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Association of sperm protein 17 with A-kinase anchoring protein 3 in flagella

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Abstract

Background: Sperm protein 17 (Sp17) is a three-domain protein that contains: 1) a highly conserved N-terminal domain that is 45% identical to the human type II alpha regulatory subunit (RII alpha) of protein kinase A (PKA); 2) a central sulphated carbohydrate-binding domain; and 3) a C-terminal Ca++/calmodulin (CaM) binding domain. Although Sp17 was originally discovered and characterized in spermatozoa, its mRNA has now been found in a variety of normal mouse and human tissues. However, Sp17 protein is found predominantly in spermatozoa, cilia and human neoplastic cell lines. This study demonstrates that Sp17 from spermatozoa binds A-kinase anchoring protein 3 (AKAP3), confirming the functionality of the N-terminal domain.

Methods: In this study in vitro precipitation and immunolocalization demonstrate that Sp17 binds to AKAP3 (AKAP110) in spermatozoa.

Results: Sp17 is present in the head and tail of spermatozoa, in the tail it is in the fibrous sheath, which contains AKAP3 and AKAP4. Recombinant AKAP3 and AKAP4 RII binding domains were synthesized as glutathione S-transferase (GST) fusion proteins immobilized on glutathione-agarose resin and added to CHAPS extracts of human spermatozoa. Western blots of bound and eluted proteins probed with anti-Sp17 revealed that AKAP3 bound and precipitated a significant level of Sp17 while AKAP4 did not. AKAP4 binds AKAP3 and expression of AKAP3 is reduced in AKAP4 knockout sperm, therefore we tested AKAP4 knockout spermatozoa for Sp17 and found that there was a reduction in the amount of Sp17 expressed when compared to wild type spermatozoa. Co-localization of AKAP3 and Sp17 by immunofluorescence was demonstrated along the length of the principal piece of the flagella.

Conclusions: As predicted by its N-terminal domain that is 45% identical to the human RIIα of PKA, Sp17 from spermatozoa binds the RII binding domain of AKAP3 along the length of the flagella.

Background

Sperm protein 17 (Sp17) is a three-domain protein that contains: 1) a highly conserved N-terminal domain that is 45% identical to the human type II alpha regulatory subunit (RII alpha) of PKA; 2) a central sulphated carbohydrate-binding domain; and 3) a C-terminal Ca^{++}/calmodulin (CaM) binding domain [1-4]. Although Sp17 was originally discovered and characterized in spermatozoa as a zona pellucida binding protein because of its ability to bind sulphated carbohydrates [1,3,5], its mRNA has

now been found in a variety of normal mouse [3] and human tissues [6] as well as in malignant somatic cells, including lymphocytes [7], primary ovarian carcinoma cells [8], and other human neoplastic cell lines [9]. Present in the head of mouse and rabbit spermatozoa, Sp17 has been shown to function immediately after the beginning of the acrosome reaction as a zona pellucida binding protein and to lose its C-terminal Ca^{++}/calmodulin (CaM) binding domain [1,2,10,11]. The role of Sp17 in flagella has never been investigated and it seems likely that the N-terminal dimer interaction domain, which is highly conserved [3] and 45% identical to the human RIIα of PKA [1] might interact with AKAPs that are abundant in the flagella. The similarity of the N-terminal domain to RIIα implicates Sp17 as a binding protein of an AKAP (A-kinase anchoring protein; [3]) and Carr et al. [12] have recently confirmed by an alignment analysis the similarity of the N-terminal of Sp17 to several other proteins from testis containing RII alpha-like AKAP binding domains (ASP (AKAP-associated protein), FSII (fibrosheathin II), ropporin).

Lymphocytes [13], spermatozoa [14-16] and cilia [17] have a variety of AKAPs. In fact all cells that have been investigated contain a variety of AKAPs [18,19], which serve to localize cAMP-dependent protein kinase (PKA) to different compartments within the cell and thereby restrict phosphorylation of target proteins and the resultant signal cascades to distinct regions within the cell. As a part of this function AKAPs are often considered to be scaffold proteins binding various components of signal transduction pathways in addition to the protein kinases [20,21]. In the flagella of spermatozoa AKAP3 (AKAP110) and AKAP4 (AKAP82) are the two most abundant proteins of the fibrous sheath (FS; [14,15,22]). AKAP4, which has two PKA regulatory sub-unit binding sites, one specific for RI alpha and one with dual specificity for RI alpha and RII alpha [14], can bind AKAP3 [16]. Two separate lines of evidence have addressed the role of AKAP3. The first suggests AKAP3 acts as a scaffold protein for RII alpha and the alpha subunit of heterotrimeric G protein, G13 [23]. The second shows that the RII alpha binding site of AKAP3 is, so far, unique in its ability to bind proteins other than RII alpha because ropporin and ASP (AKAP-associated sperm protein) have both been shown to bind AKAP3 [12]. Little is known of ASP but ropporin is known to bind rhophilin, which is a GTPase Rho binding protein fundamental to the Rho signal transduction pathway [24]. In this way AKAP3 acts as a scaffold protein for 2 independent signal transduction pathways.

In the present study we have demonstrated that Sp17 and AKAP3 are associated in spermatozoa. This interaction most likely occurs when the N-terminal domain of Sp17 binds the RII alpha binding site of AKAP3 in the fibrous sheath.

Methods

All chemicals and reagents used in this study were molecular biology grade. The entire coding region of human Sp17 was expressed as a six-histidine tagged recombinant protein in the vector pQE-30 (Qiagen Inc., Chatsworth, CA) as described [1,2]. Antibodies to recombinant human Sp17 were prepared in rabbits as described [25] using 200 μg recombinant protein emulsified in Titermax adjuvant (Sigma Chemical Co., St Louis, MO) at 0 weeks. Three further immunizations were given at weeks 4, 6 and 18 in incomplete Freund's adjuvant.

AKAP3-Sp17 binding

AKAP3 (a.a. 1–200) cDNA was synthesized from mouse testis total RNA by reverse transcription PCR and verified by sequencing. The cDNA was ligated into pGST-4T-1 expression vector (Amersham Pharmacia Biotech) and GST-fusion proteins of AKAP3, AKAP4 and GST alone expressed as described [14]. Fusion proteins were separated from crude bacterial extracts (1–3 μl) by incubation with 25 μl glutathione-agarose resin (Sigma Chemical Co., St Louis, MO) for 30 minutes, 4°C. Non-specifically bound bacterial proteins were removed by extensive washing with PBS.

Lysates were prepared from washed human spermatozoa by incubation in CHAPS/PBS buffer (127 mM NaCl, 6.7 mM Na_2PO_4, 3.25 mM NaH_2PO_4, pH 7.0 and 5 mM CHAPS (3-[(cholamidopropyl) dimethylammonio]-1-propane sulfonate)) containing Protease Inhibitor Cocktail (Sigma Chemical Co., St. Louis MO) for 30–60 minutes, 4°C. Following this, spermatozoa were sonicated, centrifuged 700 g for 5 minutes and the supernatant removed. Aliquots of spermatozoa lysate (400 μg) were then added to agarose immobilized GST-AKAP3, GST-AKAP4 or GST alone, the total volume adjusted to 0.5 ml with PBS and proteins allowed to bind for 2 hours at 4°C. After washing extensively with PBS to remove unbound proteins, SDS sample buffer containing 5% β-mercaptoethanol was added and proteins eluted by boiling for 5 minutes.

Electrophoresis and blotting

SDS-PAGE and Western blots were carried out according to methods previously described [1-3]. Blots were blocked with TBST (50 mM Tris pH 7.4, 150 mM NaCl with 0.05% Tween 20) containing 2% non-fat milk for 30–60 minutes, room temperature (RT) and probed with either rabbit anti-recombinant human Sp17 (anti-Sp17) IgG (1:2000) or normal rabbit IgG (1:2000) in TBST containing 1% non-fat milk for 1–2 hours. Antibody binding was detected with alkaline phosphatase conjugated goat anti-

rabbit IgG Fc (1:1000) in the same buffer for 1 hour at room temperature. Blots were developed with NBT/BCIP (nitro blue tetrazolium/5-bromo-4chloro-3-indolyl phosphate) as substrate. Chemiluminescent staining was performed using Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

Immunofluorescence and immunocytochemistry

Human spermatozoa (gift of Dr. S. Beyler, University of North Carolina Hospital, Chapel Hill, NC) were washed in BWW medium by centrifugation at 500 g for 5 minutes, permeabilized with 1% Triton X-100, 15 minutes, washed twice and resuspended in PBS. Aliquots of spermatozoa were air dried onto slides and then fixed in methanol for 10 minutes (-20°C). For indirect immunofluorescence, spermatozoa were probed with rat antiserum to recombinant human AKAP3 (rFSP95, gift of Dr J. Herr, University of Virginia, VA; [22]) at 1:50 dilution and anti-Sp17 IgG at 1:100. Antibody binding was detected with Alexa Fluor-488 labeled goat anti-rat IgG (Molecular Probes, Eugene OR) and Alexa Fluor-568 labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR) at 1:5000 dilution. DNA was visualized with DAPI diluted 1:250,000 from a 5 mg/ml stock.

Fluorescent images were obtained from a Zeiss Axiophot microscope equipped with Plan-apochromat 63× 1.4 NA (numerical aperture), Plan-neofluar 100× 1.3 NA and 40× 0.75 NA objectives, chroma filter sets (Chroma Technology Corp., Brattleboro, VT), and Zeiss filter set 25 (emission at 460, 530 and 610 nm) that allows detection of colocalized Alexa Fluor 488 and Alexa Fluor 568 probes (Molecular Probes, Eugene OR). Images were recorded with a Zeiss AxioCam using AxioVision software (Zeiss MicroImaging, Thornwood, NY) and exported to Adobe Photoshop® 5.0 to assemble the figures.

Fibrous sheath preparation

Mouse spermatozoa extracts were prepared from epididymal mouse spermatozoa allowed to swim out of the epididymis at 37°C, pelleted at 800 g and washed twice in PBS. Spermatozoa were lysed in sample buffer and loaded at 5 × 10^6 /well. Western blots of fibrous sheath preparations [26], prepared from CD-1 mouse epididymal spermatozoa, were kindly provided by Dr. D. O'Brien, University of North Carolina at Chapel Hill.

Results

Sp17 binds AKAP3 in flagella

Although Sp17 has been immunoprecipitated from the soluble fraction of spermatozoan lysates if they are prepared with SDS [2,10], the majority of Sp17 is present in the insoluble fraction where it is inaccessible except under strong detergent or denaturing conditions. Figure 1 demonstrates the distribution of Sp17 between the soluble and pellet fractions in a CHAPS extract of human spermatozoa. The amount of Sp17 in a soluble fraction of spermatozoa will differ depending upon the extraction conditions, detergents and buffers employed. As reported previously [4] for human spermatozoa, two groups of immunoreactive proteins are present, 22–24 kDa and 19–20 kDa (fig. 1 arrows). To investigate Sp17 in the fibrous sheath of flagella and its association with the most abundant AKAPs in spermatozoa, namely AKAP3 (AKAP110) and AKAP4 (AKAP82), which make up most of the insoluble fibrous sheath [27], we utilized recombinant proteins and in vitro pull down assays. Recombinant AKAP3, encompassing the RII binding domain (a.a. 121–141 [28]), and recombinant AKAP4, containing the dual specific RI/RII binding domain (a.a. 220–229 [14]), were synthesized as GST fusion proteins. The GST fusion proteins were immobilized on glutathione-agarose resin and added to CHAPS extracts of human spermatozoa (400 μg) that had been sonicated to release the Sp17. Western blots of bound and eluted proteins from the immobilized GST fusion proteins, probed with anti-Sp17, revealed that AKAP3 bound and precipitated a significant level of Sp17 while AKAP4 did not (Fig. 2A). As far as we could determine, most of the CHAPS-sonicated solubilized Sp17 bound to AKAP3.

This result demonstrates that Sp17 has specificity for particular AKAPs and does not indiscriminately bind all AKAPs. Moreover when recombinant GST alone was used in the binding assay, no Sp17 was precipitated indicating that GST does not interact with Sp17 (Fig. 2A). Amido black staining of mock pull down assays in which no spermatozoan lysate was added revealed similar levels of recombinant GST protein in each assay (Fig. 2A). Therefore differential levels of Sp17 binding to AKAP3 and AKAP4 were not the result of differences in the amount of AKAP protein added to the assays.

Similar binding experiments are impossible to perform with native AKAP3 and AKAP4 because these proteins are highly insoluble. Therefore we utilized spermatozoa from AKAP4 knockout (KO) mice to analyze the interaction of Sp17 with AKAPs in vivo. These mice were generated through targeted disruption of the *Akap4* gene resulting in complete loss of AKAP4 [15]. In spermatozoa from these mice, the FS forms incompletely, the principal piece is shorter and they do not exhibit progressive motility [15]. Interestingly the loss of AKAP4 also resulted in a significant reduction or absence of other proteins typically associated with the fibrous sheath: AKAP3, RII alpha and GAPDS [15]. Since AKAP4 binds AKAP3 [16] and we have shown that Sp17 binds AKAP3 in vitro, a reduction in the binding of Sp17 might therefore be expected in AKAP4 KO spermatozoa. To assess this we performed Western

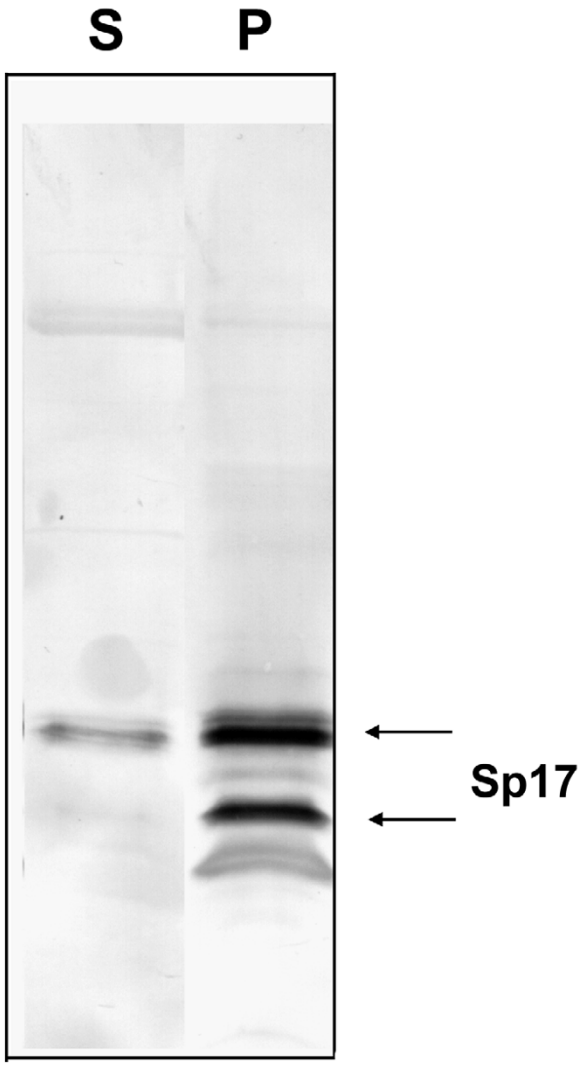

Figure 1
Distribution of Sp17 in human spermatozoa. Western blot demonstrating that Sp17 is present in both the soluble fraction (S) and the pellet (P) fraction of the CHAPS extract of human spermatozoa. As reported previously [4], the pellet fraction, which contains the majority of the Sp17 present in the lysate, has two groups of immunoreactive proteins, 22–24 kDa and 19–20 kDa (arrows).

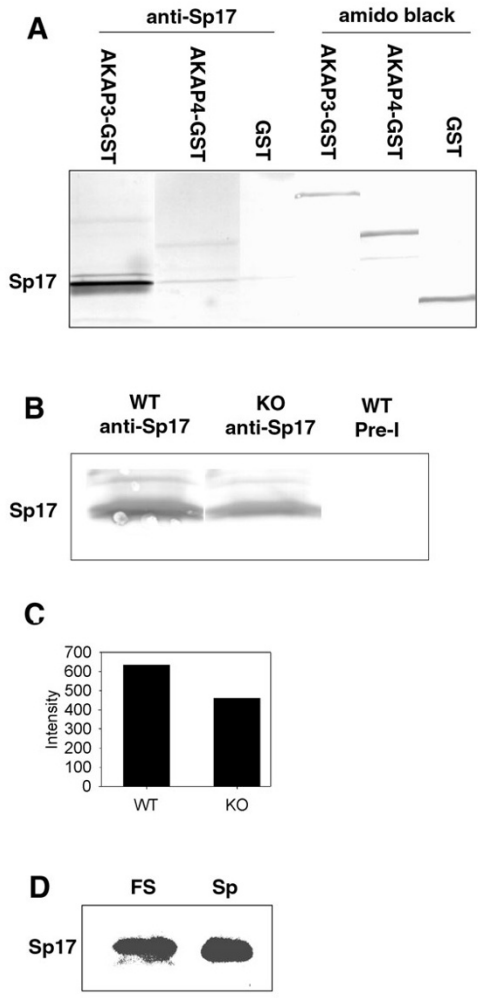

Figure 2
Binding of Sp17 to AKAP3. A. Western blot demonstrating that Sp17 is precipitated from human sperm lysates with GST-AKAP3, but not with GST-AKAP4 or GST alone. First three lanes were probed with rabbit anti-Sp17. Right hand side, (lanes 4,5,6) show amido black staining of mock pull down assays in which no spermatozoan lysate was added. Similar levels of recombinant GST proteins, GST-AKAP3, GST-AKAP4, and GST alone, were present in each assay. B. Western blot of lysates from 5 × 10^6 spermatozoa from wild type (WT) and AKAP4 knock out (KO) mice. The blot was probed with rabbit anti-Sp17 or rabbit pre-immune IgG and demonstrates a reduction in the amount of Sp17 expressed in AKAP4 KO spermatozoa when compared to wild type. C. Densitometric scanning of the Western blot in (B). A 27% reduction in signal intensity is seen in the sample from the AKAP4 knock out (KO) mice compared to the wild type (WT) sample. D. Western blot of isolated fibrous sheath proteins (FS) and SDS lysates of equivalent numbers of whole mouse spermatozoa (Sp) probed with affinity purified anti-Sp17, demonstrating that Sp17 is present in the fibrous sheath and insoluble fraction of spermatozoa.

blots of spermatozoa lysates from 5 × 10^6 wild type and AKAP4 KO spermatozoa. Probing with antibodies to Sp17 demonstrated Sp17 in both populations of spermatozoa. Significantly though there was a reduction in the amount of Sp17 present in AKAP4 KO spermatozoa when compared to wild type (Fig. 2B). To quantitate this change in protein level, densitometric scanning of the blot was per-

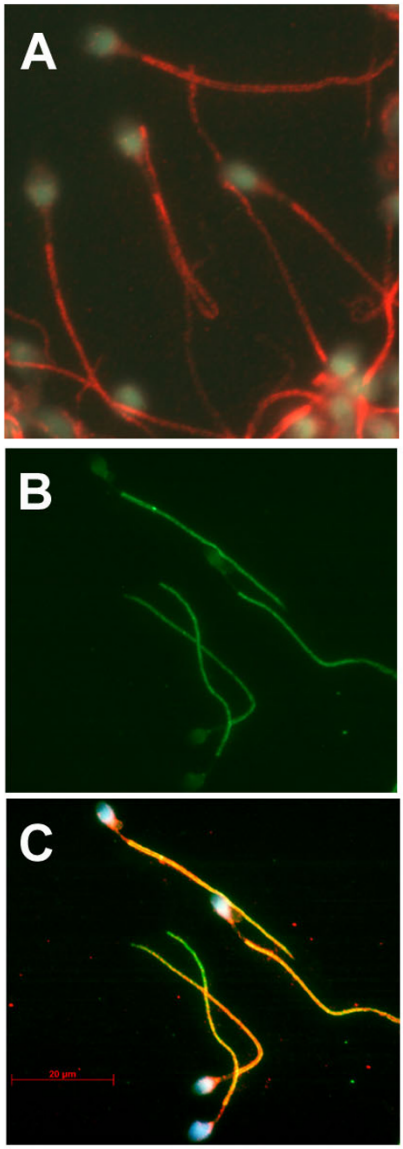

Figure 3
Immunofluorescence localization of Sp17 and AKAP3. A. Immunofluorescent localization of Sp17 in human spermatozoa. Sp17 is found in the principal piece, middle piece and in scattered patches throughout the head region. The nuclei are DAPI stained. B. Immunofluorescent localization of AKAP3 in human spermatozoa. AKAP3 is located predominantly in the principal piece of the flagella. The nuclei are DAPI stained. C. Co-localization of Sp17 and AKAP3 in human spermatozoa. AKAP3 (green), located in the principal piece, co-localizes with Sp17 in some areas (yellow) of the principal piece but not in others. Sp17 (red) is also located in the middle piece of the tail and head regions. The nuclei are DAPI stained. Scale bar is 20 μm for A, B and C.

formed using GelExpert software (NucleoTech Corporation). A 27% reduction in signal intensity was observed in AKAP4 knockout compared to wild type spermatozoa (figure 2C).

Since AKAPs 3 and 4 are located in the fibrous sheath (FS) [27] we examined whether Sp17 was also present. Isolation of the FS from spermatozoa has been achieved by its lack of solubility in successive extractions with Triton X-100, potassium thiocyanate and 4 M urea [26]. Western blots of FS preparations probed with anti-Sp17 demonstrated that Sp17 was present and similar levels of Sp17 were found in equivalent numbers of lysed spermatozoa in SDS sample buffer (Fig. 2D).

Immunolocalization

To determine whether or not AKAP3 and Sp17 co-localize in flagella, double labeling experiments with rat anti-AKAP3 and rabbit anti-Sp17 were performed. As published previously AKAP3 is found in the principal piece of human spermatozoa (FSP95; [22]) and Sp17 is found in the head and tail of monkey [29] and mouse [10] spermatozoa. As shown in figure 3A Sp17 (red) is found in the principal piece, mid piece and in scattered patches throughout the head region. AKAP3 (green, fig. 3B) is found in the principal piece of human spermatozoa. Figure 3C demonstrates the co-localization of AKAP3 and Sp17 along the length of the principal piece where the AKAP3 signal (green) and the Sp17 signal (red) merge into small aggregates of yellow staining. When spermatozoa were probed with pre-immune antibodies for either AKAP3 or Sp17, no fluorescence was detectable and spermatozoa labeled with Alexa Fluor 488 or Alexa Fluor 568 labeled goat anti-rabbit IgG or anti-rat IgG secondary antibodies only, did not fluoresce (data not shown).

Discussion

Sp17 is one of a group of proteins that share a domain with a strong homology to the N-terminus of the type II regulatory sub-unit of protein kinase A, responsible for protein dimerization and AKAP binding. Of these proteins Sp17 has the highest level of identity with RII alpha (45%) and this includes Leu^{13} (Leu^{20} of Sp17) and Phe^{36} (Phe^{43} of Sp17) defined by Li and Rubin [30] as essential for RII beta dimerization, a pre-requisite for AKAP binding. Furthermore, other amino acids that have been shown to facilitate the tethering of RII beta to AKAP, namely, Val^{20}-Leu^{21} and Phe^{31}-Leu^{33}, are also conserved in Sp17. In the present study we have demonstrated Sp17's association with AKAP3 in flagella, which is consistent with the striking conservation of Sp17's N-terminal (~90%) from marsupials to primates [2] and may be the common theme in the divergent observations of Sp17's over expression in malignant cells, and presence in spermatozoa and cilia.

The present study found significant levels of Sp17 associated with AKAP3 in the fibrous sheath and only a weak association with AKAP4 (figure 2). This demonstrates a specificity of Sp17 for particular AKAPs, although it is possible that the low affinity of AKAP4 for Sp17 could be a consequence of a sub-optimal conformation of the recombinant AKAP4 protein. However it has been observed that recombinant ropporin (full length) did not bind to recombinant AKAP4 whereas a recombinant ropporin RII binding domain did [16]. This suggests that the conformation of the recombinant AKAP4 protein is able to play a significant role in determining RII binding specificity.

Sp17's binding to AKAP3 may reflect a functional need for Ca^{++}/CaM at sites along the flagella, where Ca^{++}/CaM is known to play a role in motility [31,32]. Although all AKAPs are defined by their ability to bind the RII sub-unit of PKA, studies to address the relative affinity of RII alpha for different AKAPs have not yet been performed. Undoubtedly the affinity of AKAPs for the Sp17 RII homologue protein will be different from both RII alpha and other RII homologue proteins. AKAP3 may indeed bind other RII homologues that were not tested in this study.

Immunofluorescent double labeling experiments of Sp17 and AKAP3 (Fig. 3C) demonstrated that sub-populations of these proteins co-localize in the principal piece, and occur independently of each other as well. The similarity of solubility profiles for both Sp17 and AKAP3 proteins further suggests they are bound; they are insoluble in Triton X-100, potassium thiocyanate and 4 M urea. Our results (Fig. 2) confirm that a sub-population of Sp17 is anchored in the fibrous sheath, probably by binding to AKAP3. Nevertheless, Sp17 could bind other AKAPs such as AKAP-220 [33], TAKAP-80 [34], and sAKAP-84 [35] throughout the spermatozoon, and AKAP3 could bind the other RII homologues and RII alpha itself.

In addition to PKA or RII homologue proteins, AKAPs bind other functionally important proteins at other sites along the molecule. In the case of AKAP4, these other proteins include AKAP3, the glycolytic enzyme GAPDS and two novel proteins FSIP 1 and FSIP 2 [16]. This grouping of sequence unrelated but functionally related proteins on the AKAP is presumed to facilitate their function in the cell. As we have shown AKAP3 binds Sp17 in vitro, it is possible that Sp17 forms part of the AKAP4/RII/AKAP3/ complex through binding to AKAP3. Indeed, loss of AKAP4 resulted in reduced levels of not only AKAP3 and GAPDS [15] but also Sp17. Ropporin and ASP are also known to bind to AKAP3 via the RII binding domain [12] but whether these proteins compete with Sp17 for binding to AKAP3 or whether sub-populations of AKAP3 bind all three proteins at different sites on the fibrous sheath is not known.

Conclusions

In summary, we have demonstrated an association of Sp17 with AKAP3 in spermatozoa and because the N-terminus of Sp17 has a strong homology to the type II regulatory sub-unit of protein kinase A, responsible for protein dimerization and AKAP binding, a common theme in the divergent observations of Sp17's presence in malignant cells, cilia and spermatozoa, may be Sp17's ability to bind AKAPs.

Authors' contributions

IL carried out the molecular biology studies and participated in the study design and draft preparation. EW participated in the biochemical studies, preparation of samples for immunofluorescence and draft preparation. MO carried out the immunofluorescence, participated in the study design and prepared the final drafts of the manuscript.

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