Defects in Sperm Flagellum and Motility

Kiyoshi Miki,[*,1] **William D. Willis,**[*] **Paula R. Brown,**[*]
Eugenia H. Goulding,[*] **Kerry D. Fulcher,**[†] **and Edward M. Eddy**[*,2]

[*]*Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology,
National Institute of Environmental Health Sciences, National Institutes of Health,
Research Triangle Park, North Carolina 27709; and* [†]*Point Loma Nazarene University,
San Diego, California 92106*

A-kinase anchoring proteins (AKAPs) tether cyclic AMP-dependent protein kinases and thereby localize phosphorylation of target proteins and initiation of signal-transduction processes triggered by cyclic AMP. AKAPs can also be scaffolds for kinases and phosphatases and form macromolecular complexes with other proteins involved in signal transduction. *Akap4* is transcribed only in the postmeiotic phase of spermatogenesis and encodes the most abundant protein in the fibrous sheath, a novel cytoskeletal structure present in the principal piece of the sperm flagellum. Previous studies indicated that cyclic AMP-dependent signaling processes are important in the regulation of sperm motility, and gene targeting was used here to test the hypothesis that AKAP4 is a scaffold for protein complexes involved in regulating flagellar function. Sperm numbers were not reduced in male mice lacking AKAP4, but sperm failed to show progressive motility and male mice were infertile. The fibrous sheath anlagen formed, but the definitive fibrous sheath did not develop, the flagellum was shortened, and proteins usually associated with the fibrous sheath were absent or substantially reduced in amount. However, the other cytoskeletal components of the flagellum were present and appeared fully developed. We conclude that AKAP4 is a scaffold protein required for the organization and integrity of the fibrous sheath and that effective sperm motility is lost in the absence of AKAP4 because signal transduction and glycolytic enzymes fail to become associated with the fibrous sheath. © 2002 Elsevier Science (USA)

INTRODUCTION

Cyclic AMP (cAMP) serves as a second messenger for various biological stimuli by triggering cAMP-dependent protein kinases (PKA) to phosphorylate serine and threonine residues on other signal cascade proteins to activate or modify their functions. PKAs often are tethered to A-kinase anchoring proteins (AKAPs) associated with particular cellular components, positioning them in specific subcellular sites and providing a mechanism for defining which proteins become phosphorylated. For example, AKAP-linked signaling complexes associated with the cytoskeleton are believed to regulate fundamental cellular properties, such

as shape, movement, and division (Diviani and Scott, 2001). AKAPs also can serve as scaffolds for signal-transduction complexes that contain phosphatases, kinases, and other components that are believed to coordinate the phosphorylation status of target proteins (Edwards and Scott, 2000; Colledge and Scott, 1999). In addition, AKAP complexes may be associated with upstream activators or downstream substrates of PKA-associated signaling pathways (Colledge *et al.,* 2000; Dodge and Scott, 2000).

Mammalian sperm are unique and highly polarized cells with a haploid genome tightly packaged in the head and a flagellum that generates the motile force needed to deliver the sperm to the egg. The flagellum is divided into a middle piece, principal piece, and end piece. The middle piece is adjacent to the head and is distinguished by the presence of the mitochondrial sheath. The principal piece occupies approximately three-fourths of the length of the flagellum, while the end piece is a short segment at the tip of the flagellum. The major cytoskeletal components of the fla-

[1] Present address: Department of Cell and Developmental Biology, School of Medicine, University of North Carolina at Chapel Hill, NC 27599-7090.

[2] To whom correspondence should be addressed. Fax: (919) 541-3800. E-mail: eddy@niehs.nih.gov.

gellum are the axoneme, fibrous sheath, and outer dense fibers. The axoneme extends the full length of the flagellum and is composed of nine microtubule doublets surrounding a central pair of microtubules. Sperm are the only cell type with a flagellum, but the "nine-plus-two" complex of microtubules that forms the axoneme is also present in cilia. The fibrous sheath defines the extent of the principal piece and consists of two longitudinal columns connected by closely arrayed circumferential ribs. The outer dense fibers are associated with the microtubule doublets at the periphery of the axoneme, beginning at the sperm head and ending in the middle region of the principal piece. They taper along their length and are enclosed by the mitochondrial sheath in the middle piece and the fibrous sheath in the principal piece. The classical view has been that, while the axoneme functions as the motor of the sperm, the fibrous sheath and outer dense fibers serve a mechanical role by modulating the degree and shape of flagellar bending (Fawcett, 1975). AKAP4 is the major protein in the fibrous sheath and is the product of an X-linked gene (Moss *et al.,* 1997) expressed only in spermatids (Carrera *et al.,* 1994; Fulcher *et al.,* 1995). AKAP4 is unusual among AKAPs in having a RIα-specific binding domain distinct from the prototype RII binding domain and another domain that binds either RIα or RIIα (Visconti *et al.,* 1997; Miki and Eddy, 1998, 1999). It is assumed that AKAP4 recruits PKA to the fibrous sheath to facilitate phosphorylation of neighboring proteins that regulate flagellar function.

Sperm acquire the ability to bind to the zona pellucida, exhibit progressive motility upon release, and respond to increased levels of intracellular cAMP as they are moved through the epididymis (e.g., Lewis and Aitken, 2001). Increased production of cAMP and the phosphorylation of proteins occur during passage of sperm through the female reproductive tract (Morton and Albagli, 1973; Tash and Means, 1983; Visconti *et al.,* 1995a). This happens concurrently with the development of vigorous flagellar movement with a high-amplitude waveform, referred to as hyperactivated motility (Yanagimachi, 1970). Capacitation is the process whereby sperm gain the ability to undergo the acrosome reaction and occurs concurrently with cAMP-mediated phosphorylation of flagellar proteins (Visconti *et al.,* 1995b). Furthermore, mouse sperm require glucose to become hyperactivated and to fertilize eggs *in vitro* (Brooks, 1976; Fraser and Quinn, 1981), suggesting that glycolysis generates ATP needed for these processes. Glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS) is a germ cell-specific glycolytic enzyme bound to the fibrous sheath (Bunch *et al.,* 1998; Westhoff and Kamp, 1997). In earlier studies, glycolytic enzyme activities were found in the detergent-insoluble fraction of sperm (Mohri *et al.,* 1965; Harrison, 1971; Storey and Kayne, 1978). These and other proteins associated with the fibrous sheath are prospective direct or indirect targets for regulation via PKA-dependent phosphorylation events.

One approach used to study PKA function was to generate mice lacking the narrowly expressed RIβ or RIIβ regulatory subunits of PKA or the more widely expressed RIα or RIIα subunits. RIα and RIIα are present in the flagellum (Horowitz *et al.,* 1984; Visconti *et al.,* 1997; Johnson *et al.,* 1997; Burton *et al.,* 1999). Mice lacking RIβ or RIIβ showed specific defects in synaptic function in the hippocampus and cellular metabolism in adipose tissue, respectively, but were fertile (Brandon *et al.,* 1995; Cummings *et al.,* 1996). The RIα mutation was an embryonic lethal (unpublished results referred to in Amieux *et al.,* 1997), while RIIα-deficient mice had no obvious physiological defects and were fertile (Burton *et al.,* 1997). However, compensatory regulation of RIα protein levels in the RIIα-deficient mice (Amieux *et al.,* 1997) complicated the interpretation of these results. An alternative approach to examine PKA function was used in this study, generating mice lacking an AKAP that is synthesized only in spermatogenic cells. It was found that mutation of the *Akap4* gene disrupts sperm motility and causes male mice to be infertile. To our knowledge, this is the first use of gene targeting to explore the roles of an AKAP and the effects of disruption of PKA anchoring within a specific cell type. These studies demonstrate that AKAP4 is an important component of the fibrous sheath and is required for anchoring the signal transduction proteins and glycolytic enzymes necessary for flagellar function.

MATERIALS AND METHODS

Construction of Targeting Plasmid

The *Akap4* gene was isolated from a P1 phage library of 129Sv mouse genomic DNA (Incyte Genomics, St. Louis, MO). The DNA restriction fragment containing exons 4 and 5 was digested with *Hin*dIII and *Bgl*II and cloned into a thymidine kinase (TK) vector, pMC1TKbpA (gift of Dr. Yuji Mishina). The fragment containing exons 6 and 7 was digested with *Eco*RV and *Xba*I and then subjected to partial *Pml*I digestion to generate two fragments. The fragment containing exon 6 was subcloned into pPGKneobpAFRT2(B) (gift of Dr. Yuji Mishina) containing the *neo* gene surrounded by frt recognition sequences for *Drosophila* friptase. The fragment containing exon 7 was subcloned into the *Spe*I site outside the loxP cassette of pBS246 (Life Technologies, Grand Island, NY). Exon 6 and the neo cassette were excised from pPGKneobpAFRT2(B) and subcloned into the *Eco*RV site within the loxP cassette of pBS246. This plasmid was combined with exons 4 and 5 cloned into pMC1TKbpA to produce the gene-targeting construct pAKAP4KO (Fig. 1).

Generation of Mutant Mice

The *Akap4* gene is located on the X-chromosome and was mutated *in vivo* by using the CRE/loxP method because of concern that male chimeras produced with XY ES cells would be unable to transmit a single copy mutant allele to the next generation. Transfection of pAKAP4KO DNA and screening of targeted TC-1 embryonic stem (ES) cells (generous gift of Dr. Philip Leder) were performed as described (Dix *et al.,* 1996). Two ES cells clones with a targeted *Akap4* gene were identified and injected into C57BL/6 blastocysts to produce chimeric mice. Chimeras produced from one
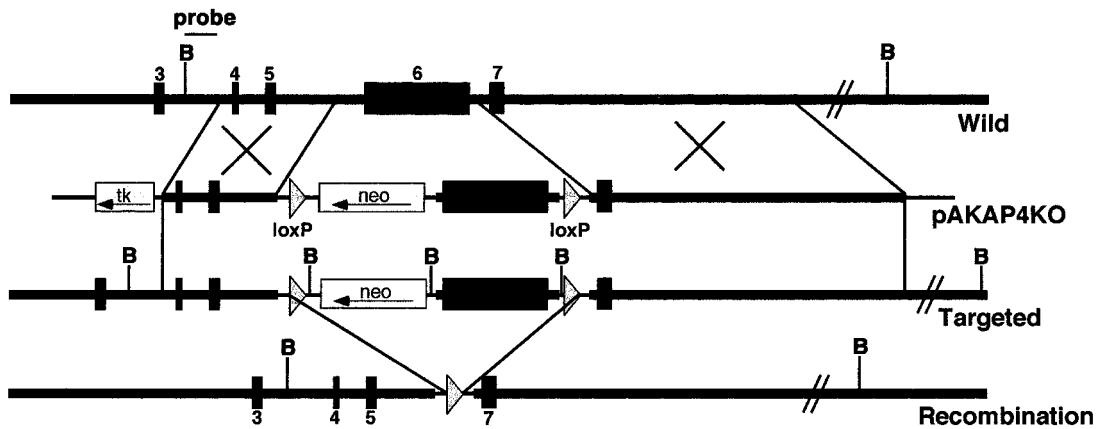
FIG. 1. Targeted disruption of the *Akap4* gene. A partial map of the *Akap4* locus and diagrams showing the gene-targeting strategy. Filled boxes indicate exons 3–7. The loxP sites in the knockout plasmid pAKAP4KO are shown by triangles. The LoxP sites are located between exon 5 and the neomycin resistance gene (neo) and between exons 6 and 7. LoxP–*Akap4* allele was created by homologous recombination in ES cells. Exon 6 encodes 92% of the processed form of AKAP4 and was excised by mating with transgenic mouse expressing Cre under the control of MMTV-LTR in oocytes. *Bam*HI sites (B) are shown on wild type, targeted, and recombined *Akap4* genes. The bar in the top margin indicates the position of the probe used for Southern analysis.

clone transmitted the targeted gene through the germ-line when bred to C57BL/6 mice. Female mice heterozygous for the mutation were bred to male transgenic mice carrying the *cre* gene under control of the mouse mammary tumor virus (MMTV) promoter [B6129-TgN(MMTV-Cre)1Mam; The Jackson Laboratory] (Wagner *et al.,* 1997). This transgene is expressed in oocytes, and recombination in female mice carrying both the targeted *Akap4* gene and the *cre* transgene resulted in deletion of the *neo* gene and exon 6 of the *Akap4* gene in oocytes (Fig. 1a). These females were bred to C57BL/6 wild type males to produce females heterozygous and males hemizygous for the mutated *Akap4* allele. The genotypes were determined by Southern blotting using a restriction enzyme fragment (*Bam*HI/*Hin*dIII) of the *Akap4* gene containing intron 3 (bp 6148–6543, GenBank Accession No. U10341). The PCR primers used to detect homologous recombination were forward 5′-AAGAGGAGGTAAGAGTGGTG-3′ and reverse 5′-GCAGCG-CATCGCCTTCTATC-3′ corresponding to sequences in intron 3 and the *neo* gene, respectively. The PCR primers used to detect recombination were forward 5′-TCCAGGTCAGAAGGCGAG-TTA-3′ and reverse 5′-CCCTGTGACTGTTCCCCAAGATT-3′ corresponding to sequences in exons 5 and 6, respectively. Because the presence of the *neo* gene in intron 5 did not disrupt fertility of the mice before recombination, it was unnecessary to use frt/friptase recombination to delete *neo*. All procedures involving animals used in this study were approved by the NIEHS Animal Care and Use Committee.

Analysis of Sperm Phenotype

Cauda epididymal sperm were collected from 3- to 4-month-old mice by incubating minced cauda epididymis in M2 medium at 37°C for 10 min. Sperm were examined by phase microscopy and photos were taken with a SPOT 2 CCD digital camera (Diagnostic Instruments, Sterling Heights, MI). The length of the principal piece was measured on the computer display, but 18% could not be measured because the tail was curled or was obscured by other

sperm or debris. The length of sperm from mutant mice was expressed relative to the length of sperm from wild type mice. Sperm viability was determined by using the LIVE/DEAD FertiLight sperm viability kit (Molecular Probes, Eugene, OR). Sperm motility was observed before and after incubation in M2 medium at 37°C for 4 h in the presence or absence of 5 mM dibutyryl cAMP. Alternatively, hyperactivated motility was induced by incubating sperm in M16 medium at 37°C for 2 h in 5% CO_2 and air. To determine fertility, six mutant males were mated continuously with two wild type females for 2 months, with the females being replaced every 2 weeks.

Electron Microscopy

Sperm were isolated for scanning electron microscopy as described above, washed in PBS, and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium phosphate buffer overnight at 4°C. Sperm were washed in the buffer and collected on Nucleopore filters or glass coverslips, subjected to critical point drying, and coated with gold/palladium. Samples were examined in a Cambridge S200 scanning electron microscope at 20 KV. For transmission electron microscopy, cauda epididymides were fixed under the same conditions, postfixed in 2% osmium tetroxide in cacodylate buffer, and embedded in Lowicryl resin. Sections were stained with uranyl acetate and lead citrate and examined in a LEO910 transmission electron microscope at 80 kV. The scanning and transmission electron microscopes used were in the Department of Laboratory Medicine and Pathology, University of North Carolina.

Preparation of Antisera to AKAP4

Oligopeptides were synthesized (Sigma-Genosys Biotechnologies, The Woodlands, TX) corresponding to amino acids 191–204 and 790–803 deduced from the mouse *Akap4* cDNA sequence reported by Fulcher *et al.* (1995), and a cysteine was added at the N

terminus for conjugation to KLH carrier protein. New Zealand White rabbits were immunized with conjugated peptide and bled for antiserum production (Covance Research Products, Denver, PA). The antisera to both peptides gave comparable results, but only the antiserum against peptide 191–204 was used in this study.

Western Blotting

Sperm were collected from the cauda epididymis, washed and suspended in PBS (140 mM NaCl, 10 mM phosphate buffer, pH 7.2), and then counted in a hemocytometer. Crude lysates were prepared from testes by homogenization in 1 ml of lysis buffer containing 140 mM NaCl, 0.1% Triton X-100, 1 mM DTT, Complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN), and 20 mM Hepes buffer, pH 7.4. Sperm were lysed directly in SDS sample buffer. Lysates corresponding to 0.5 mg wet weight testis and 5×10^3 sperm (for AKAP4, GAPDS, AKAP3, ODF2, or α-tubulin) or 1×10^5 sperm (for RIα or RIIα) were analyzed by Western blotting (Miki and Eddy, 1998). Antisera and mouse monoclonal antibodies used were rabbit anti-mouse AKAP4 (1:5000 dilution), rabbit anti-mouse GAPDS (Bunch *et al.,* 1998) (1:2000 dilution), rat anti-human AKAP3 (Mandal *et al.,* 1999) (1:1000 dilution; gift of Dr. John C. Herr), rabbit anti-ODF2 (Shao *et al.,* 1997) (1:2000 dilution; gift of Dr. Frans A. van der Hoorn), mouse anti-mouse α-tubulin (1:5000 dilution, Sigma), mouse anti-mouse PKA RIα (1:1000 dilution; Transduction Laboratories, San Diego, CA), and rabbit anti-mouse PKA RIIα (1:1000 dilution; SantaCruz Biotechnology, Inc., Santa Cruz, CA). Proteins were detected by using the ECL method (Amersham Pharmacia Biotech, Piscataway, NJ) after membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (SantaCruz) or anti-rat IgG (ICN-Cappel, Costa Mesa, CA) (Miki and Eddy, 1998). Prestained standard proteins (BioRad Laboratories, Hercules, CA) were used to estimate protein molecular weights.

Immunocytochemistry

Sperm from the cauda epididymis were washed in PBS and diluted to 10^6/ml. A suspension of sperm (30 μl) was deposited on poly-L-lysine-coated glass slides at 4°C for 30 min, treated with ice-cold PBS containing 0.5% (v/v) Triton X-100 for 1 min, fixed for 20 min in methanol at –20°C, and then air-dried. The slides were blocked with Automation Buffer (Biomeda Corp., Foster City, CA) containing 5% (v/v) normal goat serum for 30 min and incubated for 1 h with antisera to AKAP4 (1:200 dilution), GAPDS (1:400 dilution), AKAP3 (1:100 dilution), ODF2 (1:200 dilution), and/or mouse monoclonal antibody to α-tubulin (1:800 dilution; Sigma, St. Louis, MO). The slides then were washed three times with Automation Buffer for 15 min each and incubated for 1 h with FITC-conjugated anti-rabbit IgG (ICN-Cappel), anti-rat IgG (ICN-Cappel), or anti-mouse IgG (ICN-Cappel) or with TRITC-conjugated anti-mouse IgG (Sigma) at 1:200 dilution. Immunostaining was observed by fluorescence microscopy, photographed as described before, and formatted by using Photoshop software (Adobe Systems, San Jose, CA).

RESULTS

Generation of Mice Lacking AKAP4

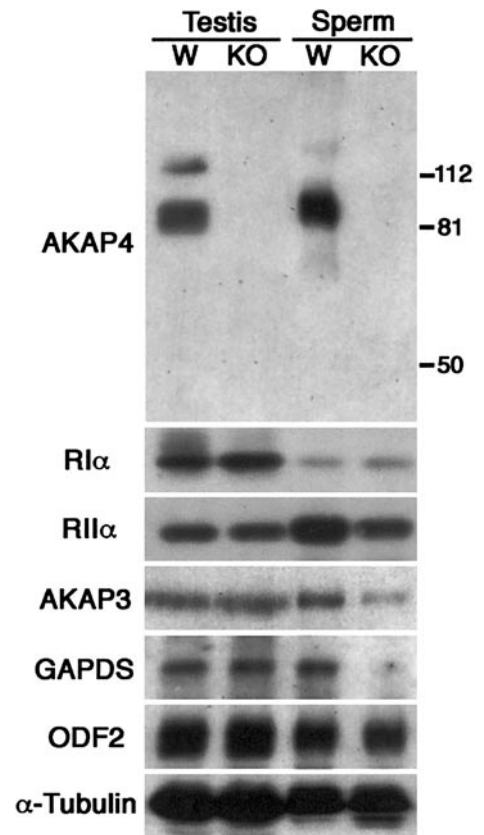Chimeras were produced with ES cells in which loxP sequences flanked a *neo* gene and exon 6 of *Akap4* (Fig. 1).

FIG. 2. Comparison of FS protein expression in testis and sperm of wild type and *Akap4⁻* mice. Testes from wild type (W) and *Akap4⁻* (KO) mice were homogenized in PBS containing protease inhibitors and solubilized in SDS sample buffer. Epididymal sperm were counted and directly solubilized in SDS sample buffer. Sperm flagellar proteins were analyzed by Western blotting using the antibody shown in the left margin. The kDa of the protein standards are shown on the right.

Female offspring were mated with males carrying a *cre* transgene to produce progeny with both the targeted *Akap4* gene and the *cre* transgene. Expression of CRE in oocytes resulted in deletion of the region between the loxP sites and produced a mutated *Akap4* gene for transmission to the next generation (data not shown). Male offspring with a mutated *Akap4* gene were infertile and AKAP4 protein was not detectable with an antiserum to an N-terminal peptide sequence (Fig. 2), but heterozygous females were unaffected and transmitted the mutation to their offspring. The infertility of all males with a mutation in the single-copy *Akap4* gene precluded generation of homozygous females.

Motility of Sperm Lacking AKAP4

Sperm were examined by phase microscopy to determine why the lack of AKAP4 caused male infertility. While the numbers and viability of sperm from mutant mice were not

TABLE 1

Characteristics of Sperm from *Akap4* Mutant and
Wild Type Mice

Parameter	Wild type	$Akap4^-$
Number of epididymal sperm $(\times 10^7)^a$	2.6 ± 0.64	4.5 ± 0.80
Viability $(\%)^b$	74 ± 6.4	71 ± 5.1
Motility $(\%)^b$	78 ± 6.4	8.2 ± 3.9
Relative length of the principle piece^c	1.0 ± 0.04	0.49 ± 0.21
Fertility^d	6/6	0/6

[a] $n = 4$ for wild type; $n = 7$ for $Akap4^-$.
[b] $n = 6$.
[c] $n = 10$ for wild type sperm; $n = 34$ for $Akap4^-$ sperm. This analysis omitted 18% of the population of mutant sperm that could not be measured due to a curly tail or obscuring cell debris. Wild type is taken as 1.0.
[d] Six males were mated with two females each for 2 months, with females being replaced every 2 weeks.

different from those of wild type males, less than 10% were motile (Table 1) and their flagellar motion was sluggish, of low amplitude, and failed to produce forward progression. The middle piece remained straight, and flagellar bending was restricted to the principal and end pieces. Wild type sperm incubated in M16 medium for 2 h developed hyperactivated motility, but sperm lacking AKAP4 incubated in parallel showed no change in the pattern or incidence of motility. Addition of 5 mM dibutyryl cAMP to sperm from mutant mice 10 min after isolation did not increase the percentage motile ($6.2 \pm 4.7\%$), but addition after 4 h did produce an increase ($34 \pm 7.6\%$). However, the type of motility produced was not different from that of untreated sperm of mutant mice that were maintained under the same conditions. These observations indicate that AKAP4 and/or other fibrous sheath proteins that require the presence of AKAP4 are involved in determining the nature and rate of flagellar activity.

Structure of Sperm Lacking AKAP4

The principal piece of sperm lacking AKAP4 was reduced in diameter compared with sperm of wild type mice (Fig. 3). In addition, the flagellum was shortened and the tip was sometimes curled or splayed apart into fine filaments (Figs. 3b–3g; Table 1). Scanning electron microscopy confirmed that the diameter of the principal piece was reduced and that the distal flagellum occasionally splits apart into several filaments (Figs. 3h and 3i). Western blotting with antibodies to an outer dense fiber protein (ODF2) and a microtubule protein (α-tubulin) demonstrated that these proteins are present in comparable amounts in sperm from mutant and wild type mice (Fig. 2). This suggests that the other cytoskeletal structures of the flagellum, the outer dense fibers and axoneme, are intact and that the smaller diameter and splaying of the flagellum was caused by a reduction in size and integrity of the fibrous sheath.

Immunofluorescence microscopy was used to determine the composition of the filaments. Antibodies to ODF2 and to α-tubulin bound to the filaments, indicating that they contain both outer dense fibers and axonemal proteins (Fig. 4). Stronger fluorescence was observed in the principal piece of sperm from mutant mice compared with sperm from wild type mice, probably due to easier access of antibody to proteins in the principal piece lacking a fibrous sheath. Superimposing images of filaments immunostained with both antibodies suggested that the ratio of the two proteins varied between filaments (Fig. 4). However, when sperm within the cauda epididymis of mutant mice were examined by transmission electron microscopy, the appearance and association of outer dense fibers and axonemes were comparable to those in sperm from wild type mice (Fig. 5). Nevertheless, the occurrence of splaying indicates that flagellar integrity is compromised in sperm lacking AKAP4.

The length of the middle piece of sperm from mutant mice was similar to that of sperm from wild type mice (Fig. 3a), but the length of the remainder of the flagellum averaged about half that of sperm from wild type mice (Figs. 3b–3g; Table 1). Damage during passage through the epididymis or during isolation is unlikely to be the cause because the viabilities of sperm freshly isolated from mutant and wild type mice were similar (Table 1). In addition, the flagella of sperm isolated from the caput epididymis of mutant mice were also shorter than flagella of sperm from wild type mice (data not shown). These observations suggest that AKAP4 has a role in flagellar elongation and/or in determining or maintaining flagellar length.

Structure of the Fibrous Sheath in Sperm Lacking AKAP4

Transmission electron microscopy was used to examine the structure of the fibrous sheath in sperm lacking AKAP4. Because of the thin principal piece and poor motility, we did not expect to find a recognizable fibrous sheath. However, the flagella of sperm in the epididymis of mutant mice contained nascent longitudinal columns adjacent to microtubule doublets 3 and 8, and thin circumferential ribs were connected to the columns (Fig. 5). The ribs were regularly spaced, uniform in size, and closely associated with the plasma membrane. This indicates that formation of the fibrous sheath begins and the basic organization is established, but the process is incomplete without the incorporation of AKAP4.

Proteins in the Principal Piece of Sperm Lacking AKAP4

The characteristic feature of AKAPs is their ability to anchor PKAs by their regulatory (R) subunit dimers. Previous studies have shown that AKAP4 has one site that can anchor RIα and another site that can anchor either RIα or RIIα (Miki and Eddy, 1998) and that another AKAP present in the fibrous sheath, AKAP3, has a site that can anchor
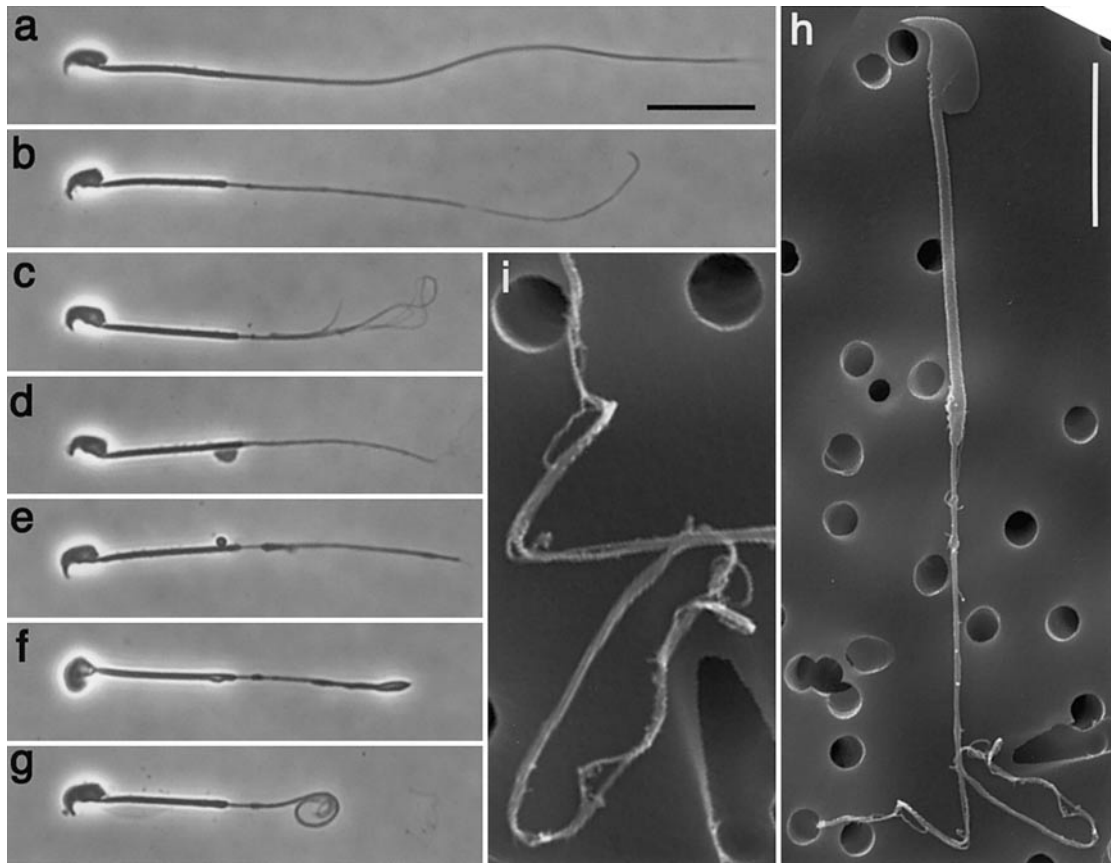
FIG. 3. Abnormal flagella of sperm from *Akap4⁻* mutant mice. Cauda epididymal sperm were gently isolated from wild type (a) and *Akap4⁻* mutant mice (b–g). Sperm in PBS were spotted onto a slide glass and observed by phase contrast microscopy. Scale bar in (a) represents 20 μm. *Akap4⁻* sperm were also observed by scanning electron microscopy (1890× magnification) (h). A higher magnification view of the distal tip of the same flagellum (i). Scale bar in (h) represents 10 μm.

RIIα (Vijayaraghavan *et al.,* 1999). Western blotting (Fig. 2) and immunofluorescence microscopy (Fig. 6) indicated that AKAP3 is present in reduced amounts in sperm lacking AKAP4, compared with sperm from wild type mice. We expected a decrease in PKA levels in sperm lacking AKAP4 and with reduced amounts of AKAP3. While RIIα levels were reduced, RIα levels appeared to be unchanged (Fig. 2).

We next determined whether AKAP4 anchors other proteins in the fibrous sheath that might be involved in regulating flagellar activity. Glycolysis is a major source of energy for sperm functions essential for fertility (Kellerman and Weed, 1970; Frenkel *et al.,* 1975; Fraser and Quinn, 1981). GAPDS is a glycolytic enzyme encoded by a gene expressed only in spermatogenic cells (Welch *et al.,* 1992) and remains tightly associated with the fibrous sheath under rigorous extraction procedures (Bunch *et al.,* 1998). GAPDS was not detectable by immunofluorescence microscopy in sperm from mutant mice (Fig. 6). In addition, Western blotting showed that GAPDS was expressed at comparable levels in the testis of wild type and mutant

mice, but was present in considerably lower amounts in sperm from mutant mice (Fig. 2). These results indicate that AKAP4 is required for GAPDS to bind to the fibrous sheath and strongly suggest that glycolysis is disrupted in sperm lacking AKAP4.

DISCUSSION

It is generally accepted that AKAPs anchor PKAs within particular cytoplasmic regions, placing them in proximity to proteins immediately downstream in signal transduction pathways. A more recent model broadens their roles to include serving as scaffolds for protein complexes involved in regulating signal transduction processes (Edwards and Scott, 2000; Colledge and Scott, 2000; Colledge *et al.,* 2000, Dodge and Scott, 2000). AKAP4 is present only in spermatogenic cells and is the predominant protein in the fibrous sheath of the sperm flagellum. The restricted temporal and spatial expression of AKAP4 provided a unique opportunity
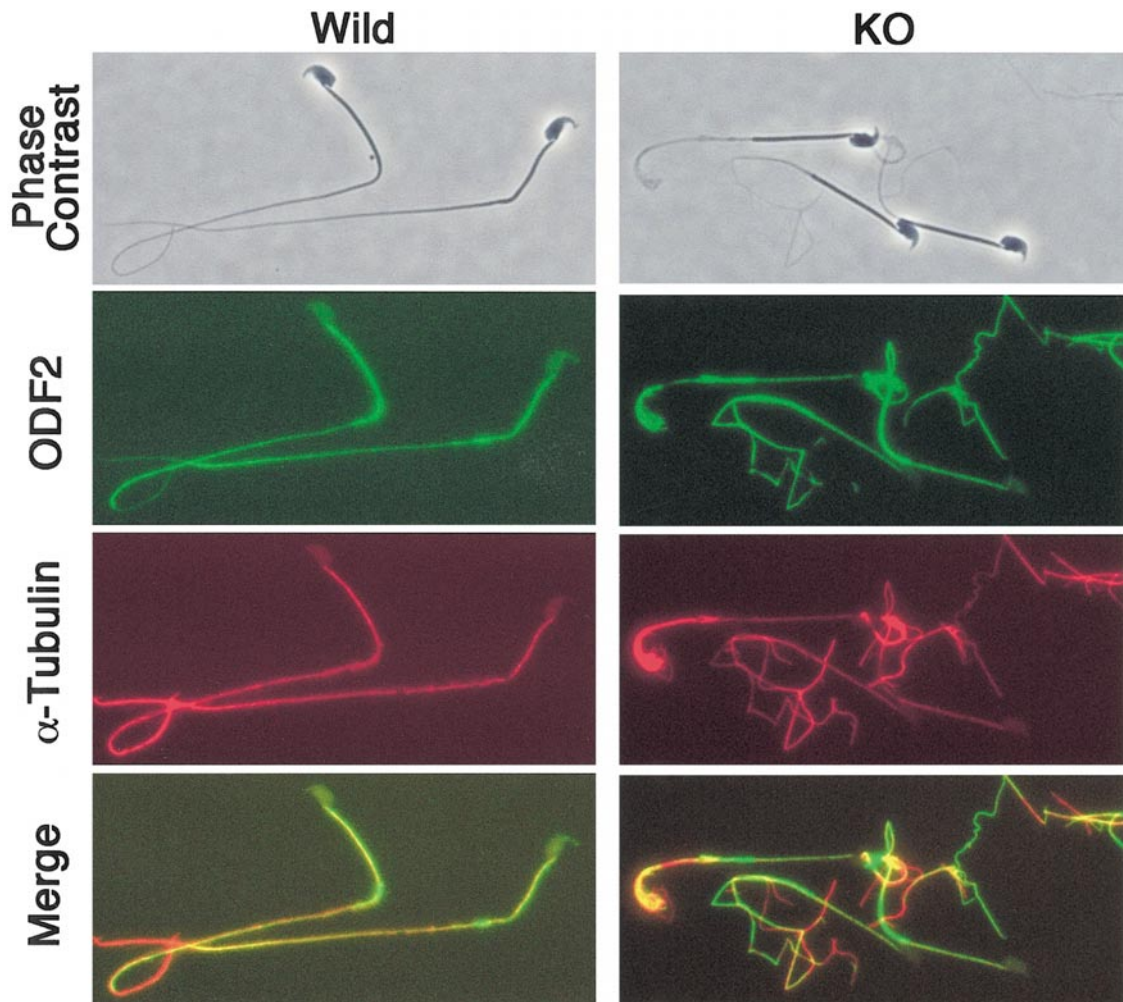
Wild   KO

FIG. 4. The presence of ODF and axoneme components in *Akap4⁻* sperm. Sperm were permeabilized and stained with antibodies to ODF2 and α-tubulin, markers of ODF and the axoneme, respectively. Images were merged by using Photoshop software.

to use gene targeting to test the scaffold model by determining how the loss of AKAP4 affects fibrous sheath composition and sperm function. These studies demonstrated that male mice lacking AKAP4 were infertile, the motility of their sperm was poor, the principal piece of the flagellum was reduced in diameter, the fibrous sheath was incompletely developed, and other proteins usually found in the principal piece region of the flagellum were either absent or reduced in amount. Possible explanations for these effects are that PKA is present in reduced amounts, that the PKA distribution is altered, that the association of other proteins with the fibrous sheath is disrupted, and/or that significant structural changes have occurred in the fibrous sheath.

PKA has an important role in the regulation of sperm function (Garbers and Kopf, 1980; Burton *et al.,* 1999), and Western analysis was used to determine whether the

amount of PKA was reduced in sperm from mutant mice. While there are genes for at least four regulatory subunits of PKA in the mouse, RIα and RIIα predominate in sperm and are present throughout the flagellum (Visconti *et al.,* 1997; Moos *et al.,* 1998; Burton *et al.,* 1999). More than half of the RII subunits in the detergent-resistant fraction of rat sperm were associated with an 80,000-molecular weight protein (Horowitz *et al.,* 1984), which was suggested to be AKAP4 (Johnson *et al.,* 1997). Detergent-insoluble RI subunits are present in mouse sperm (Visconti *et al.,* 1997), and RI subunits were associated with the fibrous sheath and outer dense fibers of boar sperm (Moos *et al.,* 1998). AKAP4 is an abundant protein in sperm, is detergent-insoluble, and has domains that bind RIα and RIIα (Miki and Eddy, 1998). We expected the lack of AKAP4 and decrease in amount of AKAP3 in sperm from mutant mice would result in reduced RIα and RIIα levels. Our Western blotting results indicated
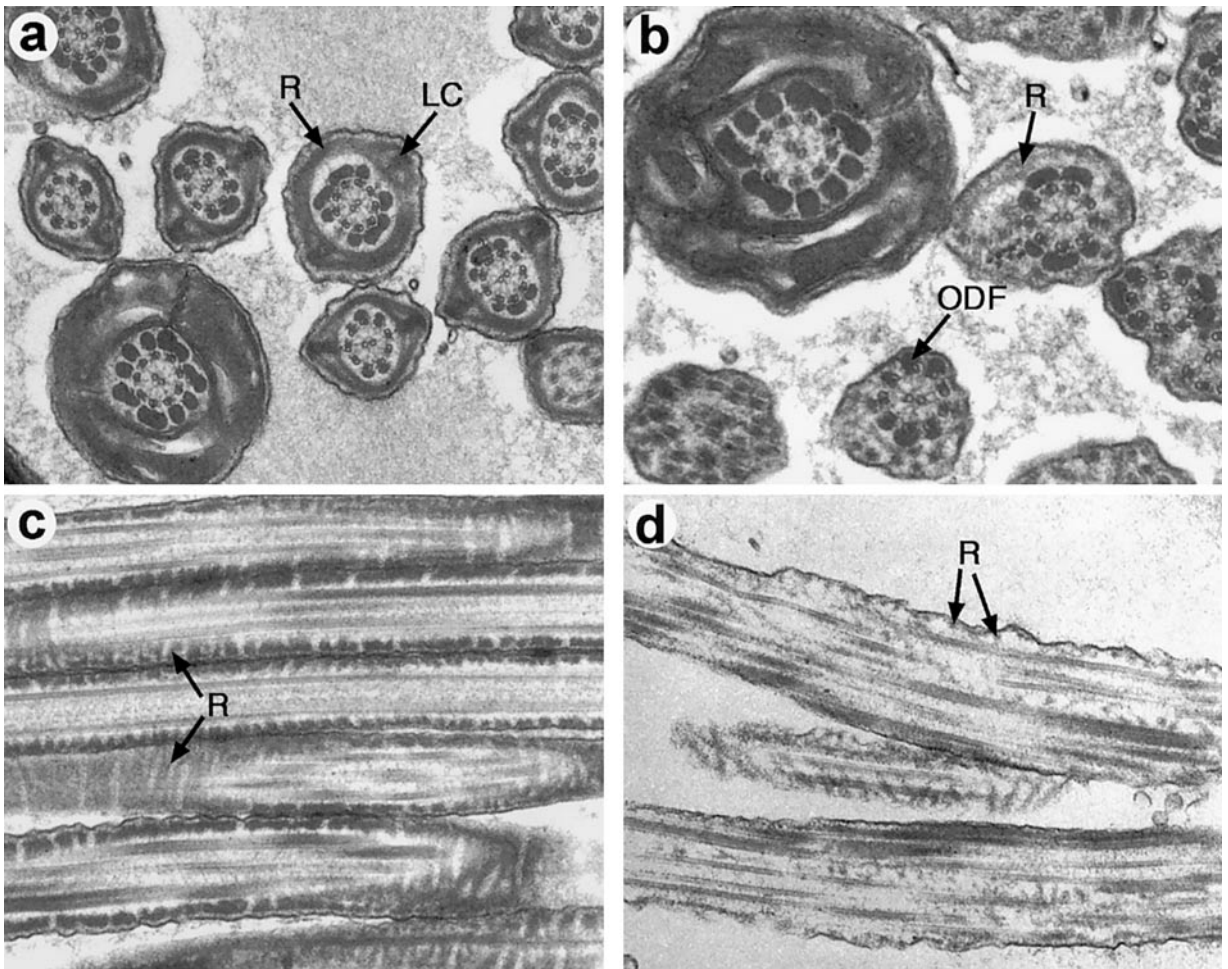
FIG. 5. Transmission electron micrographs showing incomplete developing of the fibrous sheath of sperm from mice with a mutation in the *Akap4* gene. Cauda epididymides from wild type (a, c) and hemizygous mutant mice (b, d) were fixed and sectioned for the transmission electron microscopy. Cross-sections (a, b) and sagittal sections (c, d) of flagella are shown. R, rib of the fibrous sheath; LC, longitudinal column of the fibrous sheath; ODF, outer dense fibers. Magnification, 20,000×.

that RIIα levels are reduced while RIα levels are unchanged in sperm from mutant mice. In addition, there was an increase in immunofluorescence in the middle piece and a decrease in immunofluorescence in the principal piece of sperm from mutant mice after staining with anti-RIα or anti-RIIα, compared with sperm from wild type mice (unpublished observation). Because sperm from wild type mice stain uniformly for RIα and RIIα (Visconti *et al.,* 1997; Moos *et al.,* 1998; Burton *et al.,* 1999), these observations suggest that redistribution and reduction in amount of PKA contribute to the poor motility of sperm from mutant mice.

Another cause of the sperm defects might be that PKA is no longer anchored in proximity to target proteins involved in regulating flagellar activity. However, there are conflicting data on whether or not PKA anchoring is required for sperm function. The motility of bovine sperm was disrupted by addition of a membrane-permeable peptide that reversibly inhibited PKA binding, suggesting that interaction of RII with AKAPs, and not PKA catalytic activity, is required for motility (Vijayaraghavan *et al.,* 1997). In contrast, the majority of RI and RII subunits were in the detergent-soluble fraction of mouse sperm during capacitation (Visconti *et al.,* 1997). Also, the majority of PKA was not anchored to the flagellum in mice with a targeted mutation in the gene for RIIα, but sperm motility and fertility were unaltered (Burton *et al.,* 1999). Some of these differences might be explained by the observations that RIα levels were increased in RIIα-mutant mice (Burton *et al.,* 1999) and that RIα can bind to both the RIα-specific domain and to the RIα/RIIα dual-specificity domain of AKAP4 (Miki and Eddy, 1998). RIIα has a 500-fold greater affinity than RIα for binding the Ht31 peptide *in vitro* (Burton *et al.,* 1997). If the affinity of RIIα for AKAP4 is similar *in vitro,* RIα would presumably be excluded from binding to the

RIα/RIIα dual domain when both are present, but RIα should be able to anchor PKA to both the RIα-specific and RIα/RIIα dual domain of AKAP4 in mice lacking RIIα. However, the membrane-permeable peptide might inhibit binding of both RI and RII subunits. Furthermore, it is unclear whether the binding affinity of RIα to the Ht31 peptide is an accurate indication of the binding affinity to AKAP4 *in vivo*. Taken together, these findings suggest that another cause of the sperm defects in mice lacking AKAP4 is that loss of RIα and RIIα binding sites in the flagellum results in the separation of PKA from target proteins that regulate flagellar activity.

If AKAP4 were a scaffold for localizing and grouping functionally important proteins in addition to PKA, this association would be lost in mutant mice. Sperm have a high glycolytic capacity (Brooks, 1976), utilize glucose effectively, and require glucose for hyperactivated motility (Brooks, 1976; Fraser and Quinn, 1981), fertilization (Hoppe, 1976), and capacitation (Travis *et al.,* 2001). Neither lactate nor pyruvate can substitute for glucose in these processes, and fertilization occurs *in vitro* in the presence of glucose and oligomycin, an inhibitor of oxidative phosphorylation that disrupts ATP production by mitochondria (Fraser and Quinn, 1981). The glycolytic enzymes of sperm associate as a complex insoluble in detergents that cofractionates with flagellar components (Mohri *et al.,* 1965; Harrison, 1971; Storey and Kayne, 1978). Most of the glycolytic enzymes in sperm have unique structural or function properties (reviewed in Eddy *et al.,* 1994). Genes for glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS) (Welch *et al.,* 1992), phosphoglycerate kinase-2 (PGK2) (McCarrey and Thomas, 1987), and lactate dehydrogenase-C (LDHC) (Sakai *et al.,* 1987), as well as alternate transcripts for type 1 hexokinase-S (HK1S) (Mori *et al.,* 1993), are expressed only in spermatogenic cells. GAPDS is tightly bound to the fibrous sheath (Bunch *et al.,* 1998) and loss of the enzyme in the absence of AKAP4 would block glycolysis. GAPDS was present in the spermatids of mutant mice (data not shown), but failed to accumulate in the flagellum or become incorporated into the fibrous sheath. This suggests that another effect of AKAP4 loss is the disruption of glycolysis and supports earlier proposals that glycolysis within the flagellum is important for maintenance of sperm function (Storey and Kayne, 1978; Eddy *et al.,* 1994; Travis *et al.,* 2001).

It was suggested recently that components of the Rho signaling pathway associated with the fibrous sheath may be involved in regulating flagellar function (Carr *et al.,* 2001). These include the Rho-binding protein rhophilin (Nakamura *et al.,* 1999) and ropporin, a spermatogenic cell-specific protein that binds to rhophilin and to the fibrous sheath (Fujita *et al.,* 2000). In addition, ropporin and AKAP-associated sperm protein (ASP) have N-terminal regions similar to the AKAP-binding domain of PKA RII subunits and can bind to the PKA-anchoring domain of AKAP3 (Carr *et al.,* 2001). If Rho signaling is involved in flagellar function, lack of AKAP4 and decreased amounts of AKAP3 might be expected to disrupt this pathway.

Disruption of the *Akap4* gene caused changes in the structure of the flagellum that included a thinner principal piece, incomplete development of the fibrous sheath, and a shorter flagellum. AKAP4 is present in both the longitudinal columns and circumferential ribs (Johnson *et al.,* 1997) and is synthesized late in spermiogenesis (Carrera *et al.,* 1994). The development of longitudinal column and rib anlagens (Irons and Clermont, 1982a) in sperm from mutant mice strongly suggests that AKAP4 associates with a pre-existing template to complete formation of the fibrous sheath. This is consistent with the results of a detailed study on the development of the fibrous sheath in rat. The precursors of the longitudinal columns first appeared at the distal end of the principal piece in round spermatids during the early part of spermiogenesis and continued to form in condensing spermatids until the end of spermiogenesis (Irons and Clermont, 1982a). The rib precursors did not appear until the development of elongating spermatids and continued to form along the flagellum through the early part of condensing spermatid development. The formation of the definitive fibrous sheath occurs near the end of spermiogenesis in both mouse (Sakai *et al.,* 1986) and rat (Irons and Clermont, 1982a). This is after the appearance of the ribs and concurrently with the synthesis and incorporation of AKAP4 into the fibrous sheath (unpublished observations). An implication of these data is that dysplasia of the fibrous sheath, a cause of infertility in men that is familial in many cases (Chemes, 2000), might be caused by a mutation in a gene affecting formation of the fibrous sheath template rather than in the *AKAP4* gene (Turner *et al.,* 2001).

Failure of the normal development of the axoneme severely disrupts formation of the fibrous sheath and outer dense fibers (Phillips *et al.,* 1993), but it was unknown whether incomplete development of the fibrous sheath affects the formation of the axoneme or outer dense fibers. The axoneme exists before fibrous sheath assembly begins and, as was expected, the organization of the axoneme appeared to be intact in sperm from mutant mice. However, the outer dense fibers begin to develop later than the fibrous sheath (Irons and Clermont, 1982b). While it seemed possible that outer dense fiber formation might be disrupted in sperm from mutant mice, their organization and distribution appeared to be unaltered in sperm lacking a well-developed fibrous sheath. In addition, the outer dense fibers are closely associated with the microtubule doublets of the axoneme and they remain connected when the mouse sperm axoneme is dissociated with mild trypsin treatment (Si and Okuno, 1995). In this study, the outer dense fibers and microtubules also remained connected when the flagellum split apart, indicating that the interactions between these two cytoskeletal components were maintained in sperm from mutant mice. In addition, the flagella were shortened in these sperm. The outer dense fibers extend only part way through the principal piece, suggesting that the axoneme terminates near the end of the outer dense fibers in the absence of a fully formed fibrous sheath.
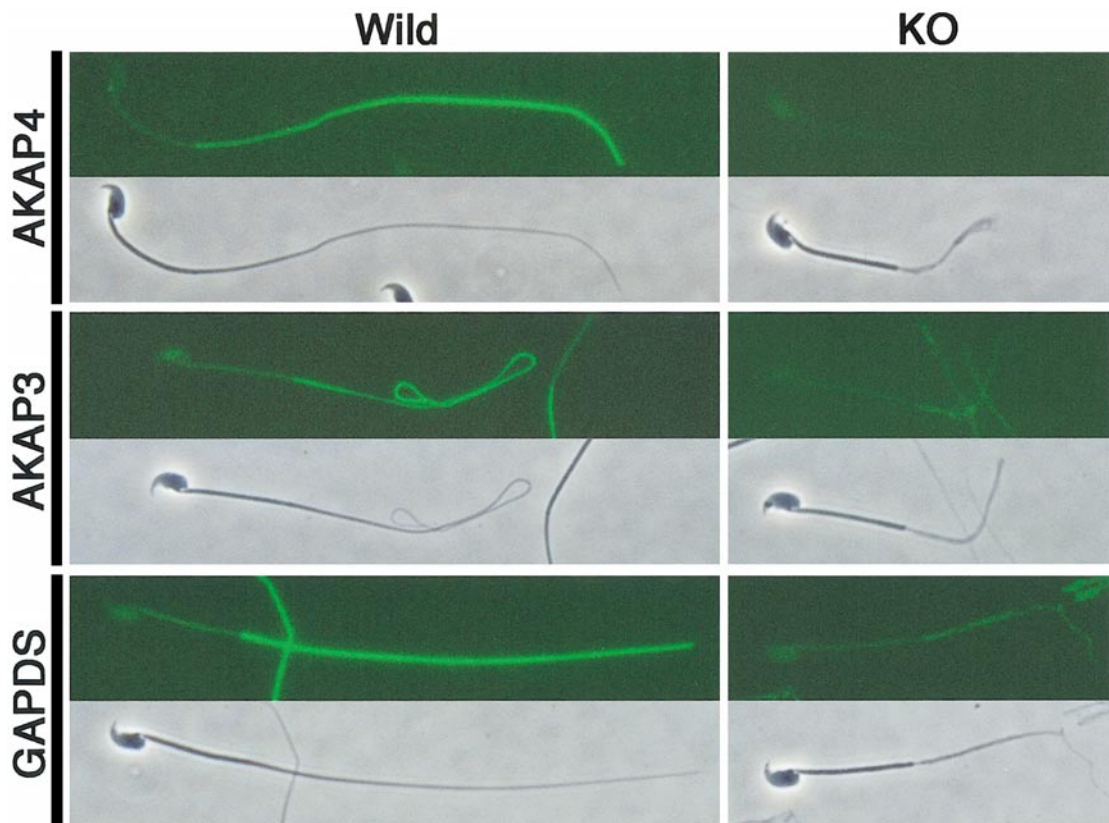
FIG. 6. The localization of FS proteins in *Akap4⁻* sperm. Wild type (W) and *Akap4⁻* (KO) sperm were permeabilized and stained with antibodies to AKAP4, GAPDS, or AKAP3. Upper panels of each set show the immunofluorescence image and lower panels show the phase contrast image of the same field.

The classical view was that the fibrous sheath has a passive structural role, that of influencing the degree and shape of flagellar bending (Fawcett, 1975). However, these studies show that absence of AKAP4 results in the loss of other proteins from the fibrous sheath and severely disrupts flagellar function. These findings are consistent with a model proposing that the fibrous sheath positions PKA in proximity to its target proteins, anchors signal transduction pathway components that regulate flagellar activity, and forms a scaffold for enzymes in the glycolytic pathway that are required for essential sperm functions. They also demonstrate that AKAP4 is essential for the fibrous sheath to accomplish its diverse responsibilities.

REFERENCES

Amieux, P. S, Cummings, D. E., Motamed, K., Brandon, E. P., Wailes, L. A., Le, K., Idzerda, R. L., and McKnight, G. S. (1997). Compensatory regulation of RIα protein levels in protein kinase A mutant mice. *J. Biol. Chem.* **272,** 3993–3998.

Brandon, E. P., Zhuo, M., Huang, Y. Y., Qi, M., Gerhold, K. A., Burton, K. A., Kandel, E. R., McKnight, G. S., and Idzerda, R. L. (1995). Hippocampal long-term depression and depotentiation are defective in mice carrying a targeted disruption of the gene encoding the RIβ subunit of cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **92,** 8851–8855.

Brooks, D. E. (1976). Activity and androgenic control of glycolytic enzymes in the epididymis and epididymal spermatozoa of the rat. *Biochem. J.* **156,** 527–537.

Bunch, D. O, Welch, J. E., Magyar, P. L., Eddy, E. M., and O'Brien, D. A. (1998). Glyceraldehyde 3-phosphate dehydrogenase-S protein distribution during mouse spermatogenesis. *Biol. Reprod.* **58**, 834–841.

Burton, K. A., Johnson, B. D., Hausken, Z. E., Westenbroek, R. E., Idzerda, R. L., Scheuer, T., Scott, J. D., Catterall, W. A., and McKnight, G. S. (1997). Type II regulatory subunits are not required for the anchoring-dependent modulation of Ca²⁺ channel activity by cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **94,** 11067–11072.

Burton, K. A., Treash-Osio, B., Muller, C. H., Dunphy, E. L., and McKnight, G. S. (1999). Deletion of type IIα regulatory subunit delocalizes protein kinase A in mouse sperm without affecting motility or fertilization. *J. Biol. Chem.* **274,** 24131–24136.

Carr, D. W., Fujita, A., Stentz, C. L., Liberty, G. A., Olson, G. E., and Narumiya, S. (2001). Identification of sperm-specific proteins that interact with A-kinase anchoring proteins in a manner similar to the type II regulatory subunit of PKA. *J. Biol. Chem.* **276,** 17332–17338.

Carrera, A., Gerton, G. L., and Moss, S. B. (1994). The major fibrous sheath polypeptide of mouse sperm: Structural and functional similarities to the A-kinase anchoring proteins. *Dev. Biol.* **165,** 272–284.

Chemes, H. E. (2000). Phenotypes of sperm pathology: Genetic and acquired forms in infertile men. *J. Androl.* **21,** 799–808.

Colledge, M., Dean, R. A., Scott, G. K., Langeberg, L. K., Huganir, R. L., and Scott, J. D. (2000). Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. *Neuron* **27,** 107–119.

Colledge, M., and Scott, J. D. (1999). AKAPs: From structure to function. *Trends Cell Biol.* **9,** 216–221.

Cummings, D. E., Brandon, E. P., Planas, J. V., Motamed, K., Idzerda, R. L., and McKnight, G. S. (1996). Genetically lean mice result from targeted disruption of the RII beta subunit of protein kinase A. *Nature* **382,** 622–626.

Diviani, D., and Scott, J. D. (2001). AKAP signaling complexes at the cytoskeleton. *J. Cell Sci.* **114,** 1431–1437.

Dix, D. J., Allen, J. W., Collins, B. W., Mori, C., Nakamura, N., Poorman-Allen, P., Goulding, E. H., and Eddy, E. M. (1996). Targeted gene disruption of Hsp70–2 results in failed meiosis, germ cell apoptosis, and male infertility. *Proc. Natl. Acad. Sci. USA* **93,** 3264–3268.

Dodge, K., and Scott, J. D. (2000). AKAP79 and the evolution of the AKAP model. *FEBS Lett.* **476,** 58–61.

Eddy, E. M., Welch, J. E., Mori, C., Fulcher, K. D., and O'Brien, D. A. (1994). Role and regulation of spermatogenic cell-specific gene expression: Enzymes of glycolysis. In "Function of Somatic Cells in the Testis" (A. Bartke, Ed.), pp. 362–372. Springer-Verlag, New York.

Edwards, A. S., and Scott, J. D. (2000). A-kinase anchoring proteins: Protein kinase A and beyond. *Curr. Opin. Cell Biol.* **12,** 217–221.

Fawcett, D. W. (1975). The mammalian spermatozoon. *Dev. Biol.* **44,** 394–436.

Fraser, L. R., and Quinn, P. J. (1981). A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. *J. Reprod. Fertil.* **61,** 25–35.

Frenkel, G., Peterson, R. N., and Freund, M. (1975). Oxidative and glycolytic metabolism of semen components by washed guinea pig spermatozoa. *Fertil. Steril.* **26,** 144–147.

Fujita, A., Nakamura, K., Kato, T., Watanabe, N., Ishizaki, T., Kimura, K., Mizoguchi, A., and Narumiya, S. (2000). Ropporin, a sperm-specific binding protein of rhophilin, that is localized in the fibrous sheath of sperm flagella. *J. Cell Sci.* **113,** 103–112.

Fulcher, K. D., Mori, C., Welch, J. E., O'Brien, D. A., Klapper, D. G., and Eddy, E. M. (1995). Characterization of Fsc1 cDNA for a mouse sperm fibrous sheath component. *Biol. Reprod.* **52,** 41–49.

Garbers, D. L., and Kopf, G. S. (1980). The regulation of spermatozoa by calcium cyclic nucleotides. *Adv. Cyclic Nucleotides Res.* **13,** 251–306.

Harrison, R. A. (1971). Glycolytic enzymes in mammalian spermatozoa. Activities and stabilities of hexokinase and phosphofruc-tokinase in various fractions from sperm homogenates. *Biochem. J.* **124,** 741–750.

Hoppe, P. C. (1976). Glucose requirement for mouse sperm capacitation in vitro. *Biol. Reprod.* **15,** 39–45.

Horowitz, J. A., Toeg, H., and Orr, G. A. (1984). Characterization and localization of cAMP-dependent protein kinases in rat caudal epididymal sperm. *J. Biol. Chem.* **259,** 832–838.

Irons, M. J., and Clermont, Y. (1982a). Kinetics of fibrous sheath formation in the rat spermatid. *Am. J. Anat.* **165,** 121–130.

Irons, M. J., and Clermont, Y. (1982b). Formation of the outer dense fibers during spermiogenesis in the rat. *Anat. Rec.* **202,** 463–471.

Johnson, L. R., Foster, J. A., Haig-Ladewig, L., VanScoy, H., Rubin, C. S., Moss, S. B., and Gerton, G. L. (1997). Assembly of AKAP82, a protein kinase A anchor protein, into the fibrous sheath of mouse sperm. *Dev. Biol.* **192,** 340–350.

Kellerman, A. S., and Weed, J. C. (1970). Sperm motility and survival in relation to glucose concentration: An in vitro study. *Fertil. Steril.* **21,** 802–805.

Lewis, B., and Aitken, R. J. (2001). Impact of epididymal maturation on the tyrosine phosphorylation patterns exhibited by rat spermatozoa. *Biol. Reprod.* **64,** 1545–1556.

Mandal, A., Naaby-Hansen, S., Wolkowicz, M. J., Klotz, K., Shetty, J., Retief, J. D., Coonrod, S. A., Kinter, M., Sherman, N., Cesar, F., Flickinger, C. J., and Herr, J. C. (1999). FSP95, a testis-specific 95-kilodalton fibrous sheath antigen that undergoes tyrosine phosphorylation in capacitated human spermatozoa. *Biol. Reprod.* **61,** 1184–1197.

McCarrey, J. R., and Thomas, K. (1987). Human testis-specific PGK gene lacks introns and possesses characteristics of a processed gene. *Nature* **326,** 501–505.

Miki, K., and Eddy, E. M. (1998). Identification of tethering domains for protein kinase A type Iα regulatory subunits on sperm fibrous sheath protein FSC1. *J. Biol. Chem.* **273,** 34384–34390.

Miki, K., and Eddy, E. M. (1999). Single amino acids determine specificity of binding of protein kinase A regulatory subunits by protein kinase A anchoring proteins. *J. Biol. Chem.* **274,** 29057–29062.

Mohri, H., Mohri, T., and Ernster, L. (1965). Isolation and enzymatic properties of the midpiece of bull spermatozoa. *Exp. Cell Res.* **38,** 217–246.

Moos, J., Peknicova, J., Geussova, G., Philimonenko, V., and Hozak, P. (1998). Association of protein kinase A type I with detergent-resistant structures of mammalian sperm cells. *Mol. Reprod. Dev.* **50,** 79–85.

Mori, C., Welch, J. E., Fulcher, K. D., O'Brien, D. A., and Eddy, E. M. (1993). Unique hexokinase messenger ribonucleic acids lacking the porin-binding domain are developmentally expressed in mouse spermatogenic cells. *Biol. Reprod.* **49,** 191–203.

Morton, B., and Albagli, L. (1973). Modification of hamster sperm adenyl cyclase by capacitation in vitro. *Biochem. Biophys. Res. Commun.* **50,** 697–703.

Moss, S. B., VanScoy, H., and Gerton, G. L. (1997). Mapping of a haploid transcribed and translated sperm-specific gene to the mouse X chromosome. *Mamm. Genome* **8,** 37–38.

Nakamura, K., Fujita, A., Murata, T., Watanabe, G., Mori, C., Fujita, J., Watanabe, N., Ishizaki, T., Yoshida, O., and Narumiya, S. (1999). Rhophilin, a small GTPase Rho-binding protein, is abundantly expressed in the mouse testis and localized in the principal piece of the sperm tail. *FEBS Lett.* **445,** 9–13.

Phillips, D. M., Pilder, S. H., Olds-Clarke, P. J., and Silver, L. M. (1993). Factors that may regulate assembly of the mammalian

sperm tail deduced from a mouse t complex mutation. *Biol. Reprod.* **49,** 1347–1352.

Sakai, I., Sharief, F. S., and Li, S. S. (1987). Molecular cloning and nucleotide sequence of the cDNA for sperm-specific lactate dehydrogenase-C from mouse. *Biochem. J.* **242,** 619–622.

Sakai, Y., Koyama, Y.-I., Fujimoto, H., Nakamoto, T., and Ya-mashina, S. (1986). Immunocytochemical study on fibrous sheath formation in mouse spermiogenesis using a monoclonal antibody. *Anat. Rec.* **215,** 119–126.

Shao, X., Tarnasky, H. A., Schalles, U., Oko, R., and van der Hoorn, F. A. (1997). Interactional cloning of the 84-kDa major outer dense fiber protein Odf84. Leucine zippers mediate associations of Odf84 and Odf27. *J. Biol. Chem.* **272,** 6105–6113.

Si, Y., and Okuno, M. (1995). Extrusion of microtubule doublet outer dense fibers 5–6 associating with fibrous sheath sliding in mouse sperm flagella. *J. Exp. Zool.* **273,** 355–362.

Storey, B. T., and Kayne, F. J. (1978). Energy metabolism of spermatozoa. VII. Interactions between lactate, pyruvate and malate as oxidative substrates for rabbit sperm mitochondria. *Biol. Reprod.* **18,** 527–536.

Tash, J. S., and Means, A. R. (1983). Cyclic adenosine 3′, 5′ monophosphate, calcium and protein phosphorylation in flagellar motility. *Biol. Reprod.* **28,** 75–104.

Travis, A. J., Sui, D., Riedel, K. D., Hofmann, N. R., Moss, S. B., Wilson, J. E., and Kopf, G. S. (2001). A novel NH(2)-terminal, nonhydrophobic motif targets a male germ cell-specific hexoki-nase to the endoplasmic reticulum and plasma membrane. *J. Biol. Chem.* **274,** 34467–34475.

Turner, R. M., Musse, M. P., Mandal, A., Klotz, K., Jayes, F. C., Herr, J. C., Gerton, G. L., Moss, S. B., and Chemes, H. E. (2001). Molecular genetic analysis of two human sperm fibrous sheath proteins, AKAP4 and AKAP3, in men with dysplasia of the fibrous sheath. *J. Androl.* **22,** 302–315.

Vijayaraghavan, S., Goueli, S. A., Davey, M. P., and Carr, D. W. (1997). Protein kinase A-anchoring inhibitor peptides arrest mammalian sperm motility. *J. Biol. Chem.* **272,** 4747–4752.

Vijayaraghavan, S., Liberty, G. A., Mohan, J., Winfrey, V. P., Olson, G. E., and Carr, D. W. (1999). Isolation and molecular character-ization of AKAP110, a novel, sperm-specific protein kinase A-anchoring protein. *Mol. Endocrinol.* **13,** 705–717.

Visconti, P. E., Bailey, J. L., Moore, G. D., Pan, D., Olds-Clarke, P., and Kopf, G. S. (1995a). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121,** 1129–1137.

Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P., and Kopf, G. S. (1995b). Capaci-tation of mouse spermatozoa. II. Protein tyrosine phosphoryla-tion and capacitation are regulated by a cAMP-dependent path-way. *Development* **121,** 1139–1150.

Visconti, P. E., Johnson, L. R., Oyaski, M., Fornes, M., Moss, S. B., Gerton, G. L., and Kopf, G. S. (1997). Regulation, localization, and anchoring of protein kinase A subunits during mouse sperm capacitation. *Dev. Biol.* **192,** 351–363.

Wagner, K. U., Wall, R. J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P. A., and Hennighausen, L. (1997). Cre-mediated gene deletion in the mammary gland. *Nucleic Acids Res.* **25,** 4323–4330.

Welch, J. E., Schatte, E. C., O'Brien, D. A., and Eddy, E. M. (1992). Expression of a glyceraldehyde 3-phosphate dehydrogenase gene specific to mouse spermatogenic cells. *Biol. Reprod.* **46,** 869–878.

Westhoff, D., and Kamp, G. (1997). Glyceraldehyde 3-phosphate dehydrogenase is bound to the fibrous sheath of mammalian spermatozoa. *J. Cell Sci.* **110,** 1821–1829.

Yanagimachi, R. (1970). The movement of golden hamster sperma-tozoa before and after capacitation. *J. Reprod. Fertil.* **23,** 193–196.