNIKYVSAESVVPSTQAVTFSPSFHNQA 750
751  IMVTKPVQEYKKEYTVQQALFCTSGIVTSIPVPLAGSALLPYHISSTACQAKAHLSSDDSNSNGDSAQVHIATKN 825
826  REEKAACLRNICLPSEHNPGNQNDFKPTNDDIEMQSSSKLPNDPAIISNFSAAVVHTIVNETLESMTSLEVTKMV 900
901  DERTDYLTKSLKEKTPPFSHCDQAVLQCSEASSNKDMFADRLSKSIIKHSIDKSKSVIPNIDKNAVYKESLPVSG 975
976  EESQLTPEKSPKFPDSQNQLTHCSLSAAKDCVPECKVSMVHGSSLETLPSCPAVTGQKSDLKESAKDQPLKKHNL 1050
1051 NSTSLEALSFGQENPFPHSHTFSSTALTCVDGLHVEDKQKVRDRNVIPDTPPSTPLVPSRASSEWDIKKLTKKLK 1125
1126 GELAKEFAPATPPSTPHNSSVGSLSENEQNTIEKEEFMLKLMRSLSEEVESSESGELPEVDVKSEHSGKKVQFAE 1200
1201 ALATHILSLATEMAASHLDNKIIQEPKVKNPCLNVQSQRSVSPTFLNPSDENLKTLCNFAGDLAAEVITEAEKIA 1275
1276 KVRNCMLFKQKKNSCYADGDEDYKVEEKLDIEAVVHPREVDPFILSLPPSSCMSGLMYKYPSCESVTDEYAGHLI 1350
1351 QILKQEGGNSELIMDQYANRLAYRSVKSGLQEAAKTTKVQCNSRMFPVPSSQVKTNKELLMFSNKEHHQEADKKR 1425
1426 QSKRNEGYFCKNQTCERTLDPYRNEVSQLYSFSTSLVHSITKDAKEELTASLVGLPKSLTDSCLFEKSGYEEDNE 1500
1501 CHVTPELPKSLQPSSQNHRFYHSTGSLNGYGCGDNVVQAVEQYAKKVVDDTLELTLGSTVFRVSETTKSADRVTY 1575
1576 AEKLSPLTGQACRYCDLKELHNCTGNSSQHFFRQGSLASSKPASNPKFSSRYQKSRIFHLSVPQIHVNLDKKAVL 1650
1651 AEKIVAEAIEKAERELSSTSLAADSGIGQEGASFAESLATETMTAAVTNVGHAVSSSKEIEDFQSTESVSSQQMN 1725
1726 LSIGDDSTGSWSNLSFEDEHQDESSSFHHLSESNGNSSSWSSLGLEGDLYEDNLSFPTSDSDGPDDKDEEHEDEV 1800
1801 EGLGQDGKTLLITNIDMEPCTVDPQLRIILQWLIASEAEVAELYFHDSANKEFMLLSKQLQEKGWKVGDLLQAVL 1875
1876 QYYEVMEKASSEERCKSLFDWLLENA 1901
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FIG. 1. Human AKAP220 sequence. (A) Cloning strategy. Clones used to obtain and confirm the complete hAKAP220 sequence are indicated. Clones 26-2, RACE1, and PCR1 were obtained by RII-overlay screening, 5′-RACE, and PCR, respectively, and subsequently sequenced both ways using the primer-walking method of dideoxy sequencing. Clone KIAA0629 (Nagase *et al.*, 1997) overlaps clone 26-2 to give the composite hAKAP220 cDNA of 9923 bp. Lines indicate the lengths of the clones and numbers at the end of the lines denote start and end points with respect to the ATG start of translation. The open reading frame (ORF) with start (+1) and stop (+5706) of translation is indicated as an open box. (B) Amino acid sequence of hAKAP220 (1901 amino acids). The sequence corresponding to the RII-binding domain identified in the rat AKAP220 sequence (Lester *et al.*, 1996) is shown in bold and underlined (amino acids 1650 to 1663). Additional putative RII-binding domains are underlined (amino acids 611 to 628 and 1539 to1556). The hAKAP220 cDNA nucleotide sequence will appear in the EMBL, GenBank, and DDBJ databases under Accession No. AF176555.

1127 and 159 to179 of AKAP220, respectively, and used at a dilution of 1/100. αAKAP220 [1112-1127] was enriched for IgG on protein A–Sepharose columns (Pharmacia, Stockholm, Sweden) and subsequently affinity-purified on columns with peptide coupled to CNBr-activated Sepharose 4B (Pharmacia) and used at 2 μg/ml. Monoclonal antibody mAb CTR453 to the centrosomal marker (Bailly *et al.*, 1989) was a kind gift from Dr. M. Bornens (Institut Curie, Paris, France) and was used at a concentration of 140 ng/ml. PKA regulatory subunits RIα, RIIα, and RIIβ were detected with anti-human mAbs (developed by Transduction Laboratories in collaboration with K.T. and T.J.) at 1 (Western blot analysis) or 2.5 μg/ml (immunofluorescence), by an antipeptide antiserum to human RIIα (Keryer *et al.*, 1999) used at a dilution of 1/100 or by polyclonal antiserum to rat heart RIIα (kindly supplied

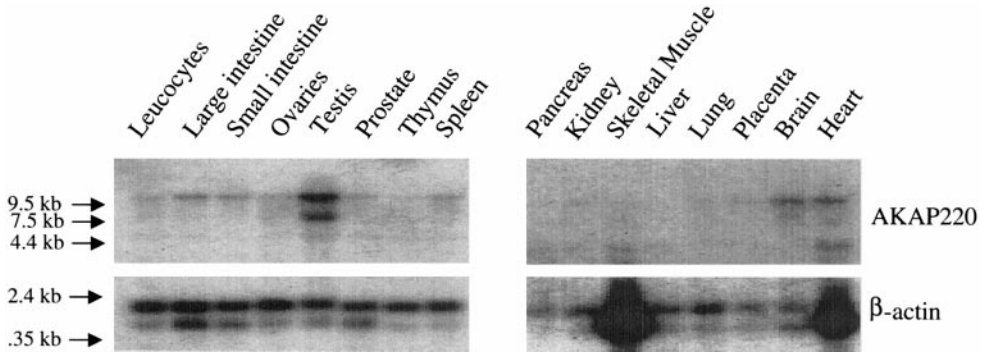


FIG. 2. Tissue distribution of hAKAP220 mRNA. Human multiple-tissue blots (Clontech, Inc.; 2 μg poly(A)$^+$ RNA per lane) were probed with a ^{32}P-labeled hAKAP220 cDNA probe (top, clone 26-2, see Materials and Methods) or a ^{32}P-labeled chicken β-actin probe (bottom). Arrows indicate migration of RNA molecular weight markers (kb).

by Dr. S. Lohmann, Universitetsklinik, Wurzburg, Germany) (dilution 1/100). S-AKAP84 was detected by an anti-AKAP149 mAb that recognizes different splice variants of the S-AKAP84/D-AKAP1 gene (Transduction Laboratories) (2.5 μg/ml). PP1 was detected by a mouse mAb reactive to all PP1 catalytic subunits (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; Cat. No. sc-7482).

Immunological procedures. Immunoblot analysis was performed as described elsewhere (Witczak *et al.*, 1999). Immunoreactive proteins were detected by horseradish peroxidase-labeled protein A (Amersham) in the second layer and developed using an ECL kit (Amersham; Cat. No. RPN2106). For immunoprecipitation, 20 μl of protein A/G PLUS–agarose beads (25% v/v, Santa Cruz Biotechnology, Inc.; Cat No. sc.2003) was added to 300 μg of 8-azido-[^{32}P]cAMP-labeled human testis protein (see below) and incubated at 4°C for 3 h to preclear the homogenates. The beads were removed, anti hAKAP220 antisera were added at a 1/25 dilution, and samples were left overnight at 4°C. Immune complexes were precipitated by incubation with 25 μl of protein A/G PLUS–agarose beads (25% v/v) for 3 h at 4°C and sedimented at 15,000*g* for 1 min and washed four times in PBS. The precipitates were subsequently boiled in SDS sample buffer and analyzed by SDS–PAGE and autoradiography or immunoblotting.

Immunofluorescence was performed on fractionated human germ cells or mature motile sperm from human donors, purified using the swim-up procedure (Wikland *et al.*, 1987). Cells were fixed on poly-L-lysine-coated glass slides with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 15 min, and immunofluorescence detection was performed as described previously (Collas *et al.*, 1996) with all antibodies used at 1/100 dilution. DNA was labeled with 0.1 μg/ml Hoechst 33342. For Triton X-100 extraction, cells were washed five times for 3 min with 1% Triton X-100 and subsequently fixed with 3% paraformaldehyde. Cells were examined on an Olympus AX70 epifluorescence microscope using a 100× objective. Photographs were taken with a Phototonic Science CCD camera and the OpenLab software (Improvision Co., Coventry, UK).

8-Azido-[^{32}P]cAMP-photoaffinity labeling. Homogenates of human testis (see above) were centrifuged at 200,000*g* to yield a soluble supernatant. The resulting pellet was dissolved in homogenization buffer containing 1% Triton X-100 and centrifuged at 200,000*g*. Fifty micrograms of protein from each fraction was incubated with 8-azido-[^{32}P]cAMP (1 μM; ICN Pharmaceuticals, Irvine, CA; sp act 50 Ci/mmol) for 1 h on ice in a solution containing 50 mM Tris, pH 7.5. Covalent attachment was achieved by using a 254-nm short-wave UV lamp at a distance of 5 cm from the solution for 15 min. All samples were boiled in SDS sample buffer and subjected to SDS–PAGE and subsequent analysis by autoradiography.

RESULTS

Characterization of the full-length hAKAP220 cDNA. A human T-cell (Jurkat) expression library was screened with the overlay technique using ^{32}P-labeled RIIα subunit. Of the clones obtained, one clone showed significant homology to the rat AKAP220 cDNA (86% identity; Lester *et al.*, 1996). The clone designated 26-2 in Fig. 1A was 2679 bp long covering major parts of the rat AKAP220 open reading frame. In-frame translation stop codons were not identified in the sequence of the human clone, neither in the 5′ nor in the 3′ end, suggesting that 26-2 was a partial clone. To obtain the remaining 5′ part of the hAKAP220 cDNA, rapid amplification of cDNA ends with human testis cDNA as a template was performed, yielding three independent identical clones (designated RACE1 in Fig. 1A) shown by sequencing to contain sequences similar to the rat AKAP220 protein and additional open reading frame and 5′ untranslated sequence. To confirm the 31-bp overlap between RACE1 and clone 26-2, a PCR was performed with human testis cDNA, using an upper primer positioned at nucleotide +2214 in the RACE1 clone and a lower primer positioned at nucleotide +3554 in the 26-2 clone and giving rise to a 1370-bp PCR product. Sequencing confirmed the correct positioning of this product in the composite sequence (PCR1 in Fig. 1A). KIAA clone 0629 present in the EST database was confirmed to contain sequences covering the remaining 3′ part of the reading frame (156 amino acids) and 3′ untranslated region (KIAA0629 in Fig. 1A). Combined, the four fragments gave rise to a composite hAKAP220 sequence of 9923 bp. This composite sequence was 86%

similar to the rat AKAP220 cDNA as determined using the BLASTN program [basic local alignment and search tool (Altschul *et al.*, 1990)], from nucleotide +1621 to +6567 corresponding to nucleotide 4847 to 9726 in the rat AKAP220 sequence. Furthermore, the open reading frame of hAKAP220 cDNA was 5706 bp with a 173-bp 5′ untranslated region containing stop codons in all three reading frames. The hAKAP220 amino acid sequence of 1901 amino acids derived from the open reading frame is shown in Fig. 1B. The calculated molecular weight of the hAKAP220 protein was 210 kDa with an isoelectric point of 5.16 and an overall net charge of −80 to −100 at physiological pH. The hAKAP220 protein contains a hydrophilic region (amino acids 1000 to 1500) and a serine-rich domain (amino acids 1600 to 1750). Peptide sequence comparison showed that the 727 N-terminal amino acids of hAKAP220 were not present in rat AKAP220, whereas amino acids 728 to 1895 in hAKAP220 showed 79% similarity to the full rat AKAP220 sequence. Despite this, the first 560 nucleotides of the 5′ untranslated region of rat AKAP220 were homologous to hAKAP220 from nucleotide 1621 to 2181, indicating that rat AKAP220 may represent a shorter splice variant or a partial cDNA. The RII binding domain identified in rat AKAP220 (Lester *et al.*, 1996) is conserved and positioned at amino acids 1650 to 1663 in hAKAP220 (bold, underlined in Fig. 1B). Analysis of the hAKAP220 peptide sequence for amphipathic helices with the common motif for RII binding domains (X{L,I,V}X3{A,S}X2{L,I,V}{L,I,V}X2{L,I,V}{L,I,V}X2-{A,S}{L,I,V}), as recently defined by Carr and co-workers (Vijayaraghavan *et al.*, 1999), identified two putative additional RII binding domains at positions 611 to 628 and 1539 to 1556 (underlined in Fig. 1B). Possible PP1 binding sites ({R,K}{V,I}XF; Schillace and Scott, 1999) are localized at positions 137 to 140 and 1195 to 1198 (bold in Fig. 1B). A putative ATP-binding domain is also present at position 1802 to 1809. Sequence alignments using the BLASTP search program (Altschul *et al.*, 1990) demonstrated that amino acids 88 to 156 and 1809 to 1900 of hAKAP220 showed 54% similarity to parts of p82 (Carrera *et al.*, 1994). Amino acids 1809 to 1900 of hAKAP220 also showed 54% similarity to a part of AKAP110 (Vijayaraghavan *et al.*, 1999) and a part of the sperm oocyte binding protein, SOB-1 (Lefevre *et al.*, 1999).

Tissue distribution of hAKAP220 mRNA. To determine which human tissues expressed the hAKAP220 mRNA, the 26-2 clone was radioactively labeled and used to probe multiple-tissue Northern blots. As shown in the top of Fig. 2, a 9.5-kb hAKAP220 mRNA is expressed at low levels in most tissues and cell types. Strong signals were seen in human testis, brain, and heart. In human testis, the probe detected two strong bands of 9.5 and 7.5 kb (arrows in Fig. 2), while an additional shorter band of 4 kb was seen in human skeletal muscle and heart. The bottom of Fig. 2 shows the same filters probed with β-actin as control.

hAKAP220 binds PKA. To examine binding of the hAKAP220 protein to the R subunit of PKA, we performed

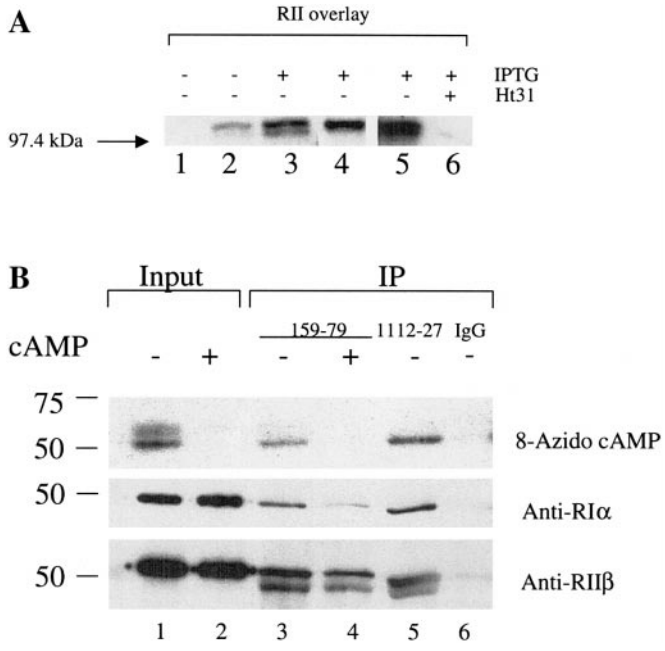


FIG. 3. Human AKAP220 binds PKA. (A) A fragment of hAKAP220 missing amino acids 1 to 912 (AKAP220 1-912) expressed in *Escherichia coli* without (lane 2) or with IPTG inducer for 3 (lane 3) or 24 (lanes 4–6) h. The AKAP220 1-912 fragment was immobilized on nitrocellulose and probed with ^{32}P-labeled RIIα subunit using the RII-overlay technique in the presence (lane 6) or absence (lanes 1 to 5) of Ht31 competitor peptide. Lane 1, nontransformed bacteria. (B) Coimmunoprecipitation of RII and RI from human testis with hAKAP220. Human testis tissue was homogenized and subjected to ultracentrifugation to yield a 200,000*g* supernatant that was subsequently photoaffinity labeled with 8-azido-[^{32}P]cAMP (top). 8-Azido cAMP labeling is shown in the absence (lanes 1, 3, and 5) or presence of unlabeled cAMP competitor (lanes 2 and 4, 100-fold excess). Lanes 1 and 2 (testis) contain 20 μg protein. Lanes 3–6 show immunoprecipitation experiments (IP) from the 8-azido-labeled testis extracts (200 μg protein). Lanes 3 and 4, IP using the N-terminal αAKAP220 [159-179] antibody; lane 5, IP using the C-terminal αAKAP220 [1112-1127] antibody; lane 6, control precipitation using affinity-purified rabbit IgG (IgG). The middle and lower blots show immunoblotting of the same filters using RIα mAb (middle) or an RIIβ mAb (bottom).

overlays using radiolabeled RIIα as a probe as described elsewhere (Bregman *et al.*, 1989). RIIα bound a protein product expressed from clone 26-2 missing amino acids 1 to 912 (AKAP220Δ1-912, Fig. 3A, lanes 2–5) in Stratagene pBk vector, whereas the Ht31 anchoring-inhibitor peptide (Carr *et al.*, 1991) fully competed RIIα binding to AKAP220Δ1-912 (Fig. 3A, lane 6).

Coimmunoprecipitation of PKA R subunits with hAKAP220. In order to investigate the nature of the PKA association with hAKAP220, we performed immunoprecipitation of 8-azido-[^{32}P]cAMP-labeled proteins from human testis using antisera generated toward the C-terminus

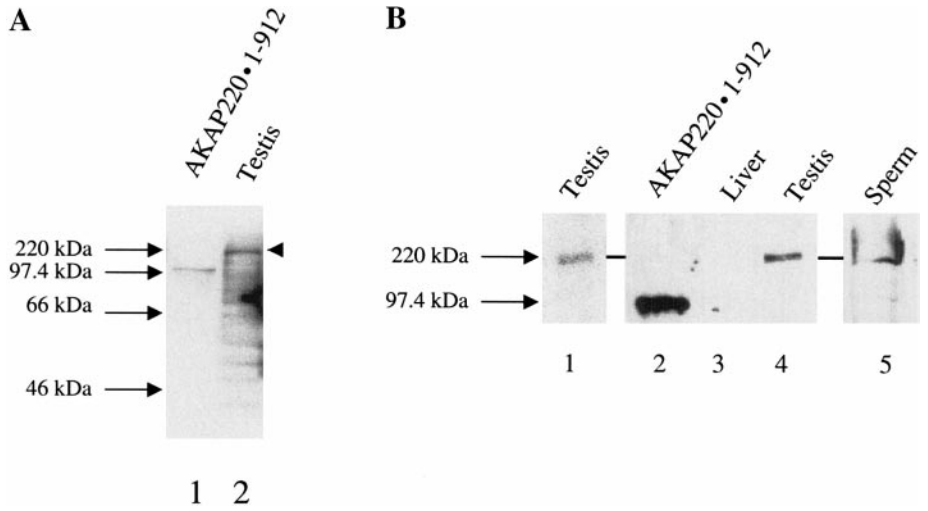


FIG. 4. hAKAP220 in human testis and sperm. (A) Overlay with ^{32}P-labeled RIIα on human testis homogenates. Lane 1, recombinant hAKAP220 missing amino acids 1 to 912 (AKAP220Δ1-912); lane 2, human testis homogenate (20 μg protein). Migration of molecular weight markers (kDa) is indicated on the left in both A and B. (B) Nitrocellulose-immobilized proteins from the supernatants of human tissue homogenates and whole sperm lysates were subjected to immunoblot analysis using the αAKAP220 [1112-1127] antibody (see Materials and Methods). Lane 2, recombinant hAKAP220 missing amino acids 1 to 912 (AKAP220Δ1-912); lane 3, human liver homogenate (20 μg protein); lane 4, human testis homogenate (20 μg protein); lane 5, human sperm lysate (10^6 sperm) aligned from a separate blot. Lane 1 shows alignment of RII overlay on the same blot as in lane 4. Immunoblotting in the presence of peptide antigen competed the signal by the αAKAP220 [1112-1127] antibody (not shown).

(αAKAP220 [1112-1127]) and N-terminus (αAKAP220 [159-179]) of hAKAP220. Figure 3B shows immunoprecipitated proteins from the soluble fraction of human testis homogenates. Precipitates were subjected to SDS–PAGE and autoradiography (top) as well as immunoblotting analyses (middle and bottom). In the input soluble fraction, two specifically 8-azido-cAMP-labeled proteins of 49 and 53 kDa were detected (Fig. 3B, top, lane 1), which by immunoblot analysis corresponded to RIα (Fig. 3B, middle, lanes 1 and 2) and RIIβ (Fig. 3B, bottom, lanes 1 and 2). Immunoprecipitation using αAKAP220 [159-179] and αAKAP220 [1112-1127] antisera demonstrated one major photoaffinity-labeled band at 49 kDa and a weaker band with slightly lower mobility (Fig. 3B, top, lanes 3 and 5). Both bands were competed with cold cAMP in the αAKAP220 [159-179] precipitates (Fig. 3B, top, lane 4). Western blot analyses showed the presence of both RIα (Fig. 3B, middle, lanes 3 to 5) and RIIβ (Fig. 3B, bottom, lanes 3 to 5). The lower intensities observed in lane 4 of the anti-RIα and anti-RIIβ immunoblots are probably due to loading differences since Western blot analysis using anti-hAKAP220 on the same blots also show a lower intensity signal in this lane (results not shown). Precipitation using affinity-purified rabbit IgG did not detect any proteins either by autoradiography (Fig. 3B, top, lane 6) or by Western blot analyses (Fig. 3B, middle and bottom, lane 6).

The hAKAP220 protein is present in testis and human sperm. In an overlay experiment of testis homogenates (Fig. 4A, lane 2) a prominent band of 220 kDa was detected together with a number of other RII-binding proteins. RIIα binding was eliminated with Ht31 peptide (results not shown), indicating that the 220-kDa protein is an AKAP. The αAKAP220 [1112-1127] antiserum was affinity purified and used for immunoblotting analysis. Immunoblotting of human liver, testis, and ejaculated sperm homogenates revealed a 220-kDa band in testis and sperm, but not in liver (Fig. 4B). The 220-kDa immunoreactive protein comigrated with the band detected by RII overlay on the same filter (lane 1). Three additional bands with faster migration were also detected. However, all bands were competed by the peptide antigen. Similar results were obtained with the N-terminal αAKAP220 [159-179] antiserum (results not shown).

hAKAP220 mRNA is expressed in human germ cells. To determine whether germ cells in the testis expressed the hAKAP220 mRNA, male human germ cells were isolated and fractionated as described under Materials and Methods. hAKAP220 mRNA was expressed at high levels in fractions 4 and 5, enriched in pachytene spermatocytes (PS) (Fig. 5, lanes 4 and 5), and at lower levels in fractions 1–3 enriched in round spermatids (RS) (lanes 1–3). Both the 7.5- and the 9.5-kb bands were detected in all germ cell fractions. Similarly, 9.7- and 7.3-kb transcripts of AKAP220 were detected in rat pachytene spermatocytes, but not in round or elongating rat spermatids or in somatic cell types (Sertoli cells, peritubular cells, Leydig tumor cells) of the rat testis (results not shown).

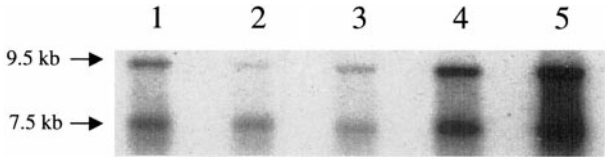


FIG. 5. hAKAP220 mRNA in germ cell fractions from human testis. Germ cell suspensions from human testis were fractionated by centrifugal elutriation, and total RNA was extracted and subjected to Northern blot analysis with ^{32}P-labeled hAKAP220 cDNA. Fractions were analyzed by flow cytometry for DNA content and separated into haploid cells [round spermatids (RS)] and tetraploid cells [pachytene spermatocytes (PS)]. Diploid cells were considered to be spermatogonia, secondary spermatocytes, or contamination by somatic cells and leukocytes. Lane 1, 57% RS, 7% PS; lane 2, 41% RS; lane 3, 16% RS, 7% PS; lane 4, 21% PS, 3% RS; lane 5, 51% PS, 4% RS. Twenty micrograms of total RNA was loaded with the exception of lane 2 (10 μg). The probe detected hAKAP220 mRNAs of 9.5 and 7.5 kb compared to molecular weight markers of 7.5 and 9.5 kb (arrows).

Dynamic localization of the hAKAP220 protein during spermatogenesis. The subcellular localization of hAKAP220 was examined in fractionated male germ cells by double immunofluorescence using the αAKAP220 [159-179] antiserum and mAb CTR453 (Bailly *et al.*, 1989), which specifically labels centrosomes in somatic cells (Fig. 6). In PS, hAKAP220 labeling was concentrated in the centrosome area (arrow in Merge + DNA, top) over a disperse cytoplasmic staining. In RS, αAKAP220 [159-179] decorated a distinct single dot colocalized with mAb CTR453 staining (RS, arrow). The RS fraction also contained a number of elongated spermatids (ES) characterized by their tails. In ES as well as in ejaculated sperm preparations, hAKAP220 labeling was restricted to the midpiece region (Fig. 6). In addition, mAb CTR453 staining was included in the midpiece area (Fig. 6, ES and Sp, arrows in Merge + DNA). Immunofluorescence using the AKAP220 [1112-1127] antiserum produced similar results in all cell types and hAKAP220 staining with both antisera was specific since it was competed with the corresponding peptides (results not shown).

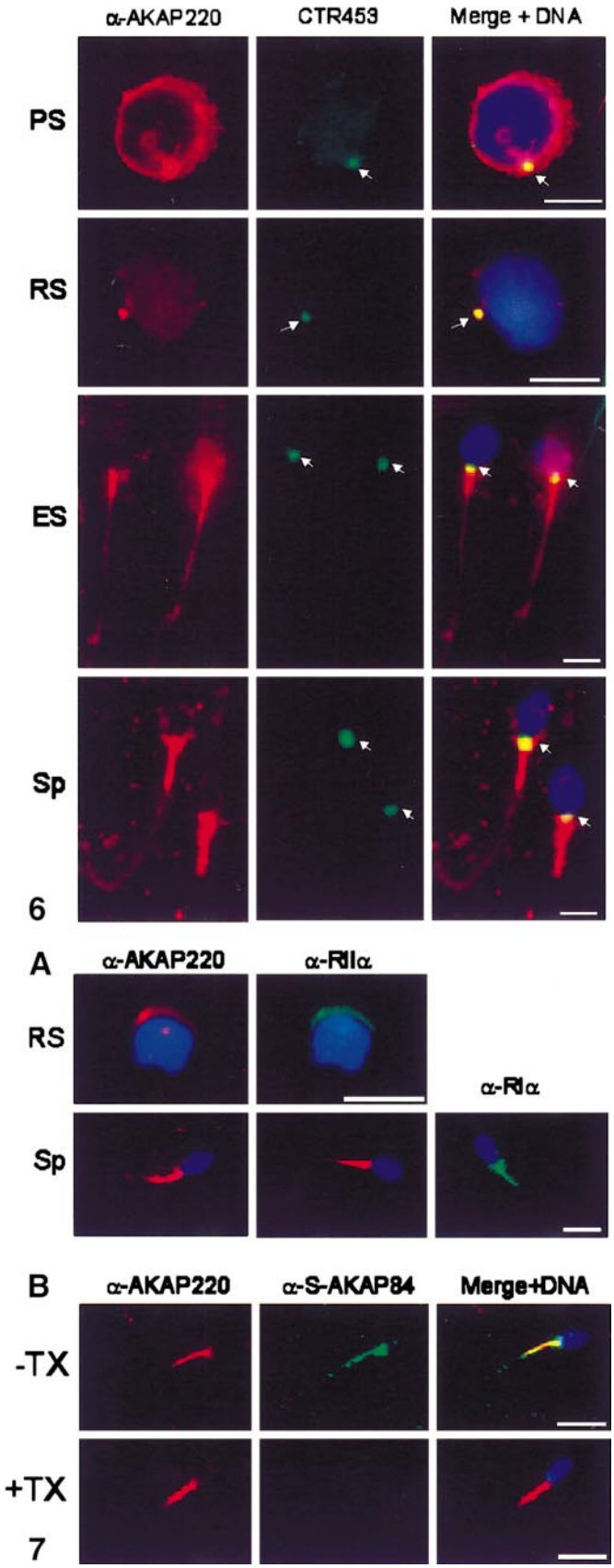
hAKAP220 colocalizes with RIIα in human germ cell centrosomes and RIIα and RIα in human sperm midpiece. Localization of hAKAP220 versus that of RIα and RIIα was examined by immunofluorescence of an RS fraction labeled with αAKAP220 [159-179] and either an RIIα mAb or an anti-RIα mAb. Both hAKAP220 and RIIα colocalized in the centrosome area of RS (Fig. 7A, RS). No RIα was detected in RS fractions (results not shown). Localization of hAKAP220, RIα, and RIIα was also determined in ejaculated sperm (Fig. 7A, Sp). Sperm were separately labeled with αAKAP220 [159-179], an anti-rat heart RIIα polyclonal antibody, or an RIα mAb. Clearly, all antibodies specifically labeled the midpiece, strongly suggesting colocalization of hAKAP220, RIα, and RIIα in this region.

hAKAP220 is detergent resistant in human sperm. Another sperm AKAP, S-AKAP84, has been shown to be localized in the midpiece of mouse elongating spermatids (Lin *et al.*, 1995) and S-AKAP84/D-AKAP1 splice variants have been shown to be targeted to outer mitochondrial- and endoplasmic reticulum membranes in somatic cells (Huang *et al.*, 1999). To address the nature of the association of S-AKAP84 and hAKAP220 with midpiece structures, human sperm were extracted with 1% Triton X-100/150 mM NaCl prior to fixation and double-immunofluorescence analysis of hAKAP220 and S-AKAP84. hAKAP220 was detected with the αAKAP220 [159-179] and S-AKAP84 with an antibody directed against human AKAP149, which recognizes human S-AKAP84 splice variants in sperm (Trendelenburg *et al.*, 1996). As expected, labeling of hAKAP220 and S-AKAP84 colocalized in the midpiece of untreated human sperm (Fig. 7B, top). Extraction of sperm with 1% Triton X-100 eliminated all S-AKAP84 labeling, whereas some hAKAP220 remained detectable (Fig. 7B, bottom). The detergent solubility of S-AKAP84 argued for its association with membranes (Lin *et al.*, 1995). In contrast, a pool of hAKAP220 was detergent resistant, suggesting association with cytoskeletal elements in the midpiece (Fig. 7B). The distinct Triton X-100 extractability of hAKAP220 and S-AKAP84 argues that the two proteins are differentially anchored in the sperm midpiece. Furthermore, immunoblot analysis of tail-enriched sperm fractions extracted with 1% Triton X-100 using anti-hAKAP220, anti-RIα, anti-RIIα, and anti-PP1 antibodies showed that all hAKAP220 and a fraction of RIα, RIIα, and PP1 was Triton X-100 insoluble (Fig. 8). This demonstrates that at least a portion of sperm tail-associated PKA R subunits and PP1 is resistant to detergent extraction and remains anchored to the midpiece/tail region after removal of the detergent-soluble S-AKAP84. Altogether, the results suggest that RIα, RIIα, and PP1 may be anchored to cytoskeletal structures in the sperm midpiece via hAKAP220.

DISCUSSION

In the present study, we report the cloning and characterization of a cDNA encoding hAKAP220, a 1901-amino-acid protein which migrates at 220 kDa and has high homology to rat AKAP220 in the C-terminal region. Using antibodies to the N- and C-termini of hAKAP220, we now account for the entire 220-kDa protein. We demonstrate that hAKAP220 is expressed at high levels throughout spermatogenesis and in mature sperm and show that hAKAP220 binds the RIα, RIIα, and RIIβ subunits of PKA in human testis. Furthermore, our results demonstrate hAKAP220 and PKA localization to germ cell centrosomes as well as detergent-resistant cytoskeletal structures in human spermatids and mature sperm midpiece, which is in contrast to the detergent-extractable S-AKAP84/D-AKAP1 associated with midpiece mitochondria.

Several AKAPs expressed in human testicular germ cells have been characterized and cloned in addition to hAKAP220. S-AKAP84 (Lin *et al.*, 1995) and/or its splice



variants D-AKAP-1 (Huang *et al.*, 1999) and AKAP121 (Feliciello *et al.*, 1998) is present in condensing spermatid midpiece. FSC-1, also known as p82 or AKAP82 or HI (Miki and Eddy, 1998; Carrera *et al.*, 1994; Turner *et al.*, 1998; Mohapatra *et al.*, 1998), and TAKAP80 (Mei *et al.*, 1997) are found in the sperm fibrous sheath. The recently described AKAP110 is localized to the fibrous sheath and acrosomal region (Vijayaraghavan *et al.*, 1999). The splice variant D-AKAP-1 of S-AKAP84 was initially characterized with a dual-specificity R binding (RI and RII) that resides in one R-binding domain (Huang *et al.*, 1997). In addition, all other germ cell AKAPs have been suggested to bind both the RI and the RII subunits of PKA either via one common or via separate binding domains. Apparently, hAKAP220 also binds RI as we were able to coimmunoprecipitate RIα with hAKAP220 antisera from testis homogenates. Thus, there seems to be an abundance of dual-specificity AKAPs in human testis. The significance of the presence and anchoring of both RI and RII in sperm is not known, but could relate to sperm motility and/or to PKA signaling events in the egg cytoplasm after fertilization (due to the fact that the entire tail is incorporated into the oocyte). The RII binding domain is conserved between the rat and the human AKAP220 proteins; consequently, it is highly likely that hAKAP220 utilizes this RII binding domain as indicated by the observed binding of RII to an expressed fragment of hAKAP220 missing amino acids 1 to 912. However, two additional putative R-binding domains were identified by

FIG. 6. Subcellular localization of hAKAP220 in human male germ cells. Top three rows: aliquots from human germ cell fractions [pachytene spermatocytes (PS), round spermatids (RS), and elongated spermatids (ES), see Materials and Methods and legend to Fig. 4] were fixed with ethanol on glass slides and subjected to immunofluorescence analysis using the αAKAP220 [159-179] antiserum (red staining). Cells were concomitantly stained with a mAb to a centrosomal marker, mAb CTR453 (green), and DNA stained with Hoechst 33342 (blue). Merged pictures are also shown (Merge + DNA). Bottom row: motile sperm (Sp) subjected to similar immunofluorescence analysis. Bars, 5 μm in all.

FIG. 7. hAKAP220 colocalizes with PKA in human germ cells. (A) Top row: an aliquot from the round spermatid (RS) fraction (lane 1 in Fig. 5) was subjected to immunofluorescence analysis (as described in Fig. 6) using the αAKAP220 [159-179] antiserum (red staining). Cells were costained with anti-RIIα mAb (green) and Hoechst 33342 (blue). Bottom row: human motile ejaculated sperm (Sp) were subjected to similar immunofluorescence analysis using the αAKAP220 [159-179] antiserum (left), polyclonal RIIα antibody (middle), or anti-RIα mAb (right). Antibody labeling is shown in red (hAKAP220 and RIIα) or green (RIα) and Hoechst DNA labeling in blue. (B) Human motile sperm were untreated (top row) or extracted with 1% Triton X-100 (bottom row) before fixation and immunofluorescence staining with the αAKAP220 [159-179] antiserum (red). Cells were costained with anti-S-AKAP84 mAb (α-AKAP149, Transduction Laboratories, Inc., green). Hoechst DNA labeling (blue) and merged picture are shown (overlap in yellow). Bars, 5 μm in all.

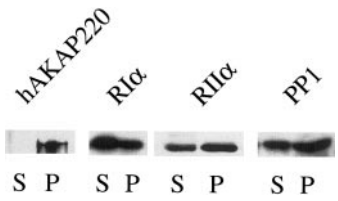


FIG. 8. Detergent extraction of human sperm tails. Sperm tails were extracted with 1% Triton X-100 for 30 min, fractionated into soluble (S) and insoluble (P) fractions, and subjected to immunoblotting using the anti-αAKAP220 [1112-1127] antibody (first from left), anti-RIα mAb (second), anti-RIIα mAb (third), and anti-PP1 (fourth) antibody.

computer analysis of the hAKAP220 amino acid sequence. Further experiments on the hAKAP220 R-binding domains are necessary to identify the binding site responsible for the binding of RIα.

In somatic cells, a centrosomal AKAP (AKAP350/450/Yotiao) was recently identified by several groups (Witczak *et al.*, 1999; Schmidt *et al.*, 1999). In germ cells hAKAP220 adds to the centrosomal AKAPs. Unpublished data indicate the presence also of AKAP450 in germ cell centrosomes.

We describe the presence of the RIIα subunit of PKA in the midpiece of human sperm that colocalizes with hAKAP220. This observation supports previous reports on RII localization in sperm (Lieberman *et al.*, 1988; Pariset *et al.*, 1989). However, using monoclonal anti-RIIα antibodies or an antipeptide polyclonal antibody to amino acids 54 to 72 of human RIIα (Keryer *et al.*, 1999), no specific labeling could be observed in human sperm (data not shown). Nevertheless, a polyclonal antibody to rat heart RIIα showed a specific and distinct signal in sperm midpiece. These results indicate that the RIIα protein present in sperm displays a sperm-specific immunoreactivity, possibly due to alternative splicing or posttranslational modification of RIIα in sperm. This observation is consistent with previous reports on bovine (Horowitz *et al.*, 1989) and human sperm (Pariset and Weinman, 1994).

The solubility properties of hAKAP220 in sperm suggest anchoring to cytoskeletal components in the midpiece. In the rat testis, however, RIα and RIIβ have been shown to be both soluble and membrane associated (Landmark *et al.*, 1993). Consequently, coimmunoprecipitation of RIα and RIIβ from the soluble fraction of human testis homogenates using hAKAP220 antisera probably reflects cytoplasmic, granular hAKAP220 binding to cytosolic and membrane-associated RIα and RIIβ in developing germ cells and somatic cells in the testis. Insufficient availability of human germ cells prevented us from examining the association of hAKAP220 and the R subunits in pachytene spermatocytes and round spermatids. However, it is likely that binding of these proteins to germ cell centrosomal structures renders them insoluble, which could explain why we were unable to coimmunoprecipitate RIIα and hAKAP220 in human testis lysates using a nonionic detergent or a cytoskeletal (RIPA) solubilization buffer (data not shown). Alternatively, hAKAP220 antisera may be denatured in RIPA buffer. Nevertheless, although an RIIα association with AKAP220 has been shown in other cell types (Lester *et al.*, 1996; Schillace and Scott, 1999), and although both proteins are colocalized in sperm midpiece and germ cell centrosomes, there is a possibility that hAKAP220 may not bind RIIα *in vivo*.

The relocalization of hAKAP220 during spermatogenesis may indicate that this protein serves a dual role in the differentiation process. The centrosomal hAKAP220/PKA complex may regulate spindle formation during meiosis and/or microtubule organization in postmeiotic germ cells since microtubule stability in somatic cells is influenced by PKA (Lane and Kalderon, 1994; Gradin *et al.*, 1998). The midpiece-associated hAKAP220 could serve to anchor PKA and/or PP1γ2 that may directly regulate the contractile machinery in the sperm axoneme. Thus, hAKAP220 may contribute to the development of motile functions during spermiogenesis.

The normal function of sperm in RIIα knockout mice questions the importance of anchoring of PKA through AKAPs in sperm (Burton *et al.*, 1999). However, other PKA functions in these mice are rescued by the upregulation of RIα (Burton *et al.*, 1997). We demonstrate that RIα and RIIα are colocalized and associated with AKAP220 in the human sperm midpiece. Thus, although RIα was not detected in mouse sperm midpiece by Burton *et al.* (1999), our results indicate that anchoring of PKA through RIα and/or RIIα may be necessary for normal human sperm function. This would explain the inhibitory effect of Ht31 on primate and bovine sperm motility (Vijayaraghavan *et al.*, 1997).

A recent paper demonstrated that AKAP220 binds PP1α in hippocampal neurons (Schillace and Scott, 1999) and the hAKAP220 protein contains several potential PP1 binding sites. Human sperm do not contain PP1α, but PP1γ2 has been implicated in regulation of sperm motility (Smith *et al.*, 1996). The presence of PP1 in the insoluble fraction of sperm tails may indicate hAKAP220–PP1 colocalization in sperm. However, a further analysis of hAKAP220–PP1 colocalization by immunocytochemistry was precluded by the lack of available suitable antibodies. Nevertheless, since AKAP220 has been shown to bind PP1 in other cell types, hAKAP220 may serve to integrate signals promoting cross-talk between different signaling effector systems in the sperm tail. Preliminary results in our laboratory suggest a centrosomal localization of hAKAP220 also in human somatic cells. Thus, hAKAP220 may serve a function in cell cycle control of both somatic cells and germ cells in addition to its putative role in spermatogenesis and sperm function.

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