FSCB, a Novel Protein Kinase A-phosphorylated Calcium-binding Protein, Is a CABYR-binding Partner Involved in Late Steps of Fibrous Sheath Biogenesis*

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We report characterization of a novel testis- and sperm-specific protein, FSCB (fibrous sheath CABYR binding), that is expressed post-meiotically and localized in mouse sperm flagella. FSCB was identified as a binding partner of CABYR, a calcium-binding protein that is tyrosine-phosphorylated during capacitation. Orthologous genes of FSCB are present in other mammals, including rat and human, and conserved motifs in FSCB include PXXP, proline-rich and extensin-like regions. FSCB is phosphorylated by protein kinase A as shown by in vitro phosphorylation assay and also by determining phosphorylation sites in native FSCB from mouse sperm. Calcium overlay assay showed that FSCB is a calcium-binding protein from sperm. FSCB is a post meiotic protein first expressed at step 11 of mouse spermatogenesis in the elongating spermatids, and it subsequently incorporates into the flagellar principal piece of the sperm. Ultrastructurally, FSCB localized to a cortical layer of intermediate electron density at the surface of the ribs and longitudinal columns of the fibrous sheath. Due to its temporal appearance during spermiogenesis and location at the cortex of the fibrous sheath, FSCB is postulated to be involved in the later stages of fibrous sheath assembly.

Hyperactivated motility along with changes in the sperm head that confer the capacity to fertilize an oocyte result from a series of time-dependent processes collectively referred to as capacitation (1, 2). Although the molecular mechanisms underlying capacitation, including hyperactivation, are incompletely understood, some fundamental processes related to capacitation have been elucidated.

The presence of a protein source such as albumin, bicarbonate, and Ca\(^{2+}\) and an energy substrate such as glucose, pyruvate, or lactate are essential to achieve in vitro capacitation (3). Capacitation is also marked by an increase in tyrosine phosphorylation through a unique signal transduction cascade involving a sperm-specific soluble adenyl cyclase, protein kinase A (PKA), and tyrosine kinase(s). A variety of factors, including Ca\(^{2+}\), HCO\(_3\)^{-}, and H\(_2\)O\(_2\) stimulate soluble adenyl cyclase leading to increased cytosolic levels of cAMP (4). This increase in cAMP then activates PKA, and the consequence is a significant increase in tyrosine phosphorylation of protein substrates localized in the flagellum such as AKAP3, AKAP4, CABYR, hsp-90, ODF2, and tubulin (3–12).

The fact that PKA is a key regulator of capacitation-associated changes such as protein phosphorylation and hyperactivation has been well established. Pharmacological stimulants that elevate intracellular cAMP, such as the phosphodiesterase inhibitors, caffeine, and pentoxifylline, enhance hyperactivated motility of sperm (13). CAMP agonists can accelerate protein tyrosine phosphorylation in sperm, whereas antagonists of PKA inhibit tyrosine phosphorylation and capacitation (14). Mice in which sperm-specific PKA\(_{\alpha}\) is knocked out are infertile, owing to the complete absence of normal sperm movement, including hyperactivated motility (15). Moreover, these mice demonstrated no increase in protein tyrosine phosphorylation during capacitation. Recently, using antibodies against the phosphorylated form of the consensus target sequence, the role of PKA-dependent phosphorylation has further been established in human (16, 17) and boar sperm (18). Increased phosphorylation is a prerequisite for the initiation of hyperactivated movement, a key attribute of the capacitated state (19, 20).

Calcium is absolutely required for capacitation and hyperactivated sperm motility. Ca\(^{2+}\) can increase flagellar bend amplitude in demembranated sperm (21, 22). Intracellular Ca\(^{2+}\) is increased in hyperactivated sperm in both the head and tail, and Ca\(^{2+}\) oscillates with each flagellar bend (23), which indicate a direct relationship between intracellular calcium and hyperac-

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3 The abbreviations used are: PKA, protein kinase A; BSA, bovine serum albumin; CABYR, calcium-binding tyrosine phosphorylation-regulated protein; CABYR CR-A; coding region A of CABYR; AKAP, a kinase-anchoring protein; FSCB, fibrous sheath CABYR-binding; EST, expressed sequence tags; PBS, phosphate-buffered saline; DTT, dithiothreitol; aa, amino acid(s); MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JAK1, Janus tyrosine kinase 1.
tivation. Four ion channel proteins, CatSper 1–4, have been shown to reside in the membrane of the principal piece of the sperm tail and to interact with one another (24). Their deletion, although resulting in sperm of apparent normal morphology, causes male infertility by preventing hyperactivated motility. Thus sperm flagellar function is characterized by complicated causes male infertility by preventing hyperactivated motility. Four ion channel proteins, CatSper 1–4, have been shown to reside in the membrane of the principal piece of the flagellum. Calcium enters the cell mainly through voltage-dependent calcium channels (VDCCs) in the membrane of the principal piece. However, the exact role and the mechanism of calcium influx for sperm motility and for constituents of signaling cascades involved in regulating sperm maturation, motility, capacitation, hyperactivation, and/or acrosome reaction (25). At present, several tyrosine as well as serine/threonine phosphoproteins have been reported in human (7, 26), hamster (27), and mouse spermatozoa, including AKAP3, AKAP4 (7, 28, 29), and CABYR (8, 30), which localize to the unique cytoskeletal compartment of the sperm’s fibrous sheath. Identification of additional elements in the fibrous sheath “interactome” may provide further insights regarding the role of phosphorylation in regulating sperm fertilizing ability.

CABYR is a polymorphic, calcium-binding, cytoskeletal protein that shows increased tyrosine (8) as well as serine/threonine phosphorylation (30) during capacitation and is localized along the entire length of the principal piece in association with the surface of the fibrous sheath (8). CABYR contains a PKA-like RII dimerization and AKAP binding domain at its N terminus, dispersed PXPF motifs, which mediate protein-protein interactions with SH3 modules, and an extensin-like domain that has been shown to bind to glycogen synthase kinase (31). Four alternatively spliced variants of CABYR have been identified in mice, originating from two coding regions (CR-A and CR-B) (32).

To further understand the function of this specific protein in the signal transduction cascade, we hypothesized that CABYR binds to an array of interacting proteins in the fibrous sheath and used co-immunoprecipitation and mass spectrometry strategies to study the interactome of CABYR. The present study describes the sequencing, cloning, expression, and characterization of a novel testis- and sperm-specific, calcium-binding protein, termed FSCB, that is expressed during the late steps of spermiogenesis, localizes in the fibrous sheath of the flagellum, and is a phosphorylation target for PKA in sperm.

**Experimental Procedures**

**Immunoprecipitation and Mass Spectrometric Analysis of CABYR-binding Proteins**—Immunoprecipitations were performed with an immunoprecipitation kit (Roche Applied Science) using 2 × 10^8 sperm for each tube and 20 μl of polyclonal anti-CABYR CR-A (CABYR coding region A protein) or pre-immune serum following instructions from the manufacturer. The protein A-agarose was pelleted at 12,000 × g for 20 s at room temperature. Immune complexes were dissociated in 200 μl of Celis buffer (9.8 M urea, 2% Nonidet P-40, 100 mM dithiothreitol (DTT) with a protease inhibitor mixture) at 4 °C for 20 min with gentle shaking, and then separated by two-dimensional gel electrophoresis and Western blotted. Immune complexes from a duplicate set of the immunoprecipitations (including anti-CABYR CR-A and pre-immune serum) were sent for mass spectrometry. These samples were washed three times with digestion buffer to remove any remaining Nonidet P-40, then reduced with DTT and alkylated with iodoacetamide before adding 1 μg of Promega-modified trypsin (Promega, Madison, WI) overnight at room temperature. The samples were acidified to 5% acetic acid and then desalted on a C18 column. ~20% of the sample was injected onto the Finnigan LTQ-FT mass spectrometer and analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular mass and product ion spectra to determine amino acid sequence in sequential scans. The data were analyzed by data base search using the Sequest search algorithm against the NR data base.

**Cloning, Sequencing, and Analysis of cDNAs**—Among the microsequences obtained in the anti-CABYR CR-A immunoprecipitates was a novel peptide, DDQISTFK, which did not match any known protein. Data base screening showed that this peptide might come from one of two hypothetical mouse proteins noted in the mouse genome. The accession numbers of these two hypothetical proteins are XP_991269 or XP_892576 (these represent the same hypothetical protein) and XP_921314. Their reference DNA sequences are XM_986175 or XM_887483 and XM_916221. Based on the genomic sequences, two pairs of primers were designed to amplify and clone truncated and full-length genes, respectively.

The primers for the truncated FSCB gene were designed to create an EcoRI site at the 5′-end and an Xhol site at the 3′-end. The forward primer was: 5′-CGG AAT TCG CCA TCT TTT GAG AAG GCT CC-3′; the reverse primer was: 5′-CCG CTC GAG GTT GCT TAA CTC TAT TTG AAT GG-3′. The primers for the full-length FSCB gene cloning were designed to create an Ndel site at the 5′-end and an Xhol site at the 3′-end. The forward primer was: 5′-GGA ATT CTC TAT GGA AGA ATG TGA AGA ACC TGA AG-3′; the reverse primer was: 5′-CCG CTC GAG GTT CTT TAA CTC TAT TTG AAT GG-3′. PCR reactions were performed with 0.2 ng of the cloned mouse testicular Marathon-ready cDNA library (BD Biosciences-Clontech, Palo Alto, CA) in a 50-μl assay system for 35 cycles using a program of one 5-min cycle at 94 °C followed by 35 cycles of denaturation, annealing, and elongation at 94 °C for 1 min, 60 °C for 2 min, and 68 °C for 2 min. PCR products were separated on a 1.5% agarose gel, subcloned into the pCR 2.1-TOPO
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vector, and sequenced in both directions using vector-derived primers.

Northern and Multiple Tissue Expression Array Analyses—Northern blots containing ~2 μg of poly (A)+ RNA per lane from 8 different mouse tissues and a dot blot containing a normalized loading of poly(A)+ RNA (65–874 ng/dot) from 22 different mouse tissues and 7 different control RNAs and DNAs were observed on BD Biosciences-Clontech. One Northern blot was probed with 20 ng of a 32P-labeled 885-bp DNA corresponding to bp 2338–3222 of the mouse FSCB cDNA open reading frame prepared by random oligonucleotide labeling. Hybridization was performed in ExpressHyb solution (BD Biosciences-Clontech) at 68 °C overnight followed by two washes in 2× SSC, 0.05% SDS at 22 °C and two washes in 0.1× SSC, 0.1% SDS at 55 °C for 30 min. The same membrane was stripped by incubating in sterile H2O containing 0.5% SDS at 100 °C for 10 min, after which the membrane was re-probed with a 32P-labeled human β-actin cDNA (20 ng), using hybridization and washing conditions described above. Blots were exposed to a phosphorimaging screen at 22 °C for 16–24 h. The phosphorimaging scans were performed on a Storm PhosphorImager (Amersham Biosciences).

The mouse multiple tissue expression array was also probed with 20 ng of 32P-labeled 885-bp DNA corresponding to bp 2338–3222 of the mouse FSCB cDNA open reading frame. The blot was hybridized in ExpressHyb containing sheared salmon testes DNA and Cot-1 DNA, incubated overnight at 65 °C, and then washed four times in 2× SSC and 1% SDS at 65 °C for 20 min followed by two additional washes in 0.1× SSC and 0.5% SDS at 55 °C for 20 min. The blot was exposed to the phosphorimaging screen at 22 °C for 16–24 h.

Expression and Purification of the Recombinant FSCB Protein and Antibody Production—cDNAs encoding truncated and full-length FSCB were cloned into the EcoRI-XhoI sites of the pET-28b(+) and pET-22b(+) expression vectors (Novagen, Madison, WI), respectively. *Escherichia coli* strains BL21(DE3) and Rosetta were transformed with the expression vectors (Novagen, Madison, WI), respectively.

Recombinant protein expression was induced for 3 h with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were then transferred to 1× binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole) containing 0.1% Nonidet P-40 and 0.1 mg/ml lysozyme on ice for 30 min, and were sonicated briefly. The insoluble pellet resulting from centrifugation at 10,000 × g for 20 min was dissolved in 8 ml urea in 1× binding buffer for 1 h on ice. After centrifugation at 10,000 × g for 20 min, the urea-soluble fraction was loaded onto an Ni2+-activated His-binding resin column (Novagen) following the manufacturer’s protocol, and the recombinant protein was eluted with 300 mM imidazole in 1× binding buffer containing 8 M urea. The affinity-purified recombinant proteins were purified further by SDS-PAGE electrophoresis using the Prep Cell (Bio-Rad). The truncated recombinant protein was used for immunization of female Lewis guinea pigs (100 μg/guinea pig) in Freund’s complete adjuvant. Animals were boosted three times at intervals of 14 days with 50 μg of recombinant truncated protein in incomplete Freund’s adjuvant, and sera were collected 10 days after the last boost.

Western Blot Analysis of Recombinant and Native FSCB in Testis and Sperm.—The truncated and full-length recombinant FSCB proteins, mouse sperm extracts, and testis extracts were analyzed by immunoblotting. Samples (20 μg/lane) were subjected to SDS-PAGE on a 4–15% Criterion gel (Bio-Rad), and the proteins were transferred to a polyvinylidene difluoride membrane, and Western blotting was carried out as described previously (8). Following blocking, the membranes were incubated with 1:8000 dilution of the guinea pig antiserum against FSCB in PBS-T (PBS, 0.05% Tween 20) for 1 h at room temperature. Control pre-immune serum from the guinea pig was used at the same dilution as the immune serum in all experiments. The membranes were washed three times with PBS-T and then incubated with the secondary antibody (1:5000 dilution of horseradish peroxidase-conjugated anti-guinea pig IgG, Jackson ImmunoResearch, West Grove, PA) for 1 h. The signal was detected either using an Enhanced Chemiluminescent Kit (Amersham Biosciences) as described (33) or using 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry, Gaithersburg, MD). Multiple mouse tissue protein extracts were also used to examine the pattern of tissue expression of FSCB protein by Western blotting using 20 μg of total proteins in each lane. One membrane was stripped and re-probed with anti-β-tubulin monoclonal antibody (clone E7, Developmental Studies Hybridoma Bank, University of Iowa) as a sample loading control.

Indirect Immunofluorescence Localization of FSCB in the Seminiferous Tubules of Mouse Testis and in the Epididymal Duct—Testes and epididymides were obtained from adult male Swiss mice. Mice were sacrificed by cervical dislocation, and testes and epididymides were removed and fixed in Bouin’s solution overnight at room temperature. The tissues were embedded in paraffin, serially sectioned at 5 μm, and the sections were placed on slides to dry. For staging of tubule sections, after deparaffinization in xylene and rehydration in a decreasing ethanol series, periodic acid-Schiff staining was performed following a standard protocol with minor modifications. Slides were rinsed in PBS for 5 min, and then placed in 0.5% periodic acid for 10 min. After a 10-min wash in distilled H2O, followed by incubation for 1 h in Schiff reagent in the dark, the slides were placed in 1% potassium metabisulfite for 2 min. The slides were then washed in distilled H2O, stained with hematoxylin (1.38 g/100 ml of distilled H2O) for 3 s. After washes in an increasing ethanol series and xylene, the slides were mounted with Permount (Fisher Scientific, Pittsburgh, PA).

For immunofluorescence staining, after deparaffinization and rehydration, adjacent sections were incubated in 2 N HCl for 20 min, washed 3 × 5 min in PBS with 0.05% Tween 20 (PBS-T), and incubated in blocking solution containing 10% normal goat serum in PBS-T for 30 min. The specimens were then incubated with anti-FSCB serum or pre-immune serum (1:400 dilution) in
PBS-T containing 1% BSA (PBS-T-BSA) for 1 h at room temperature or overnight at 4 °C, washed 3 × 5 min in PBS-T, incubated with fluorescein isothiocyanate-labeled goat anti-guinea pig IgG (1:400 dilution, Jackson ImmunoResearch) in PBS-T-BSA for 1 h, washed 3 × 5 min in PBS-T, and mounted with Slow Fade (Molecular Probes, Eugene, OR). Periodic acid–Schiff stained sections were observed by light microscopy to determine the exact stage of the cycle of the seminiferous epithelium for individual seminiferous tubules, and then the same tubule in the adjacent section was studied by fluorescence microscopy to determine the corresponding FSCB staining pattern. Individual green fluorescence images were obtained using a digital camera (Zeiss, Thornwood, NY) and compiled using Openlab software (Improvision, Inc., Boston, MA).

Indirect Immunofluorescence Localization of FSCB in Mouse Sperm—Caudal epididymal sperm were collected and counted using a hemocytometer and diluted to a concentration of 1 × 10⁶ sperm/ml. Sperm were capacitated for 1.5 h at 37 °C and 5% CO₂ following the protocol described previously (3). Capacitation medium contained 5 mg/ml BSA and 10 mM NaHCO₃. These two media constituents are known to induce capacitation, and the capacitation state is routinely assessed in our laboratory by checking motility (>80%) and capacitation-associated increase in tyrosine phosphorylation. Non-capacitated sperm were collected in the same media but lacking BSA and NaHCO₃. A 20-μl aliquot of non-capacitated and capacitated sperm suspension containing ∼2 × 10⁶ sperm was added per well onto poly-L-lysine-coated slides. The slides were dried at 40 °C and then fixed in methanol for 10 min. In some experiments no fixation was performed, and the sperm were simply air-dried onto the slide. After washing 3 × 5 min in PBS-T, the samples were blocked in 10% normal goat serum in PBS for 30 min. The guinea pig anti-FSCB serum or the pre-immune serum was diluted (1:2000) with PBS-T-BSA and incubated with the specimen for 1 h at room temperature or overnight at 4 °C, washed 3 × 5 min, incubated with fluorescein isothiocyanate-labeled goat anti-guinea pig IgG (1:400 dilution, Jackson ImmunoResearch) in PBS-T-BSA, washed 3 × 5 min, and mounted with Slow Fade (Molecular Probes). Anti-serum to mouse CAYBR CR-A protein and the corresponding pre-immune serum were also used (1:400 dilution) for comparison with the FSCB staining. The secondary antibody was Cy3-labeled goat anti-guinea pig IgG (1:400 dilution, Jackson ImmunoResearch).

Localization of FSCB in Mouse Sperm by Immunoelectron Microscopy—Caudal sperm from adult Swiss mice were washed twice by centrifugation at 550 × g in wash buffer (Ham’s F10 Nutrient Mixture (Invitrogen) with 3% sucrose). The washed sperm were resuspended in fixative consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in a wash buffer for 15 min at room temperature. After removing fixative by centrifugation and washing three times with a wash buffer, the sperm were dehydrated through a graded series of ethanols, from 40% to 100%. The cells were infiltrated with and embedded in Lowicryl K4M (EM Science, Fort Washington, PA) according to the manufacturer’s recommendations. The blocks were polymerized with UV light for 72 h at −20 °C, and Ultrathin sections of 100 nm thicknesses were cut. Non-specific sperm antibody interactions were blocked by incubating the sections in undiluted normal goat serum for 15 min at room temperature and washing once with a wash buffer. Guinea pig antiserum against FSCB and the pre-immune serum were diluted (1:100) in a wash buffer with 1% normal goat serum, 1% BSA, and 0.05% Tween 20. Lowicryl sections were incubated with diluted anti-FSCB serum or pre-immune serum at 4 °C for 16 h. After washing four times in a wash buffer, they were incubated for 1.5 h at room temperature with 5 nm gold-conjugated secondary antibody, goat anti-guinea pig IgG (Goldmark Biologicals, Phillipsburg, NJ), diluted (1:50) in a wash buffer. The sections were washed with distilled water, silver enhanced for 20 min (Aurion R-Gent SE-EM, EM Science), and stained with uranyl acetate before examination with a 100CX electron microscope (JEOL, Peabody, MA).

Calcium-binding Overlay—Calcium-binding was examined using a ⁴⁵Ca overlay assay modified from that described by Maruyama et al. (33). The experiment was replicated three times. In brief, the truncated and full-length recombinant proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Polyvinylidene difluoride membranes subsequently were stained with Ponceau-S to localize and check protein loading in each lane. The membranes were scanned and then washed three times for 10 min in PBS to remove Ponceau-S, washed three times for 20 min each in washing buffer (10 mM imidazole HCl, 60 mM KCl, and 5 mM MgCl₂, pH 6.8), and incubated with 5 μCi/ml of ⁴⁵CaCl₂ in washing buffer for 30 min at room temperature. The membranes were subsequently rinsed for 2 min in distilled H₂O followed by a 30-s rinsing in 50% ethanol and were dried at room temperature on a filter paper for 20–30 min. The membranes were then thoroughly dried by hot air from a hair dryer and exposed to a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) for 16–24 h.

In Vitro Phosphorylation of FSCB by PKA—Full-length recombinant FSCB (0.5 μg), truncated recombinant FSCB (0.5 μg), or the native FSCB immunoprecipitated from sperm was mixed with purified PKA catalytic subunit (100 units), and the mixture was incubated at 30 °C for 15 min in a phosphorylation buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 10 mM MnCl₂, protease inhibitor mix (without EDTA), 100 μM Na₃VO₄, 5 mM P₃-nitrophenyl phosphate, 40 mM β-glycerophosphate, 2 μCi of [γ-³²P]ATP, 1% Triton X-100, and 1 mg/ml BSA. The experiments included the protein alone and the PKA catalytic subunit alone controls. After 15 min of incubation the phosphorylation reaction was stopped immediately by adding Laemmli buffer. The tube contents were incubated in boiling water for 5 min, and ³²P incorporation was analyzed using SDS-PAGE followed by autoradiography. The experiment was repeated, and the same result was obtained.

Identification of FSCB and CAYBR Assemblies by Co-immunoprecipitation and Two-dimensional Western Blot—FSCB protein was found to be relatively insoluble and could not be easily dissolved and immunoprecipitated using the standard method described above. A modified immunoprecipitation strategy was developed for extracting and precipitating FSCB. Caudal epididymal sperm were collected, and ~8 × 10⁸ sperm were resuspended in 4 ml of Celis buffer with protease inhibitor...
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mixture, omitting DTT. The suspension was incubated for 0.5–1 h at 4 °C on a rocking platform and then centrifuged at 4 °C, 12,000 × g in a table-top microcentrifuge for 10 min to remove debris. The supernatant was transferred into a dialysis cassette with a 10-kDa cut-off (Pierce) and was dialyzed against 0.1× PBS for 24 h at 4 °C with two changes of the PBS solution. The dialyzed suspension was centrifuged at 4 °C, 6,000 × g, in a table-top microcentrifuge for 10 min to remove the precipitated pellet. The suspension was transferred in equal aliquots to four 1.5-ml tubes and immunoprecipitated using either anti-FSCB serum, anti-CABYR CR-A serum, or the pre-immune sera as described above. The immunoprecipitates were dissociated either in 200 μl of Celis buffer (for two-dimensional gels) at 4 °C for 20 min with gentle shaking or in 50 μl of 2× Laemmli sample buffer (for one-dimensional gels) at 100 °C for 5 min and subjected to SDS-PAGE followed by Western blot analysis.

Phosphopeptide Identification from FSCB by Immunoprecipitation and Mass Spectrometry—Mouse sperm were capacitated in vitro as described earlier, and immunoprecipitation of FSCB was performed with anti-FSCB serum and the pre-immune serum. Proteins from the immunoprecipitates were separated by SDS-PAGE, and the gels were silver-stained. The corresponding FSCB band was identified by comparison with a Western blot of an identical gel, cored, and subjected to phosphopeptide identification by mass spectrometry. Briefly, the gel piece was dehydrated in acetonitrile, rehydrated in 30 μl of 10 mM DTT in 0.1 M ammonium bicarbonate, and reduced at room temperature for 0.5 h. The DTT solution was removed, and the sample was alkylated in 30 μl of 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 0.5 h. The reagent was removed, and the gel pieces were dehydrated in 100 μl of acetonitrile. The acetonitrile was removed, and the gel pieces were rehydrated in 100 μl of 0.1 mM ammonium bicarbonate. The pieces were dehydrated in 100 μl of acetonitrile, the acetonitrile was removed, and the pieces were completely dried by vacuum centrifugation. The gel pieces were rehydrated in 20 ng/μl trypsin in 50 mM ammonium bicarbonate on ice for 10 min. Excess trypsin solution was removed, and 20 μl of 50 mM ammonium bicarbonate was added. The sample was digested overnight at 37 °C, and the peptides formed were extracted from the polyacrylamide in two 30-μl aliquots of 50% acetonitrile/5% formic acid. These extracts were combined and evaporated to 15 μl for mass spectrometric analysis. The data were analyzed by data base search using the Sequest search algorithm against the FSCB protein. Putative phosphopeptides were validated manually.

RESULTS

Identification of FSCB Peptide in CABYR Immunoprecipitates—To identify potential CABYR binding partners, immunoprecipitation of mouse sperm protein lysates was performed using antisera to mouse recombinant CABYR CR-A as well as control pre-immune serum. The immunoprecipitates were digested with trypsin and analyzed by tandem mass spectrometry. A novel peptide (DDQISTFK) was identified among the peptides from the immunoprecipitates with the anti-CABYR CR-A antibody, and this peptide was absent from the immunoprecipitates with the pre-immune serum. This peptide belonged to a hypothetical protein annotated by accession numbers, XP_991269.1, XP_892576.1, and XP_921314.1. XP_991269.1 and XP_892576.1 represented identical sequences, whereas XP_921314.1 had a 12-aa insertion between amino acids 683 and 686. These sequences predicted large proteins of 1062 and 1074 amino acids with molecular masses of 113,605 and 114,886 kDa and pIs of 4.02 and 4.01, respectively. Unigene annotated each of the three available EST sequences as originating from mouse testes. This hypothetical protein has been named fibrous sheath CABYR-binding protein (FSCB). Homologene bioinformatics revealed five orthologous EST sequences in the rat, all from testis, and 74 of 77 EST sequences of the orthologous human gene, also originating in the testis (the other 3 ESTs were from brain, muscle, and mixed tissues).

Cloning of FSCB cDNA: Generation of rec-FSCB Protein and Guinea Pig Anti-FSCB Polyclonal Antibody—Based on the mouse genomic data base sequences, two pairs of primers were designed to amplify open reading frames encoding both full-length and C-terminal truncated cDNAs of FSCB from a mouse cDNA library. The truncated 885-bp 3’-end cDNA was inserted into the pET-28b(+) expression vector and expressed in the BL21(DE3) cells. The truncated gene was predicted to express a 295-aa protein with pl of 3.99 and predicted molecular mass of ~37 kDa with both N- and C-terminal His tags. Interestingly, the actual recombinant truncated protein was seen to migrate at 80 kDa in Coomassie Blue-stained gels (Fig. 1A, lanes 1 and 2) and was recognized by anti-His (C-terminal) antibody (Fig. 1A, lanes 3 and 4). No 37-kDa form could be detected. In addition, a prominent 160-kDa His-positive doublet band of the truncated form was also observed, representing a dimer of the 80-kDa band (Fig. 1A, lane 4). The 80-kDa His tag (C-terminal)-positive band was cored for mass spectrometry,

FIGURE 1. Expression and purification of truncated (aa 780–1074 (A)) and full-length (aa 1–1074 (B)) mouse rec-FSCB, in BL21(DE3), and Rosetta host cells, respectively. Coomassie Blue staining of uninduced (lane 1) and isopropyl-β-D-galactopyranoside induced (lanes 2 and 3) FSCB protein extracts and their anti-His antibody Western blots (lanes 3 and 4) together demonstrate an induced expression of the truncated (A) and full-length (B) recombinant proteins. The truncated FSCB migrated at ~80 kDa; a prominent 160-kDa presumptive dimer (lane 4 in A) appeared as a doublet with a dominant upper band. The full-length rec-FSCB protein migrated at 270 kDa as a doublet consisting of bands of approximately equal intensity. Both truncated and full-length recombinant forms showed a prominent proteolytic cleavage product of 18 kDa. Both purified truncated (lane 5 in A) and full-length rec-FSCB proteins (lane 5 in B) appeared to be of high purity when analyzed on silver-stained gels. The silver-stained purified 18-kDa fragment of rec-FSCB is shown in the lane 6 (A).
which confirmed the expected truncated sequence. This recombinant 80-kDa protein was subsequently purified (Fig. 1A, lane 5) and injected into guinea pigs to generate the anti-FSCB serum.

For expression of the full-length recombinant protein, the entire open reading frame was amplified from the same mouse cDNA library and subcloned into the TOPO vector. The full-length open reading frame was sequenced in both directions. The 3225-bp nucleotide sequence obtained for this novel protein was submitted to the GenBank™ data base under accession number EF133693. This sequence (Fig. 2) differed from that of XP_921314.1 in six-nucleotide SNPs (single nucleotide polymorphisms) at positions 1378 (from C to T), 1443 (from A to T), 1663 (from A to C), 2636 (from C to G), 2646 (from C to T), and 2998 (from C to T). There were only four corresponding amino acid substitutions: 460 (from P to S), 555 (from T to P), 879 (from A to G), and 1000 (from P to S). The deduced protein sequence encoded by this full-length gene was 1074 aa in length (protein ID: ABL63912.1) (Fig. 2). The full-length gene was inserted into expression vector pET-22b(H11001) and expressed in the Rosetta and BL21(DE3) strains. Following induction by 0.5 mM isopropyl-1-thio-D-galactopyranoside, the level of protein expression was much higher in the Rosetta strain than in BL21(DE3) (data not shown). The predicted molecular mass of this full-length protein was 115 kDa with a pI of 4.01 (3225-bp open reading frame). With addition of the C-terminal His tag and related expressed region, the recombinant protein was predicted to have a mass of 120 kDa. However, the recombinant protein migrated at ~270 kDa as shown in Coomassie Blue-stained gels (Fig. 1B, lanes 1 and 2) and was recognized by anti-His (C-terminal) antibody (Fig. 1B, lanes 3 and 4). No significant His tag-positive bands at 120 kDa were noted. The 270-kDa full-length recombinant protein was purified (Fig. 1B, lane 5) and used for subsequent experiments. An 18-kDa His tag (C-terminal)-positive band was always found in preparations of both the truncated and full-length proteins from bacteria (Fig. 1, A and B, lane 4). This 18-kDa band was also cored and microsequenced. The peptides confirmed that this band was also a truncated C-terminal FSCB that included sequences present in the 80-kDa band. The 18-kDa band was also purified (Fig. 1A, lane 6) and used for immunizations.

Genomic Locus of FSCB—A search of the mouse genome databases located the FSCB gene on chromosome 12 at 12C1 (mouse genomic contig sequence accession number NW_001030500.1). The homologous rat gene was located on chromosome 6 (q24), whereas the human gene was located on chromosome 14 (q21.3). The loci of both rat and human FSCB genes are located at chromosomal regions syntenic to the mouse locus.

![FIGURE 2. Nucleotide and amino acid sequences of FSCB. The upper line presents the open reading frame of the DNA sequence (numbered at left). The start codon is underlined, and the termination codon is shown by an asterisk. Lower line presents amino acid sequences (numbered at right). PXXP motifs are shaded in gray. The peptide obtained by mass spectrometry from the original immunoprecipitation is shaded in black. Two potential tyrosine phosphorylation sites (aa 176–184 and 325–331) are shown in clear boxes. The potential cAMP- and cGMP-dependent protein kinase phosphorylation sites (aa 22–25, 955–958, and 1018–1021) are indicated by double underlining. Seven serine/threonine residues demonstrated to be phosphorylated in vivo by mass spectrometric phosphoprotein analysis are marked with asterisks over the amino acids.](https://www.jbc.org/content/282/47/34109)
FIGURE 3. Comparison of the deduced amino acid sequences of mouse sperm FSCB with predicted rat and human orthologues (mouse: FSCB, ID: ABL63912.1, rat: predicted similar to protein C14orf155, ID: XP_576037; human: hypothetical protein LOC84075, ID: NP_115511). The alignment was constructed using the BioEdit Sequence Alignment Editor program. The black and gray shaded areas indicate the amino acid identities and similarities, respectively, among the molecules.
Evolutionary Conservation and Motif Analysis of FSCB—A comparison of the proteins deduced from these genes is presented in Fig. 3. Motif analysis of mouse FSCB revealed 13 Pro-X-X-Pro (PXXP) consensus motifs, the cognate sites for SH3 interactions, at aa 7–10, 24–27, 49–52, 133–136, 493–496, 517–520, 565–568, 613–616, 625–628, 649–652, 673–676, 733–736, and 963–969. Screening the sequence against the PROSITE and Pfam data bases indicated the presence of one glutamic acid-rich region (aa 336–952), one extensin-like region (438–825), one proline-rich region (aa 484–763), a glutamic acid-rich region (aa 336–952), one extensin-like region (aa 438–825) and two-dimensional Western blots (Fig. 5, A and B), although in both cases several bands and spots of lower molecular masses were noted. The 270-kDa bands in testis and sperm samples confirmed the recombinant immunogen (the truncated 295-aa FSCB protein) at the apparent mass of 80 kDa and the full-length recombinant FSCB protein at apparent mass of 270 kDa in Western blot (Fig. 5A, lanes 1 and 2). When reacted with mouse sperm or testis proteins, this monospecific guinea pig antiserum to rec-FSCB recognized mainly a 270-kDa band in one-dimensional Western blots (Fig. 5, B and C), although in both cases several bands and spots of lower molecular masses were noted. The 270-kDa bands in testis and sperm samples corresponded to a similar size band of full-length rec-FSCB protein. The finding of similar migration patterns of native mouse FSCB and rec-FSCB confirmed that rec-FSCB has characteristics of the native protein. Both proteins give significantly higher apparent molecular mass than their theoretical molecular.
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Pre-immune serum did not detect any protein. In the testis lysates is also a protein of 270 kDa with a number of minor proteins of lower molecular mass visible. Probed with guinea pig pre-immune serum (lane 1) and truncated rec-FSCB protein (lane 2) resolved by one-dimensional SDS-PAGE and probed with guinea pig antiserum against FSCB. The antiserum detected a dominant 270-kDa band of full-length rec-FSCB (lane 1) as well as the 80-kDa band of the truncated protein as expected (lane 2). These proteins were not detected by the pre-immune serum (lanes 3 and 4), mouse sperm lysates resolved by one-dimensional SDS-PAGE and probed with guinea pig pre-immune serum (lane 1) and anti-FSCB serum (lane 2), respectively. Sperm FSCB migrated in a predominant 270-kDa band, with less abundant lower forms above 170 kDa, identical to the pattern of full-length recombinant FSCB (lane 1 in A). C, mouse testis lysates resolved by two-dimensional SDS-PAGE and probed with guinea pig pre-immune serum (C1) and anti-FSCB serum (C2), respectively. The predominant spot in the testis lysates is also a protein of 270 kDa with a number of minor proteins of lower molecular mass visible. Pre-immune serum did not detect any protein.

FIGURE 5. One- and two-dimensional Western blots of rec-FSCB proteins, mouse sperm lysates, and mouse testis lysates with antibody raised against the truncated form of FSCB. A, purified full-length (lane 1) and truncated rec-FSCB protein (lane 2) resolved by one-dimensional SDS-PAGE and probed with guinea pig antiserum against FSCB. The antiserum detected a dominant 270-kDa band of full-length rec-FSCB (lane 1) as well as the 80-kDa band of the truncated protein as expected (lane 2). B, mouse sperm lysates resolved by one-dimensional SDS-PAGE and probed with guinea pig pre-immune serum (lane 1) and anti-FSCB serum (lane 2), respectively. Sperm FSCB migrated in a predominant 270-kDa band, with less abundant lower forms above 170 kDa, identical to the pattern of full-length recombinant FSCB (lane 1 in A). C, mouse testis lysates resolved by two-dimensional SDS-PAGE and probed with guinea pig pre-immune serum (C1) and anti-FSCB serum (C2), respectively. The predominant spot in the testis lysates is also a protein of 270 kDa with a number of minor proteins of lower molecular mass visible. Pre-immune serum did not detect any protein.

FIGURE 6. Protein expression of FSCB in various tissues as assessed by Western blot. Twelve different mouse tissues extracts were resolved by SDS-PAGE and probed with anti-FSCB serum (A), pre-immune serum (B), and with anti-β-tubulin monoclonal antibody (C). FSCB protein was detected only in testis. Pre-immune serum did not detect any protein, and the anti-β-tubulin blot showed total protein loading in all the tissues.

Localization and Dynamic Expression Pattern of FSCB in the Seminiferous Epithelium during the Late Steps of Spermiogenesis—FSCB expression was first examined in mouse testicular sections, where the stages of the seminiferous epithelium are easily discernible within each seminiferous tubular cross-section. As shown in Fig. 7, immunofluorescent localization of FSCB in the mouse testis using the anti-FSCB serum revealed a post-meiotic pattern of expression in the late steps of spermiogenesis. The staining patterns indicated a significant dynamic and cyclic change as well as intracellular migration of FSCB during spermiogenesis. No fluorescence signal was observed in round or elongating spermatids at step 9 (stage IX) or in any spermatocytes within the seminiferous epithelium (Fig. 7A). The green fluorescence of FSCB first appeared in the elongating spermatids at step 11 (stage XI), where a diffuse staining (Fig. 7B) was noted. Gradually this fluorescence intensity became more prominent in the spermatid from step 11 (stage XI) through step 15 (stages VI) (Fig. 7F, and the staining intensity gradually increased from step 15 to step 16. Finally, staining was confined to the testicular sperm, which were present at the luminal surface of the seminiferous epithelium at stage 16 (stage VIII); concomitantly the staining eventually disappeared from the cells located apically in the seminiferous epithelium (Fig. 7G and H). The pre-immune serum did not stain the testicular sections (Fig. 7I).

Immunofluorescence of FSCB was also detected in caput, corpus, and cauda epididymis. The staining pattern revealed FSCB in sperm in the lumen of the epididymal duct. No staining was observed in any epididymal epithelial cell type (Fig. 8, A and B). Pre-immune serum did not stain sperm in the lumen of the epididymal duct (Fig. 8, C and D).

Immunofluorescence and Immunoelectron Microscopy Localized FSCB to the Fibrous Sheath of the Principal Piece of the Flagellum in Mouse Sperm—Anti-FSCB serum recognized with an intense signal the entire length of the principal piece of both non-fixed, air dried and methanol-fixed non-capacitated sperm by indirect immunofluorescence microscopy (Fig. 9, A–D).
Within the principal piece of cauda epididymal sperm FSCB showed a gradation of concentration with diminution of signal from proximal to distal direction. No immunofluorescence was noted in the mouse sperm head, connecting piece, middle piece, or end piece. The pre-immune serum did not stain sperm in any subcellular domain (Fig. 9, E and F). Furthermore, no difference in staining pattern and intensity were observed between non-capacitated and capacitated sperm, and, importantly, no immunofluorescence was observed on live motile sperm, indicating that FSCB epitopes were not accessible on the plasma membrane (data not shown). In comparison with FSCB, the pattern of CAYB CR-A localization shares some similar features with that of FSCB but also has unique characteristics. The CAYB CR-A antibody localized to the entire length of the principal piece, and, unlike FSCB, CAYB also extended into the end piece (Fig. 9, G and H). CAYB also did not show a significant attenuation of signal along the principal piece from proximal to distal but showed a more uniform immunofluorescence along the tail. Thus, although the CAYB immunofluorescence tapered as the flagellum became more slender distally, there was little reduction in the intensity of the fluorescence in the distal tail compared with the proximal principal piece.

To characterize in detail the ultrastructural localization of FSCB, freshly prepared mouse sperm were examined by immunogold electron microscopy. Most of the gold particles were located in the fibrous sheath, especially in the cortical region of intermediate electron density at the surface of both the longitudinal columns and ribs. A few gold particles were also noted at the outer surfaces of the outer dense fibers, but FSCB was not detected in the axoneme, within outer dense fibers, on the inner surface of outer dense fibers, or in the mitochondrial sheath compartment (Fig. 10, A–C). From these data we concluded that FSCB is an integral fibrous sheath component of mature mouse sperm located on the cortical surface of fibrous sheath ribs and columns. The concentration of the protein appeared greatest at the outer surface of the fibrous sheath underlying the plasma membrane.
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and immunoprecipitated native FSCB from mouse sperm were incubated with purified PKA catalytic subunit in the presence of [γ-32P]ATP. The electrophoretic pattern and corresponding autoradiograph of the phosphorylated FSCB are shown in Fig. 12. Recombinant full-length FSCB, truncated FSCB, and native immunoprecipitated FSCB were all phosphorylated in the presence of PKA (Fig. 12A, lanes 3, 5, and 9). No phosphorylation of FSCB was observed when PKA was omitted from the reaction mixture (Fig. 12A, lanes 4, 6, and 10) indicating that this phosphorylation was not a result of autophosphorylation or phosphorylation by contaminating kinases in the case of the immunoprecipitated FSCB complex. PKA catalytic subunit was also autophosphorylated (Fig. 12A, lanes 2, 3, 5, 7, and 9). Coomassie Blue staining of the gel showed that PKA concentrations were equivalent in each reaction tube (Fig. 12C). Western blots of recombinant full-length, truncated FSCB (Fig. 5A), and immunoprecipitated native FSCB from mouse sperm (Fig. 12B) revealed that the autoradiography signals in each lane correlated with the corresponding Western blot signals. Thus, both the recombinant and native FSCB were phosphorylated by PKA in vitro.

Native FSCB Phosphopeptides Identified by Mass Spectrometry—Include the Canonical PKA Phosphorylation Sites—Following capacitation in vitro, FSCB was immunoprecipitated from the mouse sperm using anti-FSCB serum or pre-immune serum, and the immunoprecipitates were separated by SDS-PAGE. The 270-kDa band that was immunoreactive with anti-FSCB serum in Western blot was cored from a parallel silver-stained gel and subjected to phosphopeptide analysis. Seven serine/threonine residues on FSCB were found to be phosphorylated in the native FSCB. Of these, six phosphorylation sites were confirmed as serine residues, whereas one of them may be either serine or threonine. Two phosphorylated sites, Ser-25 and Ser-1020, are canonical PKA sites (arginine, any residue, serine/threonine (RXS)). Table 1 lists the FSCB peptides and sites thus far identified to be phosphorylated in the native FSCB.

Identification of Binding between FSCB and CABYR—FSCB was originally identified by tandem mass spectrometry in an immunoprecipitate of mouse sperm protein extracts obtained using anti-CABYR CR-A serum. To test further the hypothesis that FSCB and CABYR interact, co-immunoprecipitations were performed with anti-CABYR CR-A serum and anti-FSCB serum. Two-dimensional Western blots confirmed that the 86-kDa acidic spot of CABYR CR-A was present in the immune complexes precipitated from mouse sperm lysates with anti-FSCB serum (Fig. 13, A and B). Moreover, in the reverse experiment, the 270-kDa acidic spot of FSCB was detected in the immune complexes precipitated from mouse sperm lysates with anti-CABYR CR-A serum (Fig. 13, C and D). The pre-immune sera did not detect either CABYR CR-A (Fig. 13B) or FSCB (Fig. 13D) in these immune complexes. As also anticipated, a number of additional spots other than CABYR CR-A or FSCB were detected in these blots including IgG heavy chain (50-kDa regions), IgG light chain (25-kDa regions), or their oligomers originating in the antisera. FSCB was detected in its expected molecular weight and pI range in the immunoprecipitate complex; however, the FSCB signal extended as a charge...
CR-A, combined with the previous detection by mass spectrometry of FSCB in immunoprecipitates, strongly suggest that FSCB and CABYR CR-A interact with each other.

The CABYR-FSCB Interactome—The mass spectrometric analysis of immunoprecipitates of mouse sperm using anti-CABYR CR-A serum not only revealed the sequence of the previously unannotated protein FSCB, but also detected sequences corresponding to several known proteins, including the protein kinases JAK1 and Unc-51-like kinase, MAP kinase 8 interacting protein 3, roporrin, and the glycolytic enzyme enolase 1 (Table 2).

DISCUSSION

FSCB Is a Novel Testis-specific Protein—Recombinant (both truncated and full-length) as well as the native FSCB proteins have considerably higher apparent molecular mass than predicted from their amino acid sequences. It is noteworthy that both truncated and full-length proteins have very acidic isoelectric points (pI), ranging from 3.99 to 4.01, and the higher migration pattern is similar to that observed for the acidic 86-kDa isoform of CABYR (8), suggesting that the anomalous migration of FSCB in gels is due to the highly acidic nature of the molecule.

Northern and dot blots revealed that the transcripts for FSCB were present only in testis, and multitissue Western blots also showed that protein expression of FSCB was limited to the testis and epididymis. At the time of submission of this manuscript GenBankTM contained only three ESTs for mouse FSCB, all of them originating from testis. Similarly, of 77 ESTs for the orthologous FSCB gene in humans, 74 ESTs originate in testis (the other 3 are from brain, muscle, and mixed tissue).

Conserved Functional Motifs in FSCB—Comparison of protein sequences in mouse, rat, and human indicated that the N and C termini of FSCB are relatively conserved. The middle is more variable, although the human and rat orthologues share similar stretches of deletion. Conserved domains include the glutamic acid-rich region, extensin-like motif, and proline-rich region, all of which have potentially important functions. It is worth noting in this context that CABYR also has an extensin-like motif, which has been shown to be involved in CABYR binding to glycogen synthetase kinase (31).

FSCB Is a Calcium-binding Protein—Recombinant FSCB (both the truncated and the full-length proteins) demonstrated the capacity to bind calcium in the calcium overlay assay. How-
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Staining for FSCB was detected only in the principal piece, where the fluorescence intensity was observed to diminish in a proximal to distal direction. Hence, localization of FSCB overlaps with the localization of CABYR, supporting the co-immunoprecipitation results. More importantly, the ultrastructural localization of FSCB to the cortical regions of the ribs and columns of the fibrous sheath is similar to that of human CABYR (8). Taken together, the immunoprecipitation and co-localization studies demonstrated that FSCB is a binding partner of CABYR in the fibrous sheath of sperm.

**TABLE 1**

| Phosphorylated sequences | Mass    | Phosphorylated amino acid residue and location |
|--------------------------|---------|-----------------------------------------------|
| RRPS*OPMVDSQQTE         | 1866.8547 | Ser-25                                       |
| ATHS*IGNIPGSK           | 1261.5994 | Ser-57                                        |
| ATHS*IGNIPGSKNYSR       | 1896.8677 | Ser-57                                        |
| SQQTSGCTGNSLIVCPK       | 2223.9655 | Ser-161 or Thr-163                            |
| EKVDQEQQYFS*ELE         | 1952.8698 | Ser-186                                       |
| LPLTTEEIPQPS*PAEE       | 1963.9368 | Ser-275                                       |
| KQVALTIDS*MLEDGOTAIE    | 2013.9715 | Ser-365                                       |
| IHLLSQDADIMEE           | 1591.7512 | Ser-1020                                      |

FSCB, a Binding Partner of CABYR—Co-localized in the Mouse Fibrous Sheath—Co-immunoprecipitation and two-dimensional Western blot analysis demonstrated that the 86-kDa CABYR CR-A isoform can be co-immunoprecipitated with anti-FSCB serum and, conversely, that the FSCB protein can be co-immunoprecipitated along with the CAYBR CR-A isoform using anti-CABYR CR-A serum. Moreover, FSCB sequences were identified by mass spectrometry in immunoprecipitates of CABYR. These data suggested that FSCB and CABYR interact in vivo. To determine the localization of FSCB, immunofluorescence and immunoelectron microscopic analyses of mouse sperm were performed using antibodies specific for FSCB.

ever, bioinformatic analysis did not identify any calcium binding motifs (for example, EF-hand like motif) in FSCB. Indeed, many calcium-binding proteins do not have calcium binding motifs, but they still bind calcium through coordination of several negative charges. Calcium-binding proteins such as calsequestrin and CAYBR, for example, contain a preponderance of acidic residues and possess very acidic pIs (34, 35). The calcium-binding property of FSCB proteins may also be due to its acidic nature.

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**FIGURE 12. In vitro phosphorylation of full-length rec-FSCB, truncated rec-FSCB, and immunoprecipitated native FSCB from mouse purified PKA catalytic subunit.** Autoradiograph (A) and the Coomassie-stained gel (C) from the in vitro phosphorylation assay are shown. HIS-tagged full-length (lanes 3 and 4), truncated FSCB (lanes 5 and 6), immunoprecipitate of mouse sperm using guinea pig pre-immune serum (lanes 7 and 8), and immunoprecipitate of mouse sperm using anti-FSCB serum (lanes 9 and 10) were incubated with (lanes 3, 5, 7, and 9) or without (lanes 4, 6, 8, and 10) purified PKA catalytic subunit in the presence of [γ-32P]ATP under standard assay conditions as described under “Experimental Procedures.” As controls, phosphorylation buffer alone (lane 1) and PKA alone (lane 2) were also included in the experiment. The molecular masses (kDa) of the standards are marked on the left, and the position of PKA catalytic subunits, heavy chain (H), and light chain (L) bands of IgG and BSA are marked on the right. Phosphorylated FSCB bands are indicated by arrowheads. The recombinant full-length, truncated FSCB, and native FSCB all were phosphorylated in the presence of PKA (lanes 3, 5, and 9), and no phosphorylation of FSCB was observed when PKA was omitted from the reaction mixture (lanes 4, 6, and 10). For comparison, Fig. 5A shows the corresponding Western blot of recombinant full-length (lane 1), and truncated FSCB (lane 2). Western blot probed with anti-FSCB serum (B) identified the native FSCB in the immunoprecipitates with anti-FSCB serum (arrow in head in lane 2) and not in the control immunoprecipitates using the pre-immune serum (lane 1).
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tyrosine and/or serine/threonine phosphorylated protein substrates of the capacitation-associated PKA pathway such as AKAP3, AKAP4, and CABYR are also present. These PKA substrates reside in the fibrous sheath and are involved in capacitation and hyperactivation (19). AKAPs in mouse sperm, for example, are phosphorylated at serine/threonine residues (41) suggesting they are directly downstream of PKA activation during capacitation. Based on these observations, we believe that FSCB is a downstream target of the cAMP/PKA-signaling pathway with an implied role in motility and hyperactivation of sperm during capacitation.

**FSCB Incorporates into the Fibrous Sheath in the Late Steps of Fibrous Sheath Biogenesis**—Spermiogenesis in mouse is divided into 16 steps, on the basis of the size and shape of the nucleus, acrosome, and spermatid cell body (42, 43). The assembly of the cytoskeletal elements, outer dense fibers and fibrous sheath, during this process shows specific temporal patterns unique to spermiogenesis (44). FSCB immunostaining was first observed in the elongating spermatids (step 11, or stage XI). The protein gradually migrated at step 15 into the sperm and disappeared from the cells located apically in the seminiferous epithelium at step 16. Coupled to the immunogold observations of FSCB on the surface of the fibrous sheath in epididymal sperm, including the inner and outer surfaces of the longitudinal columns and ribs, it may be concluded that a new molecular element of the fibrous sheath has been identified.

In comparison to the expression patterns found for sperm fibrous sheath proteins reported to date (8, 44–46), the temporal patterns of FSCB assembly, as well as its subcellular location, indicate that FSCB is likely incorporated into the fibrous sheath after the bulk of core fibrous sheath proteins have already assembled. AKAP3 and AKAP4, for example, are major constituents of the fibrous sheath (25). AKAP3 expression was first observed in the cytoplasm of-round spermatids in step 4 (stage IV) and in the flagellum of elongating spermatids in step 9 (stage IX) (47). Thus, AKAP3 expression in both cytoplasm and flagellum precedes the expression of FSCB in both locations. Moreover, this proposed temporal sequence is in accord with the ultrastructural observation that FSCB is located on the surface of the fibrous sheath. Formation of the definitive fibrous sheath occurs from distal to proximal during steps 14 and 15 in mice (48). Previous studies have shown that the precursors of longitudinal columns first appear in the rat in round spermatids, whereas precursors of the ribs are first seen in late elongating spermatids (46, 49, 50). Based on the observation in human sperm that AKAP3 localized in the ribs, but not in the longitudinal columns by immunogold labeling (7), it has been suggested that AKAP3 is incorporated into the fibrous sheath concurrent with formation of the ribs, but after formation of the longitudinal columns has begun and AKAP3 may be involved in formation of the rib precursors (47).

The temporal pattern of FSCB expression shows both similarities and differences to that reported for AKAP4. AKAP4 is present in both the longitudinal columns and ribs of the fibrous sheath (41) and constitutes ~40% of the 4 M urea-insoluble components of the fibrous sheath (51). AKAP4 was first detected in both the cytoplasm and flagella of step 14 (stage II–III) condensing spermatids and disappeared from the cytoplasm of condensing spermatids after step 15 (47). Targeted disruption of the AKAP4 gene caused defects in the formation of the fibrous sheath with shortened longitudinal columns and thin circumferential ribs accompanied by a lack of progressive motility (52). Thus, it has been suggested that the incorporation of AKAP4 into the longitudinal columns and ribs is a major step in completion of assembly of the definitive fibrous sheath (47). The temporal appearance of FSCB in the round spermatid cytoplasm at step 11 is earlier than the appearance of AKAP4 in spermatid cytoplasm, and FSCB persists longer in the cytoplasm than does AKAP4. However, FSCB appears to reach the flagellum later than...
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does AKAP4. In sum, the temporal pattern of FSCB expression, assembly, and final subcellular localization indicates that FSCB is an important constituent protein of the fibrous sheath, is assembled at the surface of a preformed fibrous sheath core (anlagen), and constitutes a step in the final assembly of this structure at the later stages of fibrous sheath biogenesis.

The CABYR-FSCB Interactome—Recovery of JAK1 and Unc-51-like kinases as well as MAP kinase-interacting protein 3 in CABYR immunoprecipitates provides new insights into the possible kinases downstream to PKA that interact in supramolecular complexes at the surface of the fibrous sheath. Recovery of roporin in the CABYR-FSCB complex confirms previous localizations of roporin in the fibrous sheath (53) while pointing to specific molecular interactions between roporin and CABYR or other interacting proteins in the complex. Confirmation of the glycolytic enzyme enolase 1 in the CABYR-FSCB complex reinforces the role of glycolysis in the fibrous sheath (40, 54) while suggesting a possible role for CABYR and/or FSCB in glycolysis.

In summary, recent studies show that the fibrous sheath functions not only as a passive mechanical peripheral cytoskeletal element of the flagellum, but also as a scaffold for constituents of signaling cascades and for glycolytic enzymes, and thus is well equipped and positioned to play a role in the regulation of sperm motility. FSCB is a newly discovered fibrous sheath constituent whose specific location, calcium- and CABYR-binding capacities, phosphorylation by PKA, and co-isolation of sperm motility. FSCB is a newly discovered fibrous sheath constituent protein of the fibrous sheath biogenesis.

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