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Sequence and Localization of the Mouse Sperm Autoantigenic Protein, Sp17[1]

Min Kong, Richard T. Richardson, Esther E. Widgren, and Michael G. O'Rand[2]

Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

ABSTRACT

The present study characterizes the sperm protein Sp17 in the mouse. Sp17 is a mammalian testis- and sperm-specific protein that has been isolated, sequenced, and characterized from rabbit testis and spermatozoa. In this study, a rabbit Sp17 cDNA probe representing the entire protein coding region was used to screen a mouse testis cDNA library to obtain the mouse Sp17 sequence. The mouse mRNA for Sp17 encodes a 149-amino acid protein with a predicted molecular weight of 17 296. The mouse Sp17 (MSp17) cDNA sequence is 82% identical to the rabbit Sp17 cDNA sequence while the MSp17 protein sequence is 74% identical to the rabbit protein sequence. The presence of native Sp17 in mouse spermatozoa and testis was demonstrated by Western blot analysis, immunoprecipitation, and immunolocalization. After SDS-PAGE, the native Sp17 has an apparent molecular mass of 24 kDa. The sequence of the native Sp17 was confirmed by Western blots of mouse testis and spermatozoa probed with two anti-peptide antibodies—one, anti-G22C, made against amino acids 61–82 in the rabbit sequence (61–83 in the mouse), and a second, anti-K18C, made against amino acids 120–136 in the C-terminal region in the human sequence (118–134 in the mouse sequence). In the absence of proteolytic inhibitors, part of the C-terminal of native MSp17 is cleaved, giving rise to an 18-kDa band. Sp17 is present in spermatocytes and spermatids in the testis. In spermatozoa, Sp17 is not available to bind antibody on the surface of live, acrosome-intact spermatozoa, but it is present on the equatorial surface of live, acrosome-reacted spermatozoa. In fixed spermatozoa, staining is observed along the length of the principal piece, weakly along the midpiece, and over the acrosomal region of the head. When the acrosome reaction begins, acrosomal staining is seen throughout the equatorial region of the acrosome. Using mimotope analysis, this study has also demonstrated that native Sp17 is a sperm autoantigen and that recombinant mouse Sp17 is immunogenic in males with a highly restricted linear epitope.

INTRODUCTION

Sp17 is a mammalian testis- and sperm-specific protein that has been isolated, sequenced, and characterized from rabbit testis and spermatozoa [1]. In the rabbit, Sp17 is a member of the RSA family (17 kDa) of rabbit sperm-specific autoantigens that binds rabbit zona pellucida as well as sulfated carbohydrates [2]. In other species (human, pig, mouse), RSA-like molecules have been identified on Western blots and by immunofluorescence with anti-RSA antibodies [3, 4]. Anti-RSA antibodies have also been shown to inhibit mouse sperm-zona binding [4]. Because RSA is a family of autoantigenic proteins and several RSA-like molecules were previously identified in mouse spermatozoa, the present study was undertaken to focus on one particular molecule and to identify its sequence and localization.

During the previous study of rabbit Sp17 [1], Northern blot analysis of mouse tissues revealed that Sp17 mRNA was not present in any of the mouse somatic tissues examined (mouse heart, brain, spleen, lung, liver, skeletal muscle, and kidney) and was specific to the testis, with an approximate size of 0.6–0.8 kb. In order to characterize Sp17 in the mouse, a rabbit Sp17 cDNA probe, representing the entire protein coding region (open reading frame), was used to screen a mouse testis cDNA library to obtain the mouse Sp17 sequence. In contrast to the rabbit, which has mRNA species of two different sizes but with identical coding regions [1], the resulting mouse cDNA clones were all derived from one mRNA species. The mouse mRNA for Sp17 encodes a 149-amino acid protein with a predicted molecular weight of 17 296. The mouse Sp17 (MSp17) cDNA sequence is 82% identical to the rabbit Sp17 cDNA sequence while the MSp17 protein sequence is 74% identical to the rabbit protein sequence. To begin our characterization of MSp17, we report the sequence of MSp17, the localization of Sp17 in testis and spermatozoa using anti-recombinant Sp17 antiserum (anti-rSp17), and the autoimmunity of MSp17 in male mice.

MATERIALS AND METHODS

Materials

The mouse germ cell cDNA library in λgt11 was a gift from Dr. David Joseph (Laboratories for Reproductive Biology, University of North Carolina at Chapel Hill). Restriction enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN) and New England Biolabs, Inc. (Beverly, MA). Radioactive labeling of DNA was carried out with use of a random priming labeling kit from Boehringer-Mannheim with ^{32}P-labeled dCTP. All DNA sequencing was done by the dideoxy chain termination method using a Sequenase II kit (United States Biochemical Corp., Cleveland, OH).

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[2]Correspondence: Dr. Michael O'Rand, Dept. Cell Biology & Anatomy, CB #7090, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599. FAX: (919) 966–1856.

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Terminal deoxynucleotidyl transferase was purchased from Gibco BRL (Life Technologies, Gaithersburg, MD). The first-strand cDNA Synthesis Kit from Clontech (Clontech Laboratories, Inc., Palo Alto, CA) was used when 5′-end and 3′-end cDNAs were constructed. Affinity-purified rabbit anti-keyhole limpet hemocyanin (KLH) to use as a control serum for immunocytochemistry, and calcium ionophore A23187 were purchased from Sigma Chemical Company (St. Louis, MO). The peroxidase substrate AEC (3-amino-9-ethylcarbazole) was purchased from Vector Laboratories, Inc. (Burlingame, CA), and Biggers, Whitten, and Whittingham (BWW) medium was purchased from Irvine Scientific (Santa Ana, CA). CD1 and B6AF1 male mice were obtained from Charles River Breeding Laboratories (Wilmington, MA) and Jackson Laboratories (Bar Harbor, ME), respectively, and housed in a University laboratory animal care facility (approved by the American Association for Accreditation of Laboratory Animal Care). All chemicals were of reagent or molecular biology grade. The pin-block used for mimotope analysis was synthesized by Chiron Mimotopes Peptide Systems, (Clayton, Victoria, Australia). The peptides G22C and K18C were synthesized at the Salk Institute (under contract NO1-HD-0−2906 with the NIH,USPHS) and were made available by the Contraceptive Development Branch, Center for Population Research, NICHD (Bethesda, MD).

Methods

Screening, cloning, sequencing, and analysis. The mouse germ cell cDNA library was screened by a random prime labeled cDNA probe that represented the protein coding region of rabbit Sp17 [1]. This probe was made by polymerase chain reaction (PCR) from a rabbit testis λgt11 library containing the Sp17 clone, with use of the sense oligonucleotide 5′TCCCCCGGGAGGCAAGTTCTTGCC3′ and the antisense oligonucleotide 5′CGCGGATCCGCCAGTGCCCTCAATTGT3′ as primers [5]. After the third screen, 24 of the 26 clones picked from the library hybridized by Southern blot analysis to the original probe used for screening. All 24 clones were subsequently analyzed by *Eco*RI, *Taq* I, and *Bsm*AI digestion, and the results indicated that all the clones were the same. Therefore four of the positive clones were selected for sequencing. Each clone was digested with *Eco*RI, and the large fragment was subcloned into M13mp18 or pBluescript II KS vector at the *Eco*RI site and sequenced. The remaining small fragment on the 5′ end, the result of an internal *Eco*RI site, resisted cloning. Therefore, to obtain the sequence upstream of the internal *Eco*RI site on the 5′ end, PCR amplification of the Sp17 λgt11 clone was carried out with use of the λgt11 forward and the reverse primers. These two primers (5′CGCGGATCCGGTGGCGACGACTCCTGGAGC3′ and 5′CGCGGATCCGACACCAGACCAACTGGTAAT3′) each contain a *Bam*HI site on their 5′ ends. The PCR product was electrophoresed by use of 2.5% Nusieve GTG 3:1 agarose (FMC Corp., Rockland, ME), and the band was excised and extracted according to the Geneclean protocol (Bio 101 Inc., La Jolla, CA). After *Bam*HI digestion of the product, the fragment was again electrophoresed, extracted using the Geneclean protocol (Bio 101 Inc.), and cloned into Mp13mp18 or pBluescript II KS vector at the *Bam*HI site for sequencing.

Sequence analyses and alignment were performed with PC/Gene and GCG software packages (Intelligenetics, Mountainview, CA and Genetics Computer Group, University of Wisconsin, Madison, WI, respectively). Comparison of sequences with proteins in the Genbank database was carried out with the FASTA search program [6].

Mouse testis RNA and poly(A)$^+$ RNA preparation. One gram of fresh mouse testis was homogenized in 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Total RNA was isolated by use of the Total RNA Separation Kit (Clontech Laboratories, Inc.) and the manufacturer's protocols. Poly(A)$^+$ RNA was purified on biotinylated oligo(dT) magnetic spheres by using a Promega (Madison, WI) PolyAT tract mRNA isolation system.

Complementary DNA construction from mRNA. The 5′ RACE protocol [7] was performed as follows: starting with 1 μg of mRNA using M-MLV reverse transcriptase and an antisense primer P1 (5′CGCGGATCCAAGCTGGTATATTGTC3′), the cDNA template was synthesized. After excess primer P1 was removed with a chroma spin-100 column (Clontech, Inc.), the cDNA was treated with terminal deoxynucleotidyl transferase in the presence of dATP. After second-strand synthesis, the amplification reaction proceeded with the dT 17 adapter primer with a 5′ *Xho* I restriction site (5′GACTCGAGTCGACATCGAT$_{17}$ 3′), the adapter primer (5′GACTCGAGTCGACATCG3′), and a P2 primer (5′CGCGGATCCTTGCTCCCTCAGAATCTCCCG3′). P1 and P2 primers were complementary to nucleotide positions 97−112 and 73−93 in the mouse Sp17 cDNA with 5′ *Bam*HI restriction sites. After second-round amplification, the PCR product was electrophoresed and extracted according to the Geneclean protocol (Bio 101 Inc.), followed by *Bam*HI and *Xho* I digestion. The cDNA was then subcloned for sequencing into pBluescript vector that was predigested with *Bam*HI and *Xho* I. To be certain that the 3′ end was complete, the 3′ RACE protocol was performed as follows: first-strand cDNA was synthesized with the dT 17 adapter primer (above) followed by the second-strand synthesis and amplification with the adapter primer and a mouse Sp17 primer (5′ATTAGCTAAGTCATCTG3′) at nucleotide position 258−274.

Bacterial expression of the entire Sp17 coding region. The entire Sp17 coding region was amplified by PCR using the two primers: the sense primer, 5′GCGGGATCCATGTCGATTCCATTTTCC3′, containing a *Bam*HI site on the 5′ end and the antisense primer, 5′GCGGGTACCTGTGGTCTCAATTGTCTGC3′, containing a *Kpn* I site on the 5′ end.

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The PCR product was cloned into the PQE-30 expression vector (Qiagen Inc., Chatsworth, CA), which was predigested with *Bam*HI and *Kpn* I. The integrity of the insert was verified by sequencing. Bacterial expression of mouse Sp17 was performed with the Qiagen expression system (Qiagen Inc.) as described previously [1]. The recombinant Sp17 fusion protein was expressed with an N-terminal Arg-Gly-Ser, followed by six histidines and Gly-Ser preceding the 149 amino acids of the entire Sp17 coding region. The fusion protein was purified by affinity chromatography on metal chelate adsorbent nickel-NTA-agarose as described by the manufacturer (Qiagen Inc.).

Electrophoresis and blotting. Fifteen percent SDS-PAGE was performed according to the method of Laemmli [8]. Two-dimensional gel electrophoresis was performed using the method of O'Farrell [9]. Isoelectric focusing (IEF) gels were run in the first dimension under reducing conditions as described previously [1, 10]. The second dimension was 15% SDS-PAGE under reducing conditions. Western blots were carried out according to the method of Towbin et al. [11] with Immobilon-P used as the transfer membrane. Immunoblotting was carried out at room temperature. After blocking for 30 min with 5% nonfat milk in TTBS (0.05% Tween 20 + Tris buffered saline), blots were incubated for 2 h with either rabbit anti-rabbit Sp17 immunoglobulin (22 μg/ml), affinity-purified rabbit anti-rabbit G22C IgG (3 μg/ml) in 2% milk [1], or mouse anti-human K18C. G22C is an internal Sp17 peptide (mouse aa 62–83). K18C is also an internal Sp17 peptide (mouse aa 118–134). Immunoreactivity was detected by incubating blots for 1 h in alkaline phosphatase-conjugated goat anti-rabbit IgG or anti-mouse IgG as the second antibody (1:1000 dilution). Blots were developed by using NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as a substrate. Southern blots were performed as described [1, 12].

Sperm and testis lysate preparation. Mouse spermatozoa were collected from the cauda epididymides and washed twice in 40 mM Tris-HCl buffer, pH 7.2, by centrifugation at 600 × *g* for 7 min at 4°C. The pellet was resuspended in SDS-PAGE sample buffer [8] containing protease inhibitor cocktail (1 mM Pefabloc SC [Boehringer-Mannheim] plus 10 μg/ml leupeptin and 2 μg/ml aprotinin) and sonicated on ice for 30 sec. After being boiled for 5 min, the sperm lysate was centrifuged (12 000 × *g*, 2 min, 25°C), and the supernatant (approximately 4 × 10^6 sperm/lane) was loaded onto the acrylamide gel. Fresh mouse testes were homogenized on ice in SDS-PAGE sample buffer (1 ml/testis) containing 1 mM Pefabloc SC, boiled, and centrifuged (12 000 × *g*, 2 min, 25°C) two times, and 20 μl of the supernatant was loaded onto the acrylamide gel. For IEF gel electrophoresis, sperm or testis samples were sonicated or homogenized in PBS containing Pefabloc SC before being mixed with the urea sample buffer and then centrifuged, and the supernatant was layered onto the IEF gel [10]. Isoelectric point (pI) standards and calibration of the gels were identical to those described previously [1].

Immunoprecipitation. For immunoprecipitation, sperm pellets (as described above) were resuspended in TSA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, protease inhibitor cocktail, 0.02% sodium azide) containing 1% deoxycholate, 1% Triton X-100 (Sigma Chemical Co.), and 1% SDS. The suspension was incubated for 30 min at 4°C with shaking, and centrifuged (12 000 × *g*, 4°C, 30 min). The supernatant was saved, and the pellet was resuspended in TSA buffer containing 1% deoxycholate and 1% Triton X-100, incubated for 30 min at 4°C with shaking, and centrifuged. The supernatant was combined with the saved supernatant (from above; final SDS concentration 0.1%) and used for immunoprecipitation. Immunoprecipitation was carried out as described [13] with mouse anti-rabbit Sp17 serum.

Immunofluorescence and immunocytochemistry. Paraffin sections (7 μm) of mouse testis fixed in Bouin's fluid were made by routine histological procedures. The detailed method was described previously [14, 15]. After being deparaffinized and rehydrated, the sections were incubated for 30 min with 0.3% hydrogen peroxide to quench any endogenous peroxidase activity and permeabilized for 8 min in 0.2% Triton X-100 to facilitate the penetration of antibody. The sections were blocked in 10% normal goat serum (NGS) for 30 min, after which antibody in PBS containing 5% NGS was added. The primary antibody was either affinity-purified rabbit anti-rabbit G22C IgG (50 μg/ml) [1] or the same concentration of affinity-purified rabbit anti-KLH IgG. The sections were incubated overnight at 4°C followed by a 1-h incubation at room temperature. After being washed for 15 min (5 changes of PBS), the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:250 for 1 h. The sections were then developed for 15 min with AEC (3-amino-9-ethylcarbazole). Sections were counterstained with Mayer's hematoxylin and mounted in equal volumes of glycerol and PBS.

Immunofluorescent localization of Sp17 was carried out on capacitated, noncapacitated, and calcium ionophore (A23187)-treated mouse spermatozoa. Spermatozoa were capacitated in BWW medium [16] containing 2% BSA at a concentration of 2 × 10^6 sperm/ml for 1.5 h at 37°C in a humid atmosphere of 5% CO_2:95% air. Ionophore treatment (15 μM final concentration) of capacitated spermatozoa was for an additional 15 min of incubation. Live labeled spermatozoa were treated directly with either anti-G22C antiserum or control preimmune serum before washing and fixation. Spermatozoa were washed in PBS by centrifugation at 500 × *g*, fixed in 1% formaldehyde or 2.0% paraformaldehyde for 30 min on ice, and washed and resuspended in PBS. Biotin-labeled goat anti-rabbit IgG (1:250; 1 h) was used for second antibody labeling, followed by avidin-

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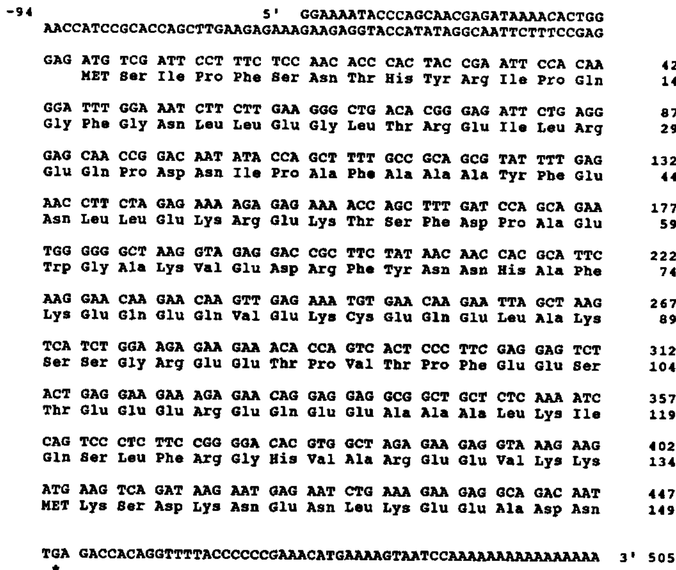

FIG. 1. Nucleotide and deduced amino acid sequence of mouse Sp17. 5′ untranslated region contains 94 nt, −94 to −1. Nucleotides 1 to 447 encode the open reading frame. This sequence is available from EMBL/GenBank/DDBJ under Accession No. Z46299. *, stop codon.

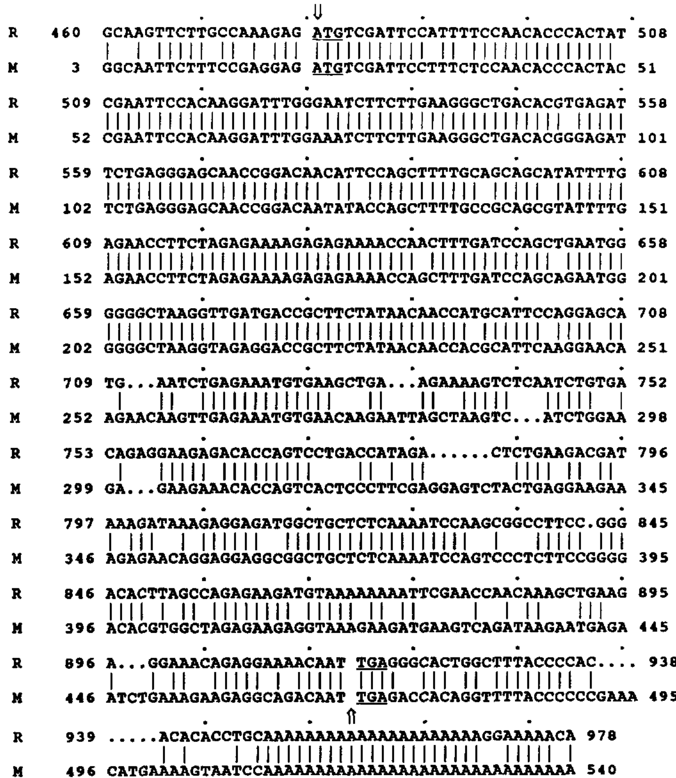

FIG. 2. Comparison of rabbit (R) and mouse (M) Sp17 cDNA sequences. They are 82% identical. ⇓ and ⇑ indicate beginning and end of protein coding region. Start and stop codons are underlined. The identity in the 5′ untranslated region (19 nt) is within the region common to both mRNAs in the rabbit sequence, and the identity in the 3′ untranslated region occurs upstream of the internal poly(A) tail region seen in the rabbit sequence.

Texas red (1:200; 30 min). Finally, spermatozoa were placed on a microscope slide in Vectashield mounting medium (Vector Laboratories, Inc.), coverslipped, and viewed with a Zeiss microscope (Thornwood, NY).

Autoimmunogenicity. To examine the autoimmunogenicity of MSp17, an analysis was carried out of the linear B-cell epitopes on the Sp17 molecule recognized by male (B6AF1) mouse anti-whole sperm and anti-MSp17. Antisera were prepared as previously described [17]. This epitope analysis used the peptide pin-block method (mimotope analysis; Chiron Mimotopes Peptide Systems) in which the 149 amino acids of mouse Sp17 were divided into 47 sequential but overlapping (7-amino acid overlap) decapeptides. Two copies of each decapeptide were synthesized attached to the pin-block so that a duplicate determination could be made within the same 96-well plate. The pin-block also contained positive and negative control peptides. The procedures used in this study and their application to sperm antigens have been described in detail [17].

RESULTS

Cloning and Sequencing of Mouse Sp17

All 24 clones selected from the mouse testis cDNA library strongly hybridized to the rabbit Sp17 probe. Four of the clones were sequenced, and a consensus sequence for mouse Sp17 was constructed (Fig. 1). The sequence contained 599 nt, 94 in the 5′ untranslated region, 447 in the open reading frame, and 58 in the 3′ untranslated region. Reverse transcription (RT)-PCR of mouse testis mRNA using the 5′ P1 and P2 primers (see *Materials and Methods*) did not reveal any additional upstream sequence. Likewise, RT-PCR of mouse testis mRNA using the 3′ primer (see *Materials and Methods*) did not reveal any additional downstream sequence. The mouse, unlike the rabbit, does not appear to contain two different mRNAs for Sp17. Additionally, unlike the rabbit sequence, the mouse sequence does not appear to have the AATAAA polyadenylation signal upstream from the poly(A) tail.

The MSp17 open reading frame encodes a polypeptide of 149 amino acids with a calculated molecular weight of 17 296 and a predicted pI of 4.6. When the MSp17 sequence is compared to the rabbit Sp17 (RSp17) sequence, there is an 82% identity (Fig. 2). This identity is continued for a short distance upstream (19 nt) and downstream (58 nt) of the coding region. Interestingly, the identity in the 3′ untranslated region is contained upstream of the internal poly(A) region seen in the rabbit sequence [1]. When the deduced protein sequence of MSp17 is compared to RSp17, a 74% identity is seen (Fig. 3). Remarkably, the first 53 amino acids are identical in both species. Consequently, as seen in the rabbit, if the MSp17 protein sequence is compared with other protein sequences in the Swiss-Prot database [6], then

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R     1 MSIPFSNTHYRIPQGFGNLLEGLTREILREQPDNIPAFAAAYFENLLEKR 50
        ||||||||||||||||||||||||||||||||||||||||||||||||||
M     1 MSIPFSNTHYRIPQGFGNLLEGLTREILREQPDNIPAFAAAYFENLLEKR 50

R    51 EKTNFDPAEWGAKVDDRFYNNHAFQEHE.SEKCEAEEKSQSVTEEETPVL 99
        |||.||||||||||:||||||||||.|:|    |||| :|  ..|  .  |||||
M    51 EKTSFDPAEWGAKVEDRFYNNHAFKEQEQVEKCE.QELAKSSGREETPVT 99

R   100 TID..SEDDKDKEEMAALKIQAAFRGHLAREDVKKIRTNKAEEETEENN 146
        .::   .|::::.|| ||||||. ||||:|||:|||::.:|.|: .||.:
M   100 PFEESTEEEREQEEAAALKIQSLFRGHVAREEVKKMRSDKNENLKEEAD 148
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FIG. 3. Comparison of rabbit (R) and mouse (M) protein sequences. They are 74% identical. Vertical lines indicate identity and dots indicate similarity of amino acids. The first 53 amino acids are 100% identical.

there is a 45% identity to the human testis cAMP-dependent protein kinase type IIα regulatory subunit (HPKA). This identity is in amino acids 8–58 of MSp17 and amino acids 2–52 of HPKA. MSp17 also contains a putative calmodulin binding site (amino acids 110–139) that is 43% identical to the binding site found in neuromodulin [18].

Recombinant Mouse Sp17

Recombinant mouse Sp17 (rMSp17) was prepared in *Escherichia coli* M15[pREP4] transformed with pQE-30 and purified from the bacterial lysate as described in *Materials and Methods*. As shown in Figure 4, SDS-PAGE of rMSp17 under reducing conditions demonstrated a major protein staining band with an apparent molecular mass of 26 kDa

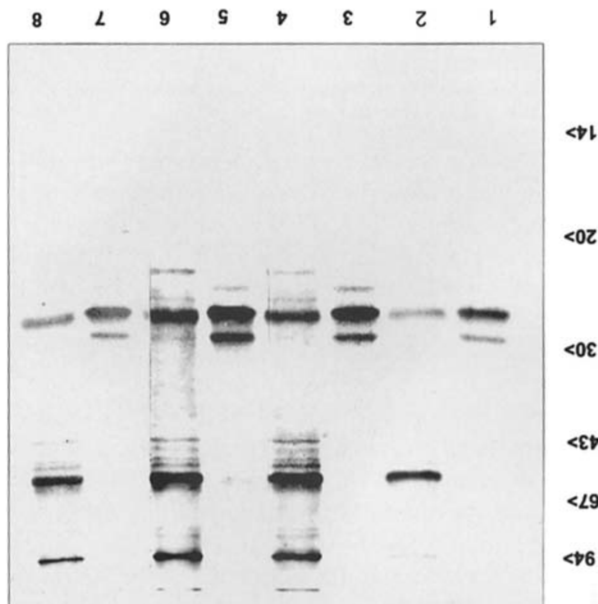
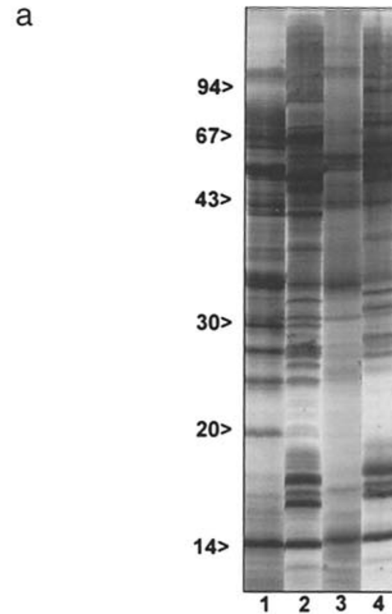


FIG. 4. Western blot analysis of recombinant mouse Sp17 after purification by nickel-NTA affinity chromatography. Two micrograms/lane loaded. In lanes 1, 3, 5, and 7, samples were reduced with β mercaptoethanol before SDS-PAGE. In lanes 2, 4, 6, and 8, samples were nonreduced. Lanes 1 and 2, amido black stain; lanes 3 and 4, immunostained with rabbit anti-RSp17; lanes 5 and 6, immunostained with mouse anti-MSp17; lanes 7 and 8, immunostained with mouse anti-RSp17. Molecular mass markers indicated in this and following figures are phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa.



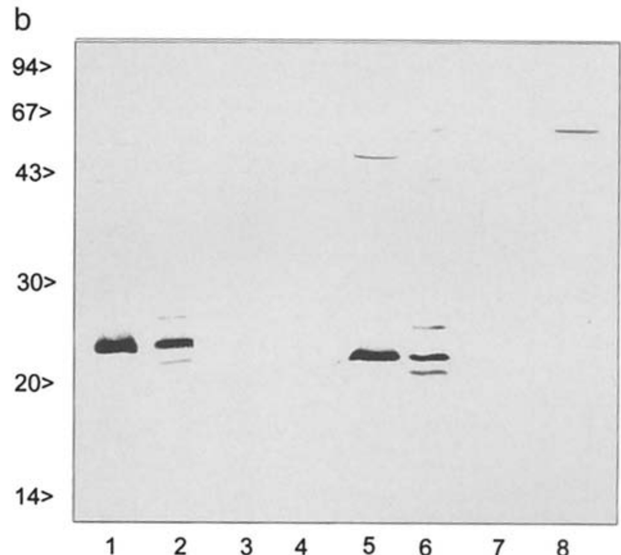


FIG. 5. Western blot analysis of mouse sperm and testis lysates. **a)** amido black staining. Lane 1, sperm lysate, reduced; lane 2, testis lysate, reduced; lane 3, sperm lysate, nonreduced; lane 4, testis lysate, nonreduced. **b)** immunostaining. Lanes 1–4, nonreduced; lanes 5–8 reduced. Lanes 1, 3, 5, and 7, sperm lysate; lanes 2, 4, 6, and 8, testis lysate. Lanes 1, 2, 5, and 6 were immunostained with rabbit anti-RSp17. Lanes 3, 4, 7, and 8 were stained with preimmune serum. See Figure 4 legend for explanation of molecular markers.

and a minor band of 29 kDa (Fig. 4, lane 1). Under nonreducing conditions, two major protein staining bands of 58 kDa and 26 kDa and a minor band of 93 kDa were observed (Fig. 4, lane 2). Western blots of the rMSp17 probed with either rabbit anti-RSp17 (Fig. 4, lanes 3 and 4), mouse anti-MSp17 (Fig. 4, lanes 5 and 6), or mouse anti-RSp17 (Fig. 4, lanes 7 and 8) demonstrated that the 29-kDa band seen under reducing conditions disappeared under nonreducing conditions and major antibody stained bands appeared at 58 kDa and 93 kDa.

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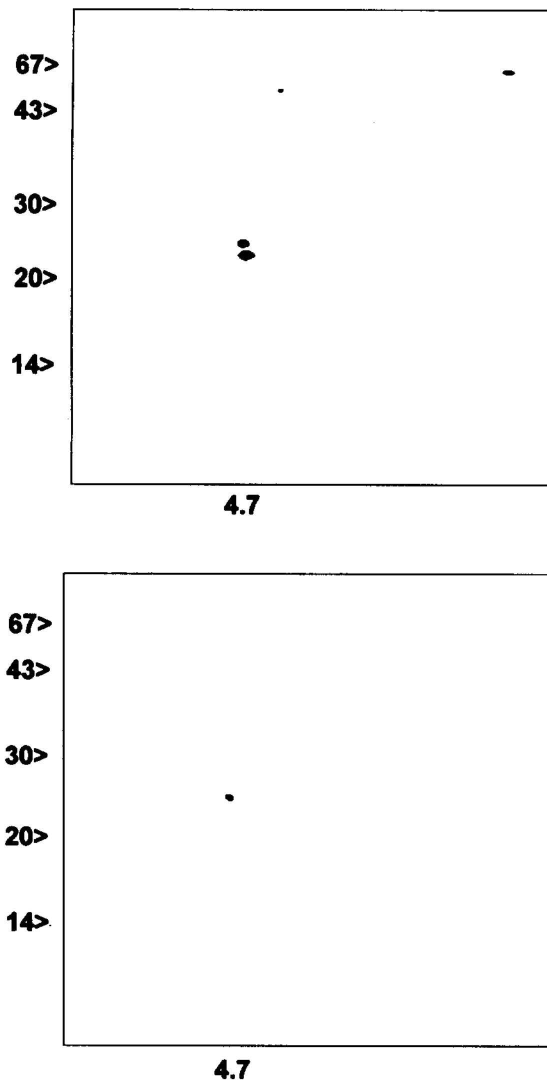

FIG. 6. Western blot analysis of mouse testis (top) and mouse sperm (bottom) lysates after 2-dimensional gel electrophoresis. Samples were isoelectric focused toward anode (top left of gel) in first dimension and by SDS-PAGE (reduced) in second dimension. See Figure 4 legend for explanation of molecular markers.

Native Mouse Sp17

To determine the presence of MSp17 in the testis and spermatozoa, Western blots of mouse testis (Fig. 5b, lanes 2 and 6) and mouse spermatozoa (Fig. 5b, lanes 1 and 5) were probed with rabbit anti-RSp17. Both testis and spermatozoa demonstrated an immunostaining band of 24 kDa, with minor bands in the testis of 23 kDa and 27 kDa. These bands were not stained by preimmune serum (Fig. 5b, lanes 3, 4, 7, and 8). Although occasional faint bands were seen at 43 and 56 kDa in spermatozoa under nonreducing conditions (Fig. 5b, lane 1), they were not present in testis (Fig. 5b, lane 2), and the lack of bands of higher molecular mass was in striking contrast to those observed in the recombinant MSp17 (compare Fig. 4, lane 4 with Fig. 5b, lanes 1 and 2). Western blots of two-dimensional gels of both mouse testis and mouse epididymal spermatozoa (Fig. 6) probed with rabbit anti-RSp17 showed reactivity with two spots at 24 kDa and 25 kDa in the testis and one spot at 24 kDa in spermatozoa. The measured pI of the spots in both the testis and spermatozoa was 4.7, while the predicted pI based on the deduced amino acid sequence for MSp17 was 4.6. These results from both testis and spermatozoa are similar to those reported for the rabbit [1]. In both cases, the observed molecular mass was not that predicted (deduced) on the basis of the amino acid sequence. Evidently both the acidic pI and the shape of the molecule influence its molecular size on SDS-PAGE [1].

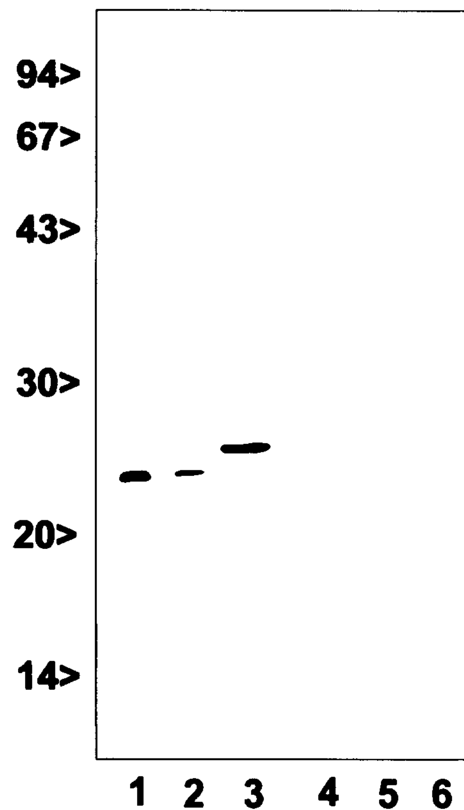

FIG. 7. Western blot analysis of recombinant mouse Sp17, mouse sperm, and mouse testis lysates immunostained with affinity-purified rabbit anti-peptide G22C antiserum. Lanes 1 and 4, sperm lysate; lanes 2 and 5, testis lysate; lanes 3 and 6, mouse recombinant Sp17, 2 µg/lane loaded. Lanes 1–3, immunostained with anti-peptide G22C; lanes 4–6, immunostained with normal rabbit IgG. See Figure 4 legend for explanation of molecular markers.

In the rabbit study [1], the sequence of the native Sp17 was confirmed by peptide sequence analysis after cyanogen bromide digestion as well as by Western blots probed with anti-peptide antibodies. In this study, the same anti-peptide antiserum, anti-G22C, made against amino acids 61–82 in the rabbit sequence (61–83 in the mouse; see Fig. 3) was used to probe Western blots of the MSp17 recombinant protein, mouse testis, and mouse spermatozoa. As shown in Figure 7, both spermatozoa (lane 1) and testis (lane 2) con-

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tained a major 24-kDa band. The recombinant bands at 26 kDa and 29 kDa (Fig. 7, lane 3) are shown for comparison. Additionally, lysates of mouse epididymal spermatozoa were probed on Western blots with a second anti-peptide antiserum, anti-K18C, made against amino acids 120–136 in the C-terminal region in the human sequence (118–134 in the mouse sequence, see Fig. 3). As shown in Figure 8 (lanes 7 and 8), spermatozoa contained only a 24-kDa band. Consequently, it may be concluded that MSp17 has a native apparent molecular mass of 24 kDa and contains the deduced protein sequence shown in Figure 3.

In a previous study with antibodies to the native rabbit RSA family, Richardson et al. [4] demonstrated not only a 24–25-kDa band but also additional protein staining bands of lower molecular mass, < 20 kDa in mouse epididymal spermatozoa. Therefore we asked if any of these previously observed bands of lower molecular size could be Sp17 breakdown products similar to those observed from native rabbit Sp17. Further experiments were conducted using mouse sperm lysates (containing twice the number of sperm, > 8×10^6 sperm/gel lane) prepared with and without a protease inhibitor cocktail (see *Materials and Methods*). As shown in Figure 8, lane 4, when enough sperm protein was loaded onto the gel, lysates prepared in the presence of inhibitors showed the major 24-kDa native Sp17 band as well as a weak band at 18 kDa. However, in lysates sonicated or homogenized before the addition of inhibitors or in samples completely lacking inhibitors, an 18-kDa band (17–19-kDa range) appeared (Fig. 8, lane 3), indicating that at least one of the lower molecular mass bands observed previously was a product of Sp17 breakdown. Interestingly, this 18-kDa proteolytic product did not stain with anti-K18C (lane 7), indicating that at least part of the C-terminal is lost as a result of proteolytic cleavage. The 18-kDa band did stain with anti-G22C antiserum (data not shown). Preimmune rabbit serum (lanes 1 and 2) and preimmune mouse serum (lanes 5 and 6) did not stain the sperm lysates.

To further confirm the presence of MSp17 in spermatozoa, a sperm lysate was prepared (see *Materials and Methods*) and mouse anti-RSp17 was used for immunoprecipitation. As shown in Figure 9, the single MSp17 band of 24 kDa, present in sperm lysates (lanes 3 and 4), was immunoprecipitated (lane 1), whereas control serum (preimmune) did not precipitate any proteins (lane 2).

Immunolocalization of Mouse Sp17

To determine the localization of MSp17 in spermatozoa, rabbit anti-RSp17 and rabbit anti-G22C were used as probes. Both antibody probes gave identical results. Live, capacitated mouse spermatozoa did not stain with either antiserum; however, live capacitated, ionophore-treated spermatozoa did stain, indicating that initially the polypeptide of Sp17 is not available to bind antibody on the surface of

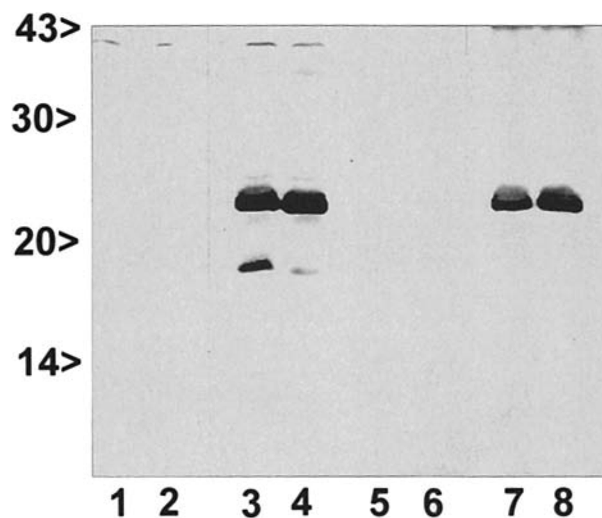

FIG. 8. Western blot analysis of mouse sperm lysates with and without protease inhibitors. 8×10^7 sperm/lane loaded. Lanes 2, 4, 6, 8, sperm lysates with protease inhibitors; lanes 1, 3, 5, 7, sperm lysates without protease inhibitors. Lanes 1 and 2, stained with rabbit preimmune serum (1:1000); lanes 3 and 4, immunostained with rabbit anti-RSp17 (1:1000); lanes 5 and 6, stained with mouse preimmune serum (1:200); lanes 7 and 8, immunostained with mouse anti-peptide K18C antiserum (1:200). Note that without inhibitors present an 18-kDa band appears (lane 3) that does not stain with anti-K18C (lane 7). See Figure 4 legend for explanation of molecular markers.

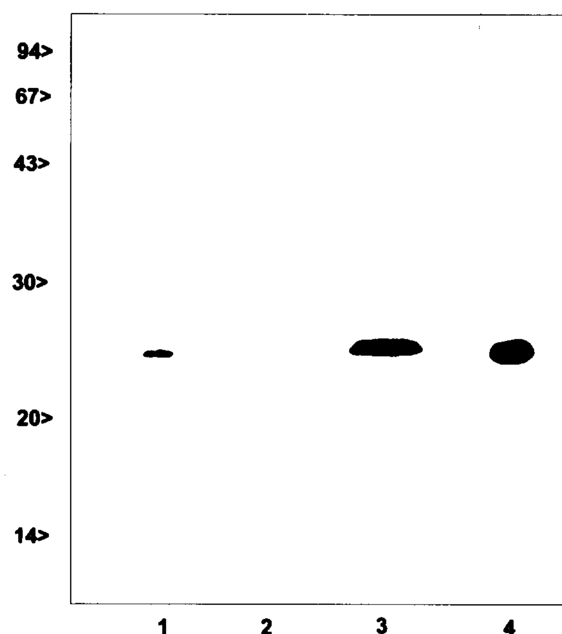

FIG. 9. Western blot analysis of immunoprecipitated mouse sperm Sp17. Lane 1, mouse sperm Sp17 immunoprecipitated with mouse anti-RSp17 and immunostained with rabbit anti-RSp17; lane 2, immunoprecipitation with mouse control serum and staining with rabbit anti-RSp17; lanes 3 and 4, sperm lysates immunostained with rabbit anti-RSp17. See Figure 4 legend for explanation of molecular markers.

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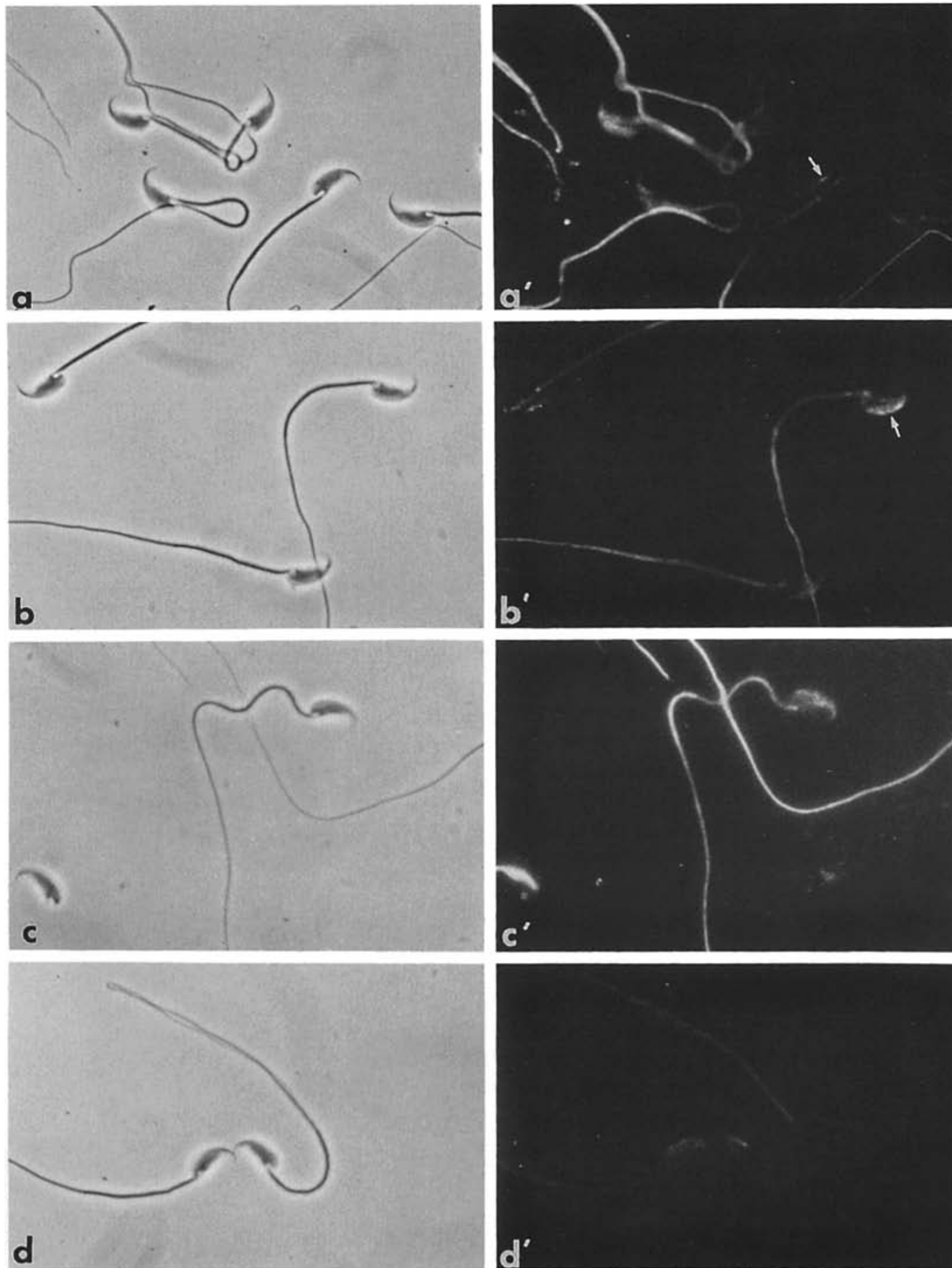

FIG. 10. Localization of Sp17 on capacitated mouse spermatozoa. **a-d**) Phase contrast images. **a'-d'**) Matching immunofluorescent images. Panels **a**, **a'**, **b**, and **b'** show paraformaldehyde-fixed spermatozoa labeled with rabbit anti-peptide G22C. Arrow in panel **a'**, small patch of label. Arrow in panel **b'**, band of fluorescence along acrosomal cap. Panels **c** and **c'** show ionophore-treated capacitated sperm labeled with rabbit anti-peptide G22C. Acrosomal staining is seen throughout equatorial region. Panels **d** and **d'** show spermatozoa labeled with preimmune serum. Original magnification 1000×; shown at 900×.

live spermatozoa but becomes available as the acrosome reaction begins. After paraformaldehyde fixation, more than 80% of capacitated or noncapacitated spermatozoa stained with both antisera. The results of staining with anti-G22C are shown in Figure 10. Figure 10a, a', b, and b' demonstrate the typical patterns observed. Staining was observed along the length of the principal piece, weakly along the midpiece, and over the acrosomal region of the head. In > 50% of the spermatozoa the acrosomal fluorescence was a small patch or spot, while in 20–30% of the spermatozoa

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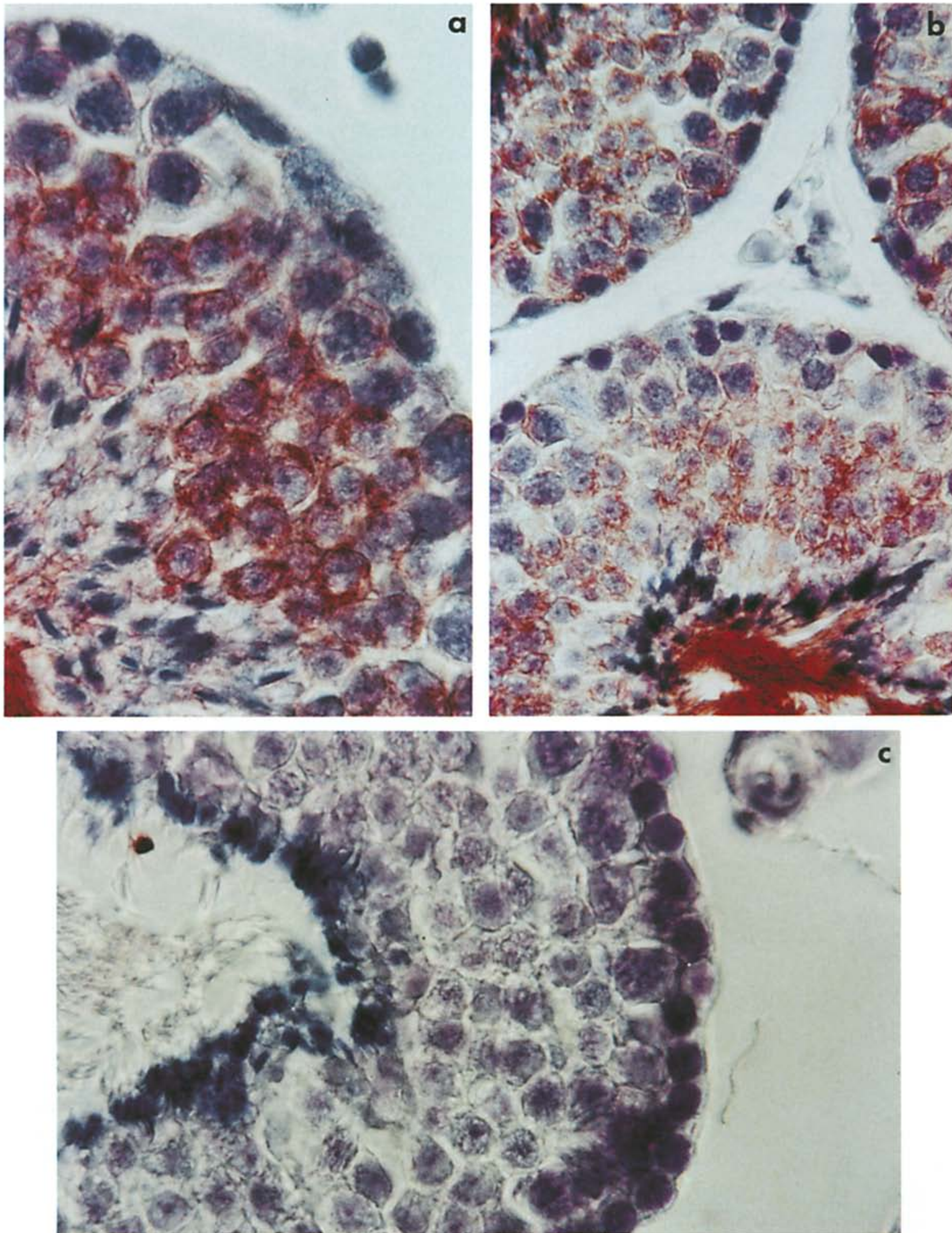

FIG. 11. Localization of Sp17 in mouse testis. **a** and **b**) Cross section of seminiferous tubule showing labeling of spermatocytes and spermatids with affinity-purified rabbit anti-peptide G22C. Note strong labeling of late spermatid tails in seminiferous lumen in **b**. Sertoli cells, Leydig cells, and peritubular cells are not labeled. **c**) Cross section of seminiferous tubule labeled with control affinity-purified anti-KLH serum. Original magnification 630×; shown at 590×.

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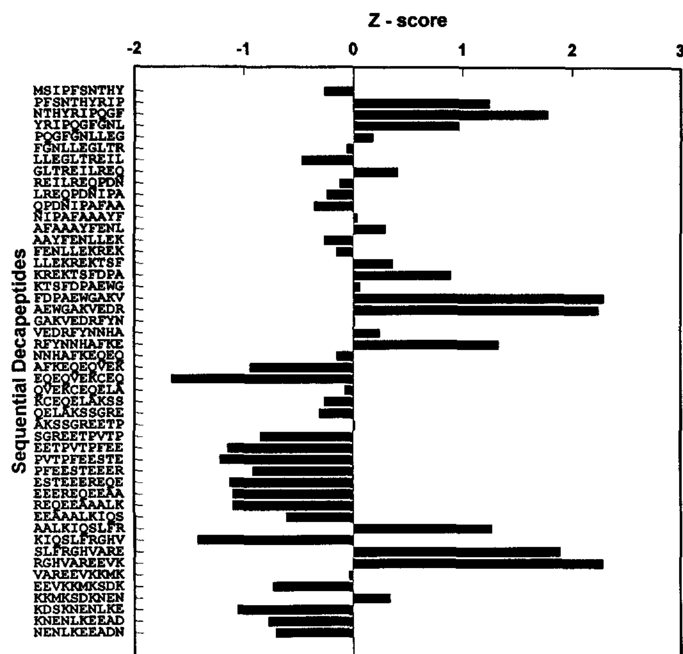

FIG. 12. Reactivity of mouse Sp17 decapeptides with male mouse anti-mouse sperm autoantibodies. Antiserum was diluted 1:5000 before use. Three major reactive regions within Sp17 sequence are 1) PFS..QGF (amino acids 4–16), 2) KRE..FKE (amino acids 49–76), and 3) SLF..EVK (amino acids 121–133). Control values for conjugated anti-mouse second antibody have been subtracted. Z score = individual peptide pin reactivity (o.d. @ 450 nm) – mean reactivity for all peptides/ standard deviation for antibody used [26].

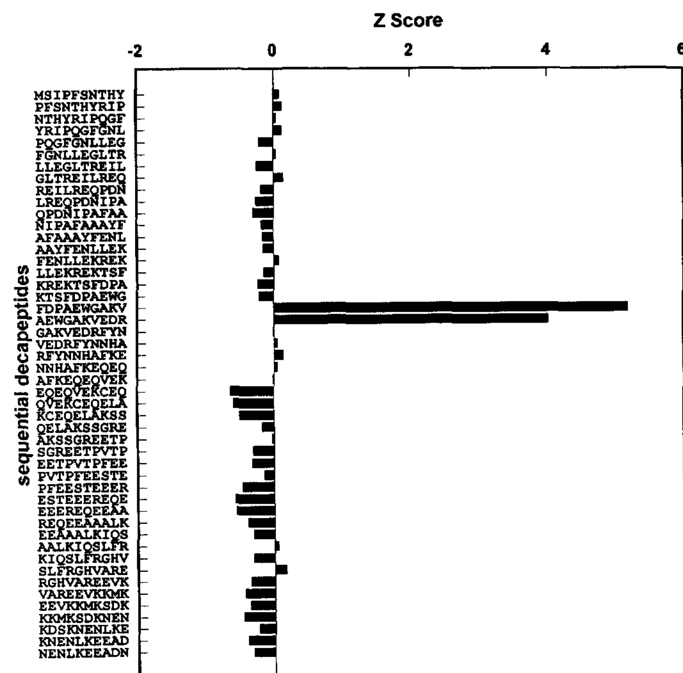

FIG. 13. Reactivity of mouse Sp17 decapeptides with male mouse anti-recombinant mouse Sp17 autoantibodies. Antiserum was diluted 1:5000 before use. Major reactive regions within Sp17 sequence are FDPAEWGAKV and AEWGAKVEDR. Control values for conjugated anti-mouse second antibody have been subtracted. For Z score see Figure 12 legend.

the acrosomal fluorescence was a band along the acrosomal cap (Fig. 10b′) and usually did not extend into the equatorial segment of the acrosome, which is quite large in the mouse (cf. [19], Fig. 15). After ionophore A23187 treatment, > 95% of the spermatozoa stained with anti-G22C, and the staining was observed along both the principal piece and middle piece (Fig. 10c′). The acrosomal staining now appeared throughout the equatorial region of the acrosome (Fig. 10c′) and was more clearly seen in detached sperm heads (Fig. 10c′). Only faint staining could be seen with preimmune or adjuvant control serum (Fig. 10, d and d′).

Localization of Sp17 in mouse testes with anti-G22C antiserum revealed that Sp17 was present in the cytoplasm of spermatocytes and spermatids (Fig. 11). The tails of late spermatids in the lumen of the seminiferous tubules were strongly labeled (Fig. 11b), similar to the reactivity observed on epididymal spermatozoa (Fig. 10). Sertoli cells and peritubular cells as well as Leydig cells and other somatic cells were not labeled with anti-G22C (Fig. 11, a and b). Preimmune serum did not label any cell types (Fig. 11c).

Autoimmunogenicity of Mouse Sp17

Finally, the autoimmunogenicity of mouse Sp17 was investigated because one characteristic of the RSA family of sperm antigens in the rabbit is their autoantigenicity. To determine whether or not autoimmunogenicity is also characteristic of mouse Sp17, male B6AF1 mice were immunized with either whole mouse epididymal spermatozoa or mouse recombinant Sp17. Sera from mice immunized with epididymal spermatozoa were used to epitope-map any linear B-cell epitope(s) of MSp17 that might be recognized by the autoantisera. As shown in Figure 12, there appeared to be three major regions within the Sp17 sequence that are strongly immunogenic: 1) PFS..QGF (amino acids 4–16, Fig. 3), 2) KRE..FKE (amino acids 49–76, Fig. 3), and 3) SLF..EVK (amino acids 121–133, Fig. 3). These three regions overlap with three of the four regions mapped in RSp17 by male rabbit anti-rabbit sperm autoantiserum [17]. To determine whether similar epitope(s) would be recognized using the recombinant protein, sera pooled from six male mice immunized with recombinant MSp17 were used. As shown in Figure 13, the male mice gave a very restricted antibody response to the mouse Sp17 peptide sequence but nevertheless recognized the decapeptide FDPAEWGAKV and, to a slightly lesser extent, the overlapping decapeptide AEWGAKVEDR. These two decapeptides contain the common linear autoantigenic B-cell epitopes of native and recombinant MSp17.

DISCUSSION

This study has described the sequence, localization, and autoimmunogenicity of the mouse sperm protein Sp17.

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MSp17 has several similarities to rabbit Sp17. The mRNA sequences of rabbit and mouse Sp17 are 82% identical, and the identity starts 19 nt upstream of the coding region and ends 58 nt downstream of the coding region. However, the mRNA for mouse Sp17 appears to be smaller than the rabbit mRNA (0.6–0.8 kb vs. 0.9 and 1.1 kb), and the mouse appears to lack two distinct mRNAs as seen in rabbits and humans [1, 20]. The mouse sequence itself lacks the poly(A) addition signal AATAAA seen in rabbit and human Sp17 but may use the alternative signal ATGAAA [21]. The significance of the lack of this AATAAA poly(A) signal, compared to rabbit and human, and the lack of two distinct mRNAs with identical coding regions is unknown. The protein sequences of rabbit and mouse Sp17 have an overall identity of 74%, with the first 53 amino acids being 100% identical. This N-terminal region of Sp17, amino acids 8–58, has a 45% identity to the human testis cAMP-dependent protein kinase type IIα regulatory subunit (HPKA). The significance of this identity is unknown; however, the N-terminal of HPKA is thought to be involved in dimer formation of the regulatory subunits [22]. MSp17 also contains a possible calmodulin binding site (amino acids 110–139) that is 43% identical to the binding site found in neuromodulin [18]. While rabbit recombinant Sp17 appears to bind calmodulin [23], we have not tested the mouse recombinant protein. The similarity of the MSp17 sequence to the rabbit Sp17 sequence and the binding of two different anti-peptide antibodies to both recombinant and native Sp17 (one to a peptide in the middle of the sequence and one to a peptide in the C-terminal region) lead us to conclude that the native 24-kDa protein identified in spermatozoa and testis contains the sequence deduced from the mRNA sequence (Figs. 1 and 3).

The presence of mouse Sp17 in spermatozoa and testis was demonstrated by Western blot analysis, immunoprecipitation, and immunolocalization. The observation that recombinant MSp17 (26 kDa) and native Sp17 (24 kDa) appear, after SDS-PAGE, to have apparent molecular weights greater than the deduced molecular weight (17 296) is consistent with studies on the rabbit Sp17. In both cases, the apparent molecular weight is greater than the deduced molecular weight. Because this occurs in the expressed bacterial protein, it is unlikely to be due to glycosylation and is most likely due to the net negative charge of the protein as well as its shape, both of which would affect its electrophoretic migration. Preliminary experiments indicate that neither phosphorylation nor dephosphorylation is responsible for the anomalous migration. Another interesting feature that we observed after nonreducing SDS-PAGE is the marked difference between native and recombinant Sp17 in their ability to form dimers or higher molecular weight aggregates (compare Fig. 4 and Fig. 5b). The most obvious reason for this would be a modified cysteine residue, perhaps via palmitylation. Studies to investigate this are currently underway.

The observation that sperm preparations that lack a cocktail of protease inhibitors contain protein bands of lower molecular size (< 20 kDa) is of interest because of our previous observations that 1) they are present in mouse epididymal sperm preparations, 2) they stain with antiserum to native rabbit RSA [4], and 3) they bind zona pellucida proteins [24]. This study has demonstrated that 18-kDa Sp17 can be only weakly detected in spermatozoa when protease inhibitors are present, but if they are not present in sonicates or homogenates of mouse epididymal sperm, then MSp17 is proteolytically cleaved to remove a C-terminal fragment. The resulting N-terminal protein has an apparent molecular mass of 17–19 kDa. The function(s) of 24-kDa Sp17 and its processed form (18 kDa) in the mouse spermatozoon has not been addressed in this study. Functional studies will be the subject of a subsequent publication. It should be noted that both the rabbit and human recombinant Sp17 have been shown to bind zona pellucida, dextran sulfate, and fucoidin [1, 20], as has native rabbit RSA [2]. We would expect that mouse recombinant Sp17 would also bind to these ligands given the close sequence similarities. However, the more important and more difficult question to address is the physiological role of Sp17 in the spermatozoon. In this regard, the localization results (Fig. 10) indicate that the Sp17 polypeptide is not available to bind antiserum on live, capacitated spermatozoa and is not inside the acrosome. Sp17 is, however, present on the equatorial surface of live, acrosome-reacted spermatozoa, indicating that it probably moves to the surface at some time immediately following the initiation of the acrosome reaction. This could be as early as cumulus passage, when progesterone may prime the surface in preparation for interaction with the zona pellucida [25]. It is during this period that changes (e.g., proteolytic cleavage, phosphorylation) might be expected to occur to Sp17 that would affect its function.

The autoimmunogenicity of MSp17 in males has been clearly demonstrated by mimotope analysis (Figs. 12 and 13). Although this method does not identify conformational epitopes, it clearly indicates that the male immune system recognizes native Sp17 in spermatozoa and a very limited region of the Sp17 recombinant molecule. The three major regions recognized by male mouse anti-sperm antibodies overlap with three of the four regions recognized in rabbit Sp17 by antiserum from male rabbits immunized with their own whole spermatozoa [17]. Thus, even though there are differences between mouse and rabbit Sp17, there seem to be common linear epitopes that are recognized as autoantigenic and that may prove to be important for the function of Sp17 during fertilization.

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