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The 12 kD FK506 Binding Protein FKBP12 Is Released in the Male Reproductive Tract and Stimulates Sperm Motility

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Abstract

Background: The 12 kD FK506 binding protein FKBP12 is a cytosolic receptor for the immunosuppressant drugs FK506 and rapamycin. In addition to its critical role in drug-induced T-cell immunosuppression, FKBP12 associates physiologically with ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptors, regulating their ability to flux calcium. We investigated a role for FKBP12 in male reproductive physiology on the basis of our identification of extremely high levels of [^{3}H]FK506 binding in male reproductive tissues.
Materials and Methods: [^{3}H]FK506 binding studies were performed to identify tissues enriched with FK506 binding sites. The abundant [^{3}H]FK506 binding sites identified in the male reproductive tract were localized by [^{3}H]FK506 autoradiography. FK506 affinity chromatography was employed to purify FKBP from epididymal fluid. Anti-FKBP12 Western analysis was used to confirm the identity of the purified FKBP. The binding of exogenous FKBP12 to sperm was evaluated by [^{32}P]FKBP12 binding studies and [^{33}P]FKBP12 autoradiography. The effect of recombinant FKBP12 on sperm motility was investigated using a Hamilton Thorne motility analyzer.
Results: Male reproductive tissues contained high levels of [^{3}H]FK506 binding. The localization of [^{3}H]FK506 binding sites to the tubular epithelium of the caput epididymis and the lumen of the cauda and vas deferens suggested that FKBP is released in the male reproductive tract. FKBP12 was purified from epididymal plasma by FK506 affinity chromatography. Radiolabeled FKBP12 specifically bound to immature but not mature sperm. In sperm motility studies, FKBP12-treated caput sperm exhibited double the curvilinear velocity of untreated controls.
Conclusions: High levels of FKBP12 are released in the male reproductive tract and specifically associate with maturing sperm. Recombinant FKBP12 enhances the curvilinear velocity of immature sperm, suggesting a role for FKBP12 in motility initiation. The highest concentrations of soluble FKBP12 in the male reproductive tract occur in the lumen of the vas deferens, a site of sperm storage and the conduit for ejaculated sperm. Preservation of mammalian sperm for reproductive technologies may be optimized by supplementing incubation or storage media with FKBP12.

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Introduction

The immunophilins are proteins that serve as receptors for the principal immunosuppressant drugs cyclosporin A, FK506, and rapamycin

(1,2). Cyclophilin was first identified as the protein receptor for cyclosporin A and was subsequently shown to have multiple isoforms of varied molecular weights (3–5). The 12 kD FK506 binding protein (FKBP) was the first member of the FKBP family to be characterized (6); subsequently, FKBPs with molecular weights of 12.6 (7), 13 (8), 25 (9), 38 (9), 52 (10), and 65 (11) kD have been identified. The cyclophilin and FKBP immunophilins possess peptidyl-prolyl isomerase activity, which is believed to contribute to proper protein folding by interconverting the *cis* and *trans* isomers of peptidyl-prolyl bonds (12,13). Although immunophilin ligands inhibit rotamase activity, a different mechanism accounts for their immunosuppressive properties. The immunosuppressant actions of cyclosporin A and FK506 derive from inhibition of the calcium-calmodulin-activated phosphatase calcineurin by the drug-immunophilin complexes (14,15). As a result, the cytosolic calcineurin substrate and transcription factor *N*uclear *F*actor of *A*ctivated *T* cells (NFAT) is not dephosphorylated upon T-cell activation and is thus unable to enter the nucleus and activate the transcription of interleukin 2 (16). The immunosuppressant effects of rapamycin are mediated by the binding of the rapamycin-FKBP12 complex to the *R*apamycin *A*nd *F*KBP12 *T*arget (RAFT1) (17), also names FRAP (18) and RAPT1 (19). RAFT1 activates protein translation by phosphorylating p70S6 kinase (20) and 4EBP1 (20,21), two key rapamycin-sensitive elements of the translation initiation signaling cascade.

Although first characterized in T cells, the immunophilins occur in a wide range of tissues with particularly high levels in the brain (22,23). Multiple neural roles of immunophilins and their ligands have been elucidated (24). FKBP12 associates physiologically with two intracellular calcium channels, the ryanodine receptor (25) and the inositol 1,4,5-trisphosphate (IP_3) receptor (26), and regulates their ability to flux calcium. FKBP13 has recently been shown to interact with a novel homologue of the erythrocyte nembrane cytoskeletal protein 4.1 named 4.1G, which is abundantly expressed in brain and throughout the body (27). The binding of FK506 to FKBP12 augments the phosphorylation state of numerous neuronal proteins including nitric oxide synthase (28) and GAP 43, a protein associated with neuronal process extension (25). FK506 and synthetic derivatives augment neuronal outgrowth in PC12 cells and sensory ganglia (29,30) and stimulate regrowth of damaged neurons with functional recovery in intact animals (30–33).

In studying the diverse roles of immunophilins, we identified extremely high levels of [^{3}H]FK506 binding in male reproductive tissues. We report that FKBP12 is abundant throughout the path of sperm maturation and show that FKBP12 stimulates the motility of maturing sperm.

Materials and Methods

[^{3}H]FK506 Binding

Tissues were dissected from sexually mature Sprague-Dawley rats, homogenized in 50 mM Trizma pH 7.4, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 40,000 × *g* for 20 min at 4°C to obtain the cytosolic fraction. The fractions were protein assayed using Coomassie reagants (Pierce), and 1–25 μg of soluble protein and 250 pM [^{3}H]FK506 (NEN DuPont) were incubated in a final volume of 0.3 ml of binding buffer containing 50 mM Trizma pH 7.4 and 2 mg/ml bovine serum albumin (BSA). After 60 min at room temperature, the reaction volume was layered over a 1.0-ml column of LH-20 Sephadex (Pharmacia) that was pre-equilibrated with binding buffer. The column was washed with 0.5 ml of buffer, and the eluates were collected, mixed with Formula 963 cocktail, and scintillation counted. Specific binding was determined by subtracting [^{3}H]FK506 binding in the presence of up to 1 μM unlabeled FK506 from total [^{3}H]FK506 bound. All binding experiments were done in duplicate and repeated three times.

FKBP12 Antibody Production

Rat FKBP12 cDNA was subcloned into the pet22b expression vector (Novagen). *E. Coli* BL21(DE3) bacteria (Novagen) were transformed and the FKBP12 fusion protein expressed and purified over a nickel column (Novagen) according to the manufacturer's protocol. New Zealand white rabbits were immunized with the FKBP12 antigen according to established protocols (Hazleton Labs, Denver, PA), except that alternating injections consisted of an FKBP12–45-nm colloidal gold (EY Labs, San Mateo, CA) conjugate to increase the immunologic response (34). Production bleeds were affinity purified by first passing the serum over affigel-10 (Biorad) columns containing pet 22b fusion protein lack-

ing the FKBP12 insert. The flowthrough was then passed over an FKBP12 affigel-10 column. After extensive washing with 10 mM Tris, pH 7.5 and 10 mM Tris, pH 7.5/500 mM NaCl, the antibodies were eluted with 100 mM glycine, pH 2.5 and 100 mM triethylamine, pH 11.5 and dialyzed against phosphate-buffered saline (PBS) and then PBS/40% glycerol for storage.

Western Analysis

Rat tissues were homogenized in 2% SDS, 1% Triton X-100, 50 mM Trizma, pH 7.4, 100 mM NaCl, 1 mM EGTA, and protease inhibitors (4 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml chymotrypsin, 2 μg/ml pepstatin, 1 mM PMSF) and incubated on ice for 20 min, with intermittent vortexing. Following table-top centrifugation at maximum speed, the supernatants were collected and protein assayed using DC reagants (Biorad). Twenty migrograms of each sample was diluted in 5× protein load buffer and subjected to electrophoresis using 18% gradient Tris-glycine minigels (Novex). Proteins were wet transferred to PVDF membrane (Millipore), blocked for 1 hr in 5% nonfat dry milk, and incubated with a 1:500 dilution of the FKBP12 antibody, or a 1:500 dilution of the antibody preadsorbed with the FKBP12 fusion protein, in 3% BSA/PBS overnight at 4°C. Blots were then washed in 5% milk once for 15 min and then twice for 5 min, followed by a 1-hr incubation at room temperature with a 1:5000 dilution of anti-rabbit secondary antibody (Amersham). After washing in 5% milk once for 15 min and then twice in 0.1% Tween/PBS for 10 min, the blots were developed by chemiluminescence using the Renaissance kit (NEN Dupont) according to the manufacturer's protocol.

[^{3}H]FK506 Autoradiography

Tissues of the male urogenital tract were dissected from sexually mature male Sprague-Dawley rats and freshly frozen in TissueTek (Sakura Finetek). Then 20-μm sections were cut onto Superfrost Plus slides (Fisher) using a cryostat. [^{3}H]FK506 autoradiography was performed as previously described (23). Briefly, slides were preincubated for 60 min in 50 mM Hepes, 2 mg/ml BSA, and 0.02% Tween-20, pH 7.4, followed by a 60-min incubation with 1 nM [^{3}H]FK506 (86.5 Ci/mmol) in the same buffer. Control sections were additionally incubated with 1 μM unlabeled FK506 to define nonspecific binding. Slides were rinsed for 4 × 5 min in fresh ice-cold buffer, air dried, and apposed to NB-2 emulsion-coated coverslips for a 2- to 3-week exposure.

FK506 Affinity Chromatography

FK506 was chemically derivatized and coupled to affigel-10 (Biorad) as previously described (1). FK506 was a generous gift of Dr. Seiji Hashimoto, Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Tsukuba, Japan. To obtain epididymal fluid, the epididymis was removed from sexually mature male rats, the connective tissue was carefully dissected away, and the specimen rinsed and then immersed in PBS. Under a dissecting microscope, the epididymal tubules were pierced using a 27-gauge needle and sperm were allowed to diffuse freely from the tubules until the medium became turbid. The epididymis was then removed, the turbid fluid table-top centrifuged on high for 15 min at 4°C, and the supernatant (epididymal fluid) collected. The epididymal fluid was incubated at a concentration of 1 mg/ml with 100 μl of a 50% FK506 matrix slurry pre-equilibrated with 50 mM Trizma, pH 7.4, 100 mM NaCl, and 0.1% Tx-100 and brought to a final volume of 500 μl with this buffer. Specific binding to the matrix was assessed by additionally adding 100 μM soluble FK506 to control samples. The final vehicle (ethanol) concentration in each tube was 2.0%. After rotating the samples for 2 hr at 4°C, the matrix was washed by incubating 3 × 20 min with 1 ml of 0.05% Tween/PBS at 4°C. The pelleted matrix was resuspended in PBS/protein load buffer, boiled for 2 min, eletrophoresed on 18% Tris-glycine gels, and either silver stained or wet transferred to PVDF and subjected to anti-FKBP12 Western analysis as described above.

FKBP12 Labeling

[^{32}P]- and [^{33}P]-labeled FKBP12 fusion proteins were generated by first expressing a glutathione S-transferase (GST) FKBP12 fusion protein containing two upstream protein kinase A (PKA) consensus phosphorylation sites; the vector employed has been described previously (17). Ten nanograms of purified GST-PKA-FKBP12 was mixed with 40 units of PKA and 100 mCi of either [γ-^{32}P]-ATP or [γ-^{33}P]-ATP (NEN DuPont) in a buffer containing 20 mM Hepes, pH 7.7, 100 mM NaCl, 12 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). After 1.5 hr at 37°C, the incubation

mixture containing labeled fusion protein was dialyzed twice against 1 L of thrombin cleavage buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$). The labeled fusion protein was cleaved by adding an equal volume of thrombin cleavage buffer containing 2 mg/ml thrombin and incubating at room temperature for 2 hr. The thrombin was inactivated by adding an equal volume of a stop solution consisting of 1 mM DTT, 1 mM PMSF, and 100 units/ml of antithrombin III. The specific activity of the probes was estimated at 1×10^5 cpm/pmol of the protein.

[^{32}P]FKBP12 Binding to Sperm

Caput and cauda sperm were obtained by piercing the caput and cauda epididymal tubules with a 27-gauge needle under a dissecting microscope. The sperm were allowed to diffuse freely from the tubules until the medium became turbid. The turbid fluid was collected and centrifuged at $600 \times g$ in a clinical centrifuge, and the pelleted sperm were washed three times in room temperature PBS. The washed sperm (10^6/ml) were then incubated with [^{32}P]FKBP12 (20,000 cpm) in PBS containing 1 mM DTT, 1 mM EGTA, and 2 mg/ml BSA for 1 hr at 4°C. The sperm were pelleted, washed two times with ice-cold incubation buffer, mixed with Formula 963, and scintillation counted. Specific binding was determined by subtracting [^{32}P]FKBP12 binding in the presence of 1 μM FK506 from total [^{32}P]FKBP12 bound. All binding experiments were done in duplicate and repeated three times.

[^{33}P]FKBP12 Autoradiography

Caput and cauda sperm were isolated from the epididymis as described above except that rat fertilization medium (RFM) (35) was used as the dispersal buffer (107.6 mM NaCl, 1.2 mM $MgSO_4$, 5.5 mM glucose, 4.3 mM KCl, 1.2 mM KH_2PO_4, 4.5 mM $CaCl_2$, 24.1 mM sodium lactate, 0.5 mM sodium pyruvate, 0.5 μg/ml streptomycin sulfate, 0.6 μg/ml benzylpenicillin, 31.5 mM Hepes, 0.4% BSA, pH 7.4). Sperm (10^6/ml) were incubated in RFM containing [^{33}P]FKBP12 (50,000 cpm) with or without 1 μM FK506 for 2 hr at 37°C, washed with RFM, wet mounted onto Superfrost plus slides, air dried, and apposed to NB-2 emulsion for a 2-week exposure.

Sperm Motility Analysis

Sperm were recovered from the caput and corpus regions of the epididymis as described above and allowed to disperse into 2 ml of Hank's balanced salts solution (HBSS) buffered with 4.2 g/L HEPES and 0.35 g/L $NaHCO_3$, and containing 2.0 g/L BSA, 0.9 g/L D-glucose, 0.1 g/L sodium pyruvate, and 0.025 g/L soybean trypsin inhibitor (pH 7.4). Sperm samples (100 μl) were treated with 10 nM human recombinant FKBP12 (Sigma) and analyzed at 15, 30, and 60 min post-addition for alterations in sperm motion parameters. To analyze sperm motility, samples were further diluted in HBSS and a-10 μl sample was placed on a glass slide between 75-μm thick stainless steel spacers and then coverslipped. Sperm were videotaped and analyzed using a Hamilton Thorne 2000 analyzer equipped with software version 6.5R. A minimum of 200 sperm were analyzed per sample. For each field, 13 frames were analyzed at a rate of 19 frames/sec. Sperm were considered motile when path velocity exceeded 20 μm/sec and progressively motile if their linear index was greater than 50.

Results

Identification of High Levels of [^{3}H]FK506 Binding Activity in Male Reproductive Tissues

In initial studies monitoring [^{3}H]FK506 binding in various organ systems, immune tissues such as the thymus were found to contain approximately the same levels of high-affinity soluble binding activity (Kd = 0.6 nM) as other peripheral tissues, such as the liver, lung, and heart (23). [^{3}H]FK506 binding levels in various brain regions, however, were 2–15 times greater than values observed in peripheral tissues, with whole brain binding 100 times the levels found in the periphery (23). We have found that tissues of the male reproductive tract display 2–40 times the [^{3}H]FK506 binding levels seen in other peripheral tissues (Table 1). The vas deferens has approximately 30% of the binding levels of whole brain, and thus contains the second highest levels of [^{3}H]FK506 binding sites in the body. [^{3}H]FK506 soluble binding levels in the testis are approximately half those of the vas deferens. Binding levels in the head or caput of the epididymis, where immature sperm reside, are similar to levels in the testis and about five times higher than the levels observed in the tail or cauda of the epididymis, where sperm reach maturity. The prostate, seminal vesicle, and coagu-

Table 1. [^{3}H]FK506 binding in the male reproductive tract and other selected tissues

Rat Tissue (soluble fraction)	B_{max} (pmol/mg)
Testis	57
Caput epididymis	54
Corpus epididymis	7.0
Cauda epididymis	14
Vas deferens	120
Prostate	18
Seminal vesicle	46
Coagulating gland	29
Brain	400
Thymus	3.1
Liver	3.7
Lung	2.6
Heart	3.6

The soluble fractions of male reproductive and other selected tissues (1–25 μg protein) were incubated with 250 pM [^{3}H]FK506 in binding buffer (50 mM Trizma pH 7.4, 2 mg/ml BSA) for 60 min at room temperature. The reaction volume was layered over a 1.0-ml column of LH-20 Sephadex, the column was washed with 0.5 ml of buffer, and the eluates were collected and counted. Specific binding was determined by subtracting [^{3}H]FK506 bound in the presence of up to 1 μM unlabeled FK506 from total [^{3}H]FK506 bound. All binding experiments were done in duplicate and repeated three times.

lating glands, which secrete proteinaceous fluid into the seminal plasma, also display substantial levels of [^{3}H]FK506 binding.

In all of the tissues assayed for soluble [^{3}H]FK506 binding activity, Scatchard analysis revealed a linear binding curve reflecting a single binding site with a Kd in the range of 0.5–1.0 nM, which is similar to that of FKBP12, the most abundant FK506 binding protein. Western blot analysis using our FKBP12 antibody (27) demonstrated that the highest levels of FKBP12 in the body are found in whole brain (Fig. 1). Consistent with the [^{3}H]FK506 binding data, the vas deferens contained the next highest level of FKBP12 protein, with other peripheral tissues, such as the liver, heart, and spleen, displaying much lower amounts of FKBP12. In all five tissues, a discrete 12 kD band was evident, although in the spleen two additional faint bands were detected and may reflect FKBP12.6 (7) and FKBP13 (36). The immunoreactivity was abolished by preabsorbing the antibody with the FKBP12 fusion protein.

Localization of [^{3}H]FK506 Binding Along the Path of Sperm Maturation

To localize [^{3}H]FK506 binding activity in male reproductive tissues, we conducted [^{3}H]FK506 autoradiography (Fig. 2). The caput (head) and cauda (tail) of the epididymis displayed strikingly different localizations of [^{3}H]FK506 binding. In the caput, there was selective labeling of the principal cells that line the lumen of the tubules (Fig. 2A). In the cauda, however, there was exclusive labeling of the luminal contents of the epididymal tubules (Fig. 2B). In the vas deferens, there was intense labeling of the lumen, with lower levels observed in the lining epithelium; the layers of smooth muscle in the wall of the vas deferens were completely negative (Fig. 2C). No labeling was observed in control sections that were treated with 1 μM unlabeled FK506 to block specific binding (data not shown).

The concentration of [^{3}H]FK506 binding activity in the principal cells of the caput epithelium and the apparent transfer of activity to the lumen of the distal epididymis and vas deferens is a common pattern observed in the epididymis in association with caput epididymal secretion (37,38). Many epididymal proteins, such as sulfhydryl oxidase and glycosidases, are well known to be secreted by the caput epididymis and subsequently become associated with luminal sperm, influencing the sperm maturation process (39–42). This suggested that FKBP12 may be synthesized in the principal cells and then released into the epididymal tubules, where the protein becomes associated with luminal sperm. If this model is correct, FKBP12 should be present in the epididymal fluid.

Identification of FKBP12 in Epididymal Fluid and Interaction with Caput Sperm

To determine if FKBP12 is present in epididymal secretions, we isolated epididymal fluid from sexually mature male rats and passed it over an FK506 column (Fig. 3). A 12 kD protein selectively bound to the FK506 matrix (Fig. 3, left panel). When the epididymal fluid was preincubated with soluble FK506, binding of the 12 kD protein to the matrix was completely abolished. Western blot analysis confirmed that the 12 kD protein was FKBP12 (Fig. 3, right panel).

If FKBP12 interacts with sperm in a manner

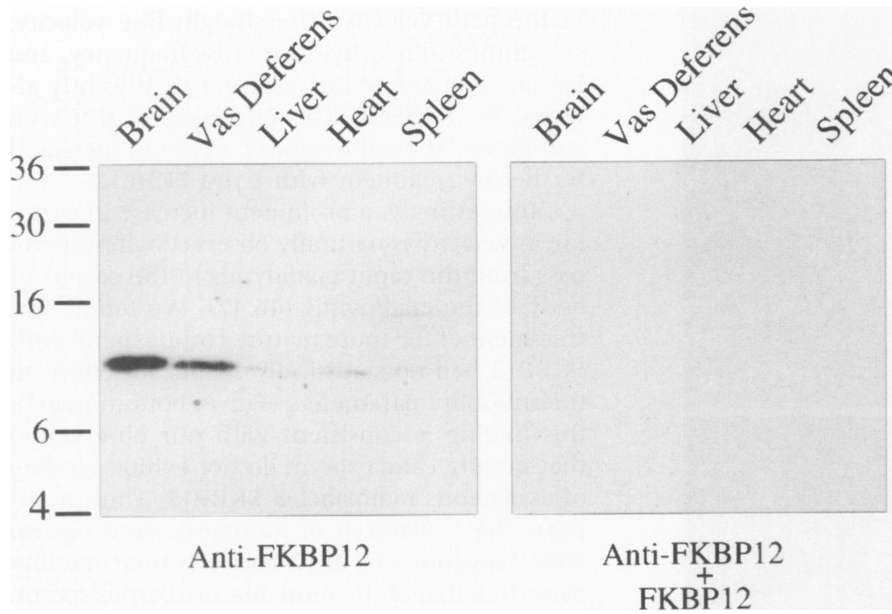

Fig. 1. FKBP12 Western analysis. Rat tissues were solubilized in 2% SDS, 1% Triton X-100, 50 mM Trizma pH 7.4, 100 mM NaCl, 1 mM EGTA, and protease inhibitors. Western analysis demonstrates high levels of FKBP12 in the brain and vas deferens (left panel). Other selected peripheral tissues such as the liver, heart, and spleen contain much lower amounts of FKBP12. The immunoreactivity is abolished by preabsorbing the antibody with the FKBP12 fusion protein (right panel).

similar to other proteins secreted by the epididymis, it should bind to sperm in a saturable fashion. Thus, the binding of endogenous FKBP12 to cauda epididymal sperm should prevent further binding of exogenous FKBP12. To investigate FKBP12 interactions with sperm, we monitored [^{32}P]FKBP12 binding to sperm purified from the caput and cauda epididymis. [^{32}P]FKBP12 bound specifically to caput sperm but not to cauda sperm (Fig. 4). Binding specificity was defined by the ability of 1 μM FK506 to inhibit [^{32}P]FKBP12 binding to sperm. The inhibition of [^{32}P]FKBP12 binding to caput sperm by FK506 further suggests that the FKBP binding site on sperm simulates the structure of FK506, as has been described for other FKBP–protein interactions. For example, leucyl-proline 1400–1401 of the IP_3 receptor has recently been shown to mediate the constitutive binding of FKBP12 to the calcium channel and is believed to have structural characteristics similar to the pyranose methyl moiety of FK506 (43).

Selective binding of exogenous-labeled FKBP12 to caput but not cauda sperm was also observed by autoradiographic analysis (Fig. 5). Caput and cauda sperm were incubated in rat fertilization medium containing [^{33}P]FKBP12 for 2 hr at 37°C. The sperm were then washed and mounted onto slides for autoradiographic exposure. Whereas caput sperm were coated with [^{33}P]FKBP12 (Fig. 5A), no binding to cauda sperm was evident (Fig. 5C). Again, the binding of [^{33}P]FKBP12 to caput sperm was specifically blocked by the addition of 1 μM FK506 to the incubation medium (Fig. 5B).

FKBP12 Stimulates Caput Sperm Motility

Sperm acquire the potential to swim as they pass from the caput to the cauda epididymis. The selective binding of exogenous FKBP12 to immature caput sperm but not to mature cauda sperm prompted us to evaluate the influence of FKBP12 on sperm motility. Caput sperm were isolated from the epididymal tubules and incubated in the presence or absence of 10 nM and 1 μM FKBP12. Videotaped images of the sperm were digitized and analyzed in real time using a Hamilton Thorne 2000 motility analyzer (44,45). The velocity of sperm movement is described in terms of curvilinear velocity, which reflects the point-to-point speed over the entire path of sperm movement, and the path and straight-line velocities, which assess the velocities over the average path of movement, and the strictly linear path of movement, respectively. The straightness of sperm movement is defined as the straight-line velocity divided by the path velocity, whereas the linearity of movement is defined as the straight-line velocity divided by the curvilinear velocity. The frequency of the tail beat is reflected by the measurement of beat cross frequency and the degree of head movement is assessed by measuring the lateral head amplitude. Treatment with 10 nM FKBP12 for 15 min resulted in a doubling of the curvilinear velocity

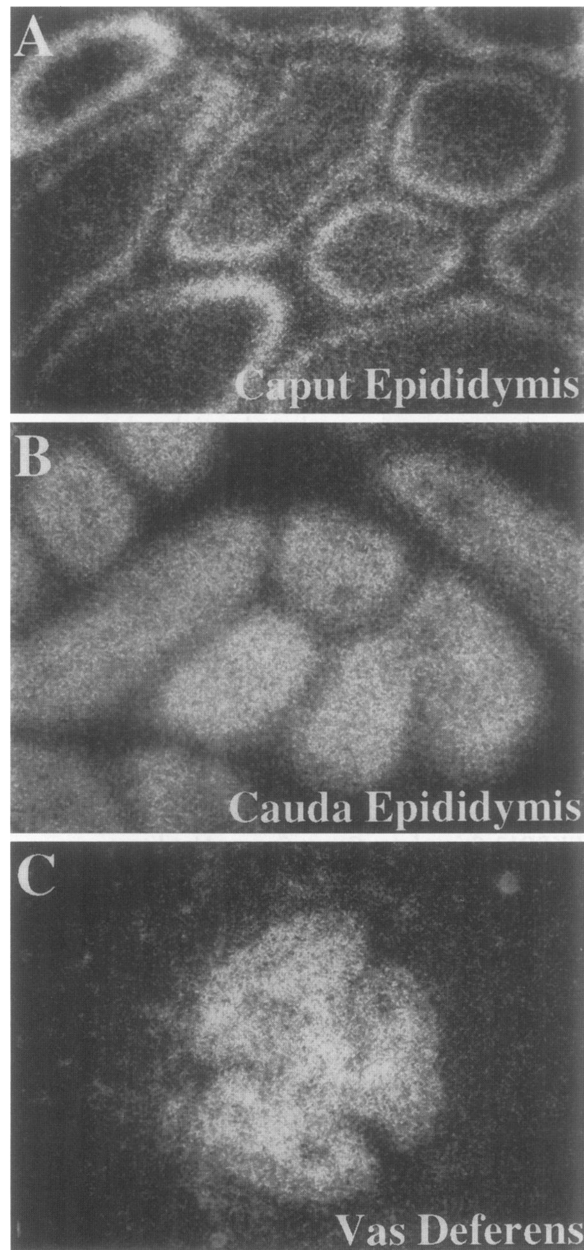

Fig. 2. Localization of [^{3}H]FK506 binding along the path of sperm maturation. [^{3}H]FK506 autoradiography was performed on 20-μm fresh frozen sections of rat caput and cauda epydidimides and vas deferens. (A) In the caput or head of the epididymis, there is selective labeling of the principal cells that line the tubular lumen. (B) In the cauda, there is exclusive labeling of the tubular lumen, where the sperm are located. (C) In the vas deferens, there is intense labeling of the lumen, with lower levels present in the lining epithelium. The layers of smooth muscle in the wall of the vas deferens are completely negative.

of caput sperm (Fig. 6, top panel). A smaller but statistically significant increase was also evident in the path velocity. The straight line velocity, straightness, linearity, beat cross frequency, and lateral head amplitude were not significantly affected by FKBP12. The effects of FKBP12 on curvilinear and path velocity were not increased further by treatment with 1 μM FKBP12.

Interestingly, a prominent increase in curvilinear velocity is naturally observed when sperm pass from the caput epididymis to the corpus or body of the epididymis (46,47). We found that treatment of the more mature corpus sperm with FKBP12 had no statistically significant effect on their motility parameters (Fig. 6, bottom panel); this finding is consistent with our observation that mature cauda sperm do not exhibit binding of exogenous radiolabeled FKBP12. Thus, it appears that treatment of immature caput sperm with exogenous FKBP12 converts their motility pattern to that of the more mature corpus sperm.

Discussion

In the present study, we have identified high levels of FKBP12 in male reproductive tissues. On the basis of [^{3}H]FK506 binding activity and Western analysis, the vas deferens was found to have the second highest levels of FKBP12 in the body. Along the path of sperm maturation, [^{3}H]FK506 binding is initially restricted to the principal cells of the caput epididymis and then localizes to the lumen of the cauda epididymis and vas deferens. This differential localization of [^{3}H]FK506 binding sites in the male reproductive tract, coupled with the identification of FKBP12 in epididymal fluid, suggests that FKBP12 is secreted by the caput epididymis into the epididymal tubules. The finding that exogenous radiolabeled FKBP12 binds to immature caput sperm but not to mature cauda sperm further indicates that secreted FKBP12 saturates FKBP12 binding sites on maturing sperm. A shift of FKBP12 from the soluble to the insoluble sperm-bound fraction is supported by the over 5-fold drop in [^{3}H]FK506 soluble binding activity seen in cauda versus caput epididymal tissue (Table 1). Interestingly, soluble [^{3}H]FK506 binding increased dramatically in the vas deferens compared with the cauda epididymis, suggesting that high levels of soluble FKBP12 are again released into the tubular lumen. The identification of FKBP12 in epididymal fluid suggested that this protein may be involved in the sperm maturation process. Indeed, we found that incubation of immature caput sperm with 10 nM FKBP12 stimulated cur-

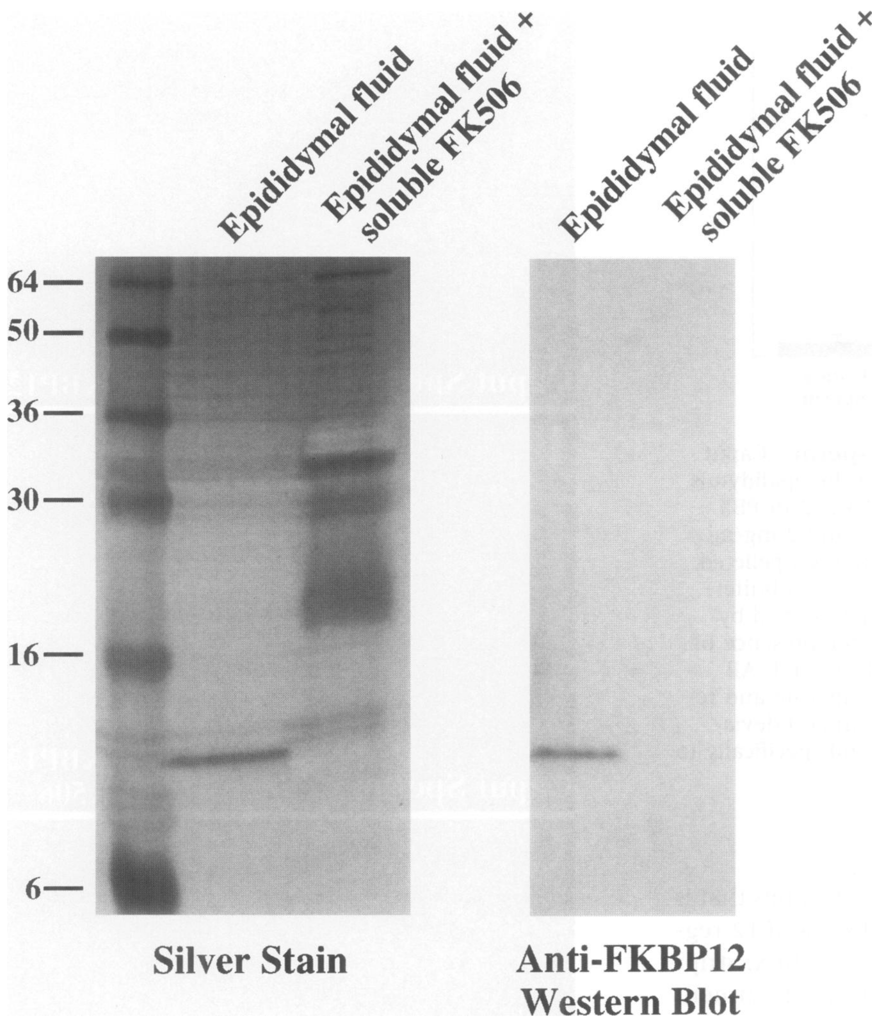

Fig. 3. Identification of FKBP12 in epididymal fluid by FK506 affinity chromatography. Epididymal fluid isolated from the epididymis was incubated with an FK506 matrix in the presence or absence of 100 μM soluble FK506. The washed matrix was resuspended in PBS and protein load buffer, boiled, and electrophoresed on 18% Tris-glycine gels. A silver stain of the gel demonstrated prominent binding of a 12 kD protein to the FK506 matrix (left panel). When the epididymal fluid was preincubated with soluble FK506, binding of the 12 kD protein to the matrix was completely abolished. Anti-FKBP12 Western analysis of a duplicate experiment confirmed that the 12 kD protein was FKBP12 (right panel).

vilinear velocity to levels seen in more mature corpus sperm.

Sperm acquire the potential to swim and fertilize eggs during a maturation process that occurs as sperm move along the epididymis. The epididymis is well known to secrete multiple factors involved in modification of the lipid content, protein composition, and glycosylation state of the sperm plasma membrane (48). The synthesis and secretion of several epididymal proteins have been shown to be androgen-dependent (49,50). After injecting mice with ^{35}S-labeled methionine, more than 50% of labeled protein in the epididymis is found in the epididymal fluid; furthermore, levels of labeled, secreted protein are approximately two to four times higher in the caput than in the corpus or cauda of the epididymis (37). These in vivo labeling experiments have additionally shown that many of the ^{35}S-labeled proteins become tightly bound to epididymal sperm; whereas caput sperm are coated with labeled protein, cauda sperm, which are presumably saturated with unlabeled protein, remain unlabeled despite being surrounded by soluble ^{35}S-labeled protein (39). The results of our binding experiments using radiolabeled FKBP12 are consistent with these previously observed patterns of interaction between secreted epididymal proteins and maturing sperm.

FKBP12 is a well-known intracellular protein whose functions associated with IP_3R and ryanodine receptor regulation and FK506-induced T-cell immunosuppression rely on its cytosolic localization. There is some evidence, however, that FKBP12 can be secreted by specific cells. Bang et al. (51) reported that mast cells sensitized with IgE release FKBP12 upon stimulation with anti-IgE. In addition, extracellular FKBP12 can induce intracellular calcium release from ryanodine-sensitive Ca^{2+} stores in neutrophils (51). The authors proposed that ryanodine receptors may be functionally linked to an extra-

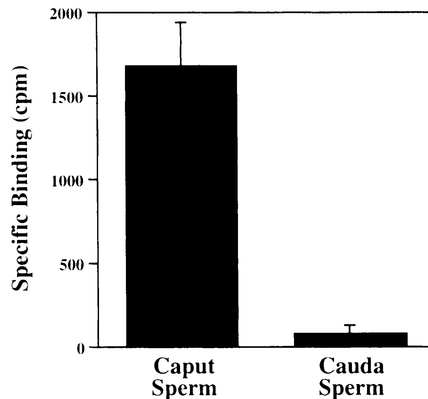

Fig. 4. [^{32}P]FKBP12 binding to sperm. Caput and cauda sperm were isolated from the epididymis, washed, and incubated with [^{32}P]FKBP12 in PBS containing 1 mM DTT, 1 mM EGTA, and 2 mg/ml BSA for 1 hr at 4°C. The sperm were then pelleted, washed two times with ice-cold incubation buffer, and counted. Specific binding was determined by subtracting [^{32}P]FKBP12 binding in the presence of 1 μM FK506 from total [^{32}P]FKBP12 bound. All binding experiments were done in duplicate and repeated three times. Error bars are standard deviations of the mean. [^{32}P]FKBP12 bound specifically to caput sperm but not to cauda sperm.

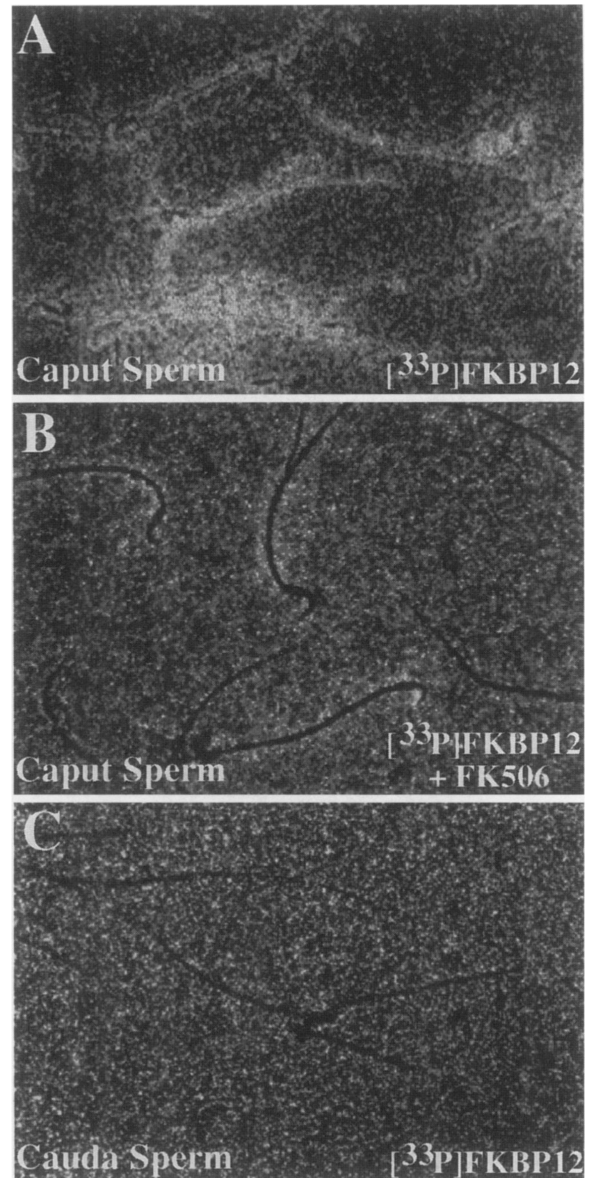

Fig. 5. [^{33}P]FKBP12 autoradiography of caput and cauda sperm. Caput and cauda sperm were isolated from the epididymis and incubated in rat fertilization medium (RFM) containing [^{33}P]FKBP12 for 2 hr at 37°C. The sperm were then washed with RFM, wet-mounted onto Superfrost plus slides, air dried, and apposed to NB-2 emulsion for a 2-week exposure. (A) Caput sperm are labeled by [^{33}P]FKBP12. (B) The labeling of caput sperm by [^{33}P]FKBP12 is specifically blocked by the addition of 1 μM FK506 to the incubation medium. (C) Cauda sperm are completely negative.

cellular membrane receptor in neutrophils that is activated by FKBP12. Within cells, FKBP12 regulates IP_3 receptor–mediated calcium flux; stripping FKBP12 from IP_3 receptors by treatment with FK506 renders the calcium channels "leaky" to calcium (4). In addition, we have localized IP_3 receptors to the acrosome and proximal tail of mammalian sperm (52). Interestingly, the levels of intracellular calcium are two to three times higher in caput sperm than in cauda sperm, and this increased calcium concentration is believed to inhibit caput sperm motility (53). Thus, it is plausible that binding of FKBP12 to maturing sperm decreases intracellular calcium levels either by interacting with a cell surface receptor or by enhancing the intracellular calcium storage capability of "leaky" IP_3 receptors. In the latter case, however, the topology of intracellular or plasma membrane IP_3 receptors would require that FKBP12 somehow gain access to the sperm cytosol. Another possibility is that FKBP12 exerts a maturing effect on sperm by peptidyl prolyl isomerization of sperm surface proteins, thereby enabling proper protein folding. Because sperm do not actively synthesize new proteins, remodeling of the plasma membrane is achieved by the actions of many secreted enzymes, including sulfhydryl oxidase (42) and glycosidases (40), which are present in the epididymal fluid.

How is FKBP12 secreted? FKBP12 lacks a defined signal peptide that would target it to the

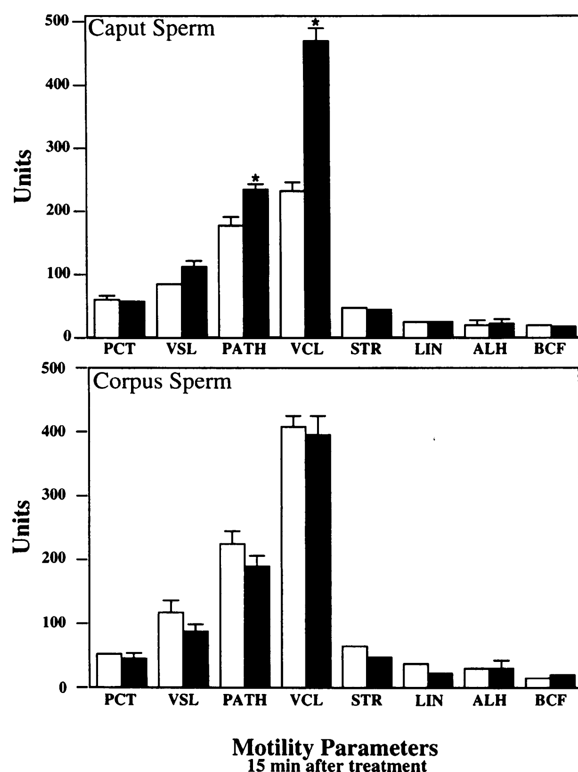

Fig. 6. Stimulation of caput sperm motility by FKBP12. Caput and corpus sperm were isolated from the epididymal tubules and dispersed in Hank's balanced salt solution (HBSS) buffered with 15 mM Hepes and 4 mM sodium bicarbonate, and containing 0.2% BSA, 5 mM D-glucose, 10 mM sodium pyruvate, 1 mM nonessential amino acids, and 25 μg/ml soybean trypsin inhibitor. Samples (100 μl) of sperm were treated with 10 nM FKBP12 and videotaped for motility analysis at 15-, 30- and 60-min time points. Videotaped images were digitized and analyzed in real time using a Hamilton Thorne 2000 motility analyzer. After a 15-min incubation with 10 nM FKBP12 (solid bars), caput sperm exhibited double the curvilinear velocity (VCL) of control caput sperm (top panel). A smaller but statistically significant increase was also evident in the path velocity (PATH). The straight line velocity (VSL), straightness (STR), linearity (LIN), beat cross frequency (BCF), and lateral head amplitude (ALH) were not significantly affected by FKBP12. Treatment of the more mature corpus sperm with FKBP12 had no statistically significant effect on their motility parameters compared with control corpus sperm (bottom panel). The treatment of immature caput sperm with FKBP12 stimulated their curvilinear velocity to the level seen in normal corpus sperm. Units presented are as follows: PCT = percent; VSL, PATH, VCL = μM/sec; STR, LIN = ratios; ALH = μM; BCF = 1/sec. Open bars represent vehicle. Error bars represent standard deviations of the mean. The asterisks represent statistical significance.

endoplasmic reticulum (ER) for translation and ultimate secretion by the classical exocytotic pathway. Interestingly, the mouse vas deferens protein (MVDP), which is a major androgen-dependent protein of vas deferens fluid, likewise has no signal peptide (54). This prompted a study of the ultrastructural localization of MVDP in the epithelium of the vas deferens. Immunoelectron microscopy identified MVDP throughout the epithelial cytoplasm but not within the ER, golgi, or vesicles; instead, the protein was abundant in apical protrusions of the cell membrane and the luminal fluid, consistent with a mechanism of apocrine secretion (i.e., release of secretory material through apical plasma membrane blebs rather than by exocytosis of secretory granules) (54). Androgen-dependent apocrine secretion has been observed in many male reproductive tissues (e.g., coagulating gland, seminal vesicle, ampulla of the vas deferens) in a variety of species (55–57) and may explain how FKBP12 is released into the male reproductive tract. Another possibility is that an alternative splice form of FKBP12 exists that contains an upstream targeting sequence, thereby enabling secretion by the classical exocytotic pathway.

FKBP12 stimulates caput sperm motility at low nanomolar concentrations, with the greatest effect seen in curvilinear velocity. Curvilinear velocity is the most sensitive measure of sperm movement as it takes into account the entire path traversed by the sperm. The levels of FKBP12 in the male reproductive tract are certainly high enough for FKBP12 to function as an endogenous regulator of sperm motility. Further support for the relevance of FKBP12 in sperm physiology derives from toxicologic studies of FK506. Male rats that received subcutaneous FK506 developed a dose-dependent decrease in sperm counts and motility (58). Whereas no abnormal histologic changes were observed in the testis, degeneration of sperm cells was seen in the epididymis. Further, when FK506 was discontinued, sperm counts and motility returned to normal. Thus, the decreased sperm counts and motility were believed to result from a direct action of the drug on sperm in the epididymis rather than on sperm production in the testis. On the basis of our findings, it is plausible that saturation of epididymal FKBP12 by FK506 disrupted the physiologic function of FKBP12 as a mediator of sperm maturation.

The highest concentrations of soluble FKBP12 in the male reproductive tract occur in the vas deferens, which is a site of sperm storage

and the conduit for ejaculated sperm. Release of FKBP12 by the vas deferens may ensure optimal preservation of sperm for fertilization. Indeed, multiple factors present within the epididymal and vas deferential fluid are believed to influence the postejaculatory functions of sperm (59–62). Maintenance of sperm motility is a major priority in artificial insemination. Our data suggest that incubation or storage of sperm with supplementary FKBP12 may optimize sperm viability.

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