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Processing of the Sperm Protein Sp17 during the Acrosome Reaction and Characterization as a Calmodulin Binding Protein

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In this study we have demonstrated that the native rabbit sperm protein, Sp17, is a 22- to 24-kDa triplet of proteins in washed ejaculated rabbit spermatozoa and is unaffected by capacitation. However, during the acrosome reaction, Sp17 is processed from a 22- to 24-kDa triplet of proteins to a triplet of proteins at 17–19 kDa by the removal of amino acids from the C-terminal. Recombinant rabbit Sp17 (rRSp17) can also be proteolytically processed by acrosome-reacted spermatozoa in a similar manner. Protease inhibitors prevent the proteolytic processing of Sp17. Both forms of native Sp17 remain associated with acrosome-reacted spermatozoa and are solubilized by ionic detergents. Previously, sequence analysis of Sp17 revealed that Sp17 amino acids 108–137 were 52% identical to the calmodulin binding domain of neuromodulin and contained an IQ motif found in other calmodulin binding proteins. In this study, a truncated recombinant Sp17, rRSp17CB, which lacks amino acids 118–146, including the potential calmodulin binding site, was made. Recombinant rabbit Sp17, but not rRSp17CB, binds to calmodulin in the presence of Ca^{2+} or EDTA, under reduced or nonreduced conditions in biotinylated-calmodulin overlay assays. In DSS crosslinker experiments, calmodulin bound to rRSp17 in a 1:1 ratio but not to rRSp17CB. Additionally, biotinylated rRSp17 interacts with native sperm calmodulin. We propose that the processing of native Sp17, by removing a C-terminal fragment during the acrosome reaction, might be a mechanism to regulate the calmodulin binding activity of Sp17 and provide calmodulin at specific sites after the acrosome reaction.
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Key Words: **Sp17; acrosome reaction; calmodulin binding; proteolytic processing.**

INTRODUCTION

Proteolytic processing is a mechanism to regulate the function and localization of many proteins during development, including sperm proteins that function during capacitation and fertilization. The processing may occur in the testis or during sperm passage through the epididymis. Mouse sperm fibrous sheath protein A kinase anchor protein (AKAP) is synthesized as a precursor pro-AKAP82 and proteolytically cleaved to a prodomain protein and AKAP82 (Carrera *et al.*, 1994). The processing may assist AKAP82 assembly into the fibrous sheath (Johnson *et al.*, 1997) and expose the RII (protein kinase A type II regulatory subunit) binding site in AKAP82 (Visconti *et al.*, 1997). Fertilin is proteolytically processed in the testis (α subunit) and epididymis (β subunit) (Blobel *et al.*, 1992). The cleavage event not only localizes and sequesters both subunits to the posterior head (Hunnicutt *et al.*, 1997) but also exposes the domain responsible for fusion to the egg plasma membrane (Lum and Blobel, 1997). Other sperm proteins such as Sp-10 (Foster *et al.*, 1994) and rat sperm surface antigen 2B1 (Jones *et al.*, 1996) are also processed during epididymal transit. Proteolytic processing also occurs during the acrosome reaction when hydrolytic enzymes are released from the acrosome. One prominent example is the serine protease acrosin. Acrosin is synthesized as an inactive proacrosin that is converted to the enzymatically active acrosin during the acrosome reaction (Baba *et al.*, 1989). A GPI-linked hyaluronidase, PH-20, is endoproteolytically processed during the acrosome reaction to expose its secondary zona pellucida (ZP) binding site (Hunnicutt *et al.*, 1996b). The processing of GPI-linked PH-20 also generates a soluble form of PH-20 that has hyaluronidase activity at acidic pH (Cherr *et al.*, 1996; Hunnicutt *et al.*, 1996a).

The sperm protein Sp17 has been cloned and sequenced from rabbit (Richardson *et al.*, 1994), mouse (Kong *et al.*,

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1995), human (Lea *et al.*, 1996), baboon (Adoyo *et al.*, 1997), and macaque (Lea *et al.*, 1998) testis. Sp17 is the designation for the 17-kDa member of the rabbit sperm autoantigen family of proteins (O'Rand *et al.*, 1988) and was first described as a ZP binding protein because of its ability to bind heat-solubilized rabbit ZP and dextran sulfate in ELISA (Richardson *et al.*, 1994). Sp17 is a cytoplasmic protein in acrosome-intact spermatozoa (Richardson *et al.*, 1994), but during the acrosome reaction binds rabbit ZP proteins R45 and R55 (Richardson *et al.*, 1994; Yamasaki *et al.*, 1995). Furthermore, when transfected into COS cells, Sp17 will bind rabbit ZP to the surface of the COS cell (Yamasaki *et al.*, 1995). Rabbit Sp17 is a polypeptide of 146 amino acids (aa) with a calculated molecular weight of 16,891 in which aa 8–58 are 43% identical to the human testis cAMP-dependent protein kinase type IIα (PKA) regulatory subunit and aa 108–137 are 52% identical to the calmodulin (CaM) binding site (aa 29–58) of human GAP-43. Sp17 also contains an "IQ motif," IQXXXRGXXXR, found in other CaM binding proteins (Cheney and Mooseker, 1992). Proteolysis of CaM binding proteins which leads to constitutively or irreversibly activated proteins or the removal of the CaM binding site is well documented (Wang *et al.*, 1989; Nairn and Picciotto, 1994; Barnes and Gomes, 1995). Consequently, the potential functions of the PKA and CaM binding domains, and whether Sp17 may be susceptible to proteolytic cleavage, are of interest in the context of sperm physiology and fertilization. In this study we investigated the potential CaM binding site of Sp17 to better understand its structure and function. We also examined the processing of Sp17 during sperm capacitation and the acrosome reaction.

MATERIALS AND METHODS

Materials

All chemicals and reagents were of molecular biology grade. Restriction enzymes, CaM, and protease inhibitors were purchased from Boehringer Mannheim (Indianapolis, IN). McCoy's 5A medium and biotinylated CaM were purchased from Gibco BRL, Life Technologies (Gaithersburg, MD). Crosslinkers DSS (disuccinimidylsuberate) and DSP (dithiobis(succinimidylpropionate)) and Ultralink Immobilized Streptavidin Plus were purchased from Pierce (Rockford, IL). All other chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO). DNA was sequenced by the dideoxy chain termination method using a Sequenase II kit (United States Biochemical Corp., Cleveland, OH) or at the UNC-CH Automated DNA Sequencing Facility on a Model 373A DNA sequencer (Applied Biosystems) using the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Heat-solubilized rabbit zonae (HSRZ) and mouse and rabbit antisera were made as described previously (Richardson *et al.*, 1994). Mouse anti-recombinant rabbit Sp17 (rRSp17) was made against the full-length rRSp17. Rabbit anti-R22C was made against human Sp17 aa 11–31. The epitope of mouse anti-recombinant rabbit Sp17-19K (rRSp17-19K, aa 61–146) was determined using mimotope analysis on a rabbit Sp17 peptide pin-block (Clayton, Victoria, Australia) as described by Lea *et al.* (1997).

Bacterial Expression and Purification of Recombinant Proteins

The coding region of Sp17 was cloned into the vector pQE-30 (Qiagen, Inc., Chatsworth, CA) and rRSp17 purified using the Ni-NTA column (Qiagen) as previously described (Richardson *et al.*, 1994). To make rRSp17CB (aa 1–117) and rRSp17-19K (aa 61–146), DNA was amplified by PCR using specific primers containing 5′ *Bam*HI and 3′ *Kpn*I restriction sites. The sense primer for rRSp17CB was 5′CGCGGATCCATGTCGATTCCATTTTCC3′ and the antisense primer was 5′CGGGGTACCTCAGATTTT-GAGAGCAGCCATCTCC3′. The sense primer for rRSp17-19K was 5′CGCGGATCCGGGGCTAAGGTTGATGACCGCTTC3′ and the antisense primer was 5′CGGGGTACCGCCAGTGCCCT-CAATTGT3′. The amplified PCR products were cloned into pQE-30 predigested with *Bam*HI and *Kpn*I, and the correctness of the inserts was verified by DNA sequencing. The recombinant DNA was transformed into cells and purified using the method stated above.

Electrophoresis and Blotting

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blots were carried out as previously described (Kong *et al.*, 1995). All Coomassie blue, amido black, and antibody (Ab) staining was done at room temperature. For Ab staining of native Sp17 from sperm lysates, blots were blocked with 3% BSA in TBS (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl) for 30 min, incubated with first Ab in 1% BSA in TBST (TBS with 0.05% Tween 20), and detected with alkaline phosphatase-conjugated goat anti-mouse Ig (immunoglobulin) or anti-rabbit IgG F_c (1:1000 dilution) (ICN, Costa Mesa, CA) using NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as a substrate.

Rabbit Spermatozoa Preparation

Rabbit spermatozoa were collected by artificial vagina. Only ejaculates that showed good motility (>80%) were used for further preparation. The number of spermatozoa and the percentage of acrosome-reacted spermatozoa were counted under the light microscope after each treatment (O'Rand and Fisher, 1987).

To prepare washed sperm lysates, ejaculates were washed with McCoy's 5A medium twice and centrifuged at 700*g* for 5 min and the sperm pellet was resuspended in sample buffer (SB) (Richardson *et al.*, 1994). The suspension (5×10^7 spermatozoa/70 μl SB) was sonicated for 20 s and shaken for 30 min at room temperature. The lysate was sonicated, rocked again, and centrifuged at 12,000*g* for 5 min at room temperature. The supernatant was boiled for 5 min and examined by SDS–PAGE.

To make capacitated sperm lysates, rabbit ejaculates were centrifuged through a Percoll gradient according to the method described previously (O'Rand and Fisher, 1987), and lysates were made as stated above. To induce the acrosome reaction, capacitated rabbit spermatozoa were suspended in complete McCoy's medium (0.18 mg/ml sodium pyruvate and 0.5 mg/ml BSA in McCoy's 5A medium) and treated with Ca^{2+} ionophore A23187 (100 μM for 30 min or 5.5 μM for 3 h) or with HSRZ (3 h) at 37°C in a humid CO_2 incubator (5% CO_2:95% air) (O'Rand and Fisher, 1987). For treatment control, DMSO (dimethyl sulfoxide) was added to capacitated spermatozoa under the same conditions. To obtain a higher percentage of acrosome-reacted spermatozoa in the preparation, gentle homogenization of capacitated spermatozoa was performed as described by O'Rand and Metz (1976).

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To examine the location of Sp17 after the acrosome reaction, capacitated, Ca^{2+} ionophore A23187-induced acrosome-reacted spermatozoa were centrifuged at 700*g* for 5 min. The supernatant was mixed with SB, boiled for 5 min, and loaded on the gel. Pellets containing acrosome-reacted spermatozoa were treated with different detergents in buffers containing 50 mM Tris–HCl, pH 7.5, and 150 mM NaCl and rocked for 30 min at room temperature. After centrifugation (700*g*, 5 min), the supernatant of the detergent extraction was resuspended in SB, boiled, and examined by SDS–PAGE.

Immunoprecipitation

Rabbit spermatozoa were collected and washed as described above. Immunoprecipitation was performed following the procedure described previously (Kong *et al.*, 1995) using mouse anti-rRSp17, preimmune serum, or anti-KLH (keyhole limpet hemocyanin) control serum.

Processing of rRSp17 by CHAPS-Treated Spermatozoa

Washed rabbit spermatozoa were treated with CHAPS/Tris buffer (50 mM Tris, pH 7.4, 150 mM NaCl, and 50 mM CHAPS (3-[(cholamidopropyl)dimethylammonio]-1-propane sulfonate)) for 30 min and the percentage of acrosome-reacted spermatozoa was determined at the end of the incubation. Using this procedure, sperm preparations that were >90% acrosome reacted were obtained. Ten micrograms of rRSp17 was added to 5×10^7 spermatozoa in CHAPS/Tris buffer and rocked for 10, 20, or 30 min at room temperature. For controls, rRSp17 was incubated in CHAPS/Tris buffer without spermatozoa or with spermatozoa treated with Tris buffer only (no CHAPS; 50 mM Tris, pH 7.4, and 150 mM NaCl) for 30 min. For inhibition experiments, rRSp17 was incubated with CHAPS/Tris-treated spermatozoa in the presence of 0.1× protease inhibitors (2 μg/ml Pefabloc SC, 1 μg/ml leupeptin, and 0.2 μg/ml aprotinin), 1× protease inhibitors, 10× protease inhibitors, 0.1 mM benzamidine, 1 mM benzamidine, or 10 mM benzamidine for 30 min at room temperature. At the end of the incubation, all samples were centrifuged at 700*g* for 5 min and the supernatant was resuspended in SB and boiled for Western blotting after SDS–PAGE. Blots were stained with amido black. To examine whether rRSp17 fragments had intact N-terminals, selected amido black-stained lanes were cut in half, reprobed with mouse monoclonal ^{MRGS}·His Ab (Qiagen) and compared to the original amido black staining.

Biotinylated-CaM Overlay Assay

The CaM overlay method was modified from Baum *et al.* (1993). rRSp17 and rRSp17CB were electrophoresed on 10–20% Tris-Glycine Ready gels (Bio-Rad, Hercules, CA) under reducing or nonreducing conditions and Western blotted. The overlay assays were carried out at room temperature. For incubation in the presence of calcium, the blot was blocked in buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, and 1% nonfat milk for 30 min and then incubated overnight in the same buffer with 1μg/ml biotinylated CaM. Blots were washed in buffer (three times, 5 min each), incubated in peroxidase-conjugated neutrolite-avidin (1:1000, 1 h; Molecular Probes, Inc., Eugene, OR), and washed (five times in the buffer without 1% non-fat milk), and the signal was detected with ECL (enhanced chemiluminescence; Amersham, Arlington Heights, IL). For incubations in the presence of EDTA, the same method was used except the buffer was 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 2 mM EDTA.

Crosslinking of rRSp17, rRSp17CB, and CaM

Recombinant RSp17 (400 pmol), rRSp17CB (400 pmol), and CaM (800 pmol) were incubated together in PBS for 45 min at room temperature. DMSO or DSS dissolved in DMSO (final concentration 0.5 mM) was added to the solution and the incubation continued for another 30 min. The reaction was stopped by adding 1 M Tris–HCl, pH 7.5 (final concentration 40 mM) for 15 min. Results were examined by PAGE on a 10–20% Tris-Glycine Ready gel and stained with Coomassie blue.

Interaction of rRSp17 and Native Sperm CaM

To investigate the interaction of rRSp17 with native sperm CaM, a modified method from Gong *et al.* (1995) was used. Recombinant RSp17 was biotinylated using EZ-Link NHS-biotin (Pierce). Washed rabbit spermatozoa were extracted with TSA (50 mM Tris, pH 7.5, 150 mM NaCl, 0.02% sodium azide, and protease inhibitors; Kong *et al.*, 1995) containing 0.2% NP-40 for 1 h at 4°C. After centrifugation, the supernatant was collected and incubated overnight at 4°C with an equal volume of TSA containing 20 μg biotinylated-rRSp17. DSP (final concentration 2 mM) was then added for 1 h. Biotinylated rRSp17 and any associated proteins were precipitated by adding streptavidin. The precipitate was washed with TSA three times, suspended in SB containing 5% 2-mercaptoethanol, boiled, analyzed by SDS–PAGE, and Western blotted. The blot was stained with monoclonal anti-CaM (Upstate Biotechnology, Lake Placid, NY) and incubated in peroxidase-conjugated goat anti-mouse Ig (1:1500; ICN) and the signal was detected with Chemiluminescence Reagent *Plus* (NEN Life Science, Boston, MA).

RESULTS

Immunoprecipitation of Native Rabbit Sp17

To study native RSp17, mouse anti-rRSp17 was used to immunoprecipitate Sp17 from ejaculated rabbit spermatozoa. A triplet of proteins of 22–24 kDa and a faint band of aggregated Sp17 (asterisk) were immunoprecipitated by mouse anti-rRSp17 (Fig. 1, lane 3) but not by mouse anti-KLH (lane 2) or by preimmune serum (lane 1). The triplet of native rabbit Sp17 proteins is similar to those reported previously by 2-D PAGE of rabbit spermatozoa (Richardson *et al.*, 1994) as well as to those reported in human sperm lysates (Lea *et al.*, 1996).

Sp17 during Capacitation and the Acrosome Reaction

After capacitation, Sp17 remained as a 22- to 24-kDa triplet (Fig. 2A, lane 2). However, when capacitated spermatozoa were treated with Ca^{2+} ionophore A23187 (37% acrosome reacted), a 17- to 19-kDa triplet was generated (lanes 3 and 4). The 17- to 19-kDa triplet was present under both reducing and nonreducing conditions (lanes 3 and 4);

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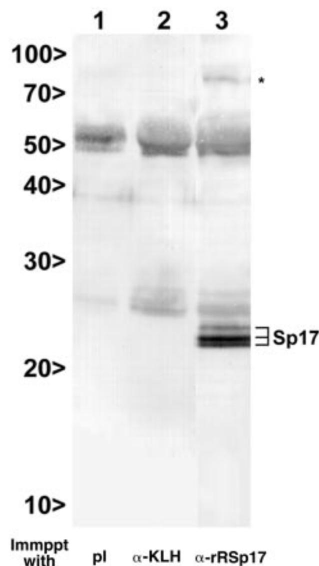

FIG. 1. Western blot of Sp17 immunoprecipitated from washed rabbit spermatozoa. Sperm lysate was immunoprecipitated with mouse anti-rRSp17 preimmune serum (lane 1), mouse anti-KLH (lane 2), and mouse anti-rRSp17 (lane 3). The blot was stained with rabbit anti-rRSp17 (1:4000). Immunoglobulin heavy and light chains were present in all three lanes. The asterisk indicates the aggregated Sp17 that was immunoprecipitated by mouse anti-rRSp17. Molecular weight marker is the 10-kDa protein ladder from Gibco BRL.

however, it was not observed without Ca^{2+} ionophore (DMSO only, 3% acrosome reacted) present in the incubation medium (lanes 5 and 6), indicating that the triplet was generated during the acrosome reaction. Decreasing amounts of the 22- to 24-kDa triplet and increasing amounts of the 17- to 19-kDa triplet as well as smaller molecular weight bands were observed when the percentage of acrosome-reacted spermatozoa was increased to 99% (lane 7). The same processing is observed in HSRZ-induced acrosome-reacted spermatozoa (Fig. 2B, lanes 1 and 2). Consequently, it would appear that Sp17 is processed during the acrosome reaction.

Three antibodies were used to analyze the 17- to 19-kDa triplet: anti-rRSp17 (full-length rRSp17), anti-R22C (Sp17 aa 11–31), and anti-rRSp17-19K (Sp17 aa 61–146). All three antibodies recognized the 22- to 24-kDa triplet (Fig. 3, lanes 2, 3, and 4); however only anti-rRSp17 and anti-R22C recognized the 17- to 19-kDa triplet (lanes 2 and 4). Anti-rRSp17-19K did not recognize the 17- to 19-kDa triplet (lane 3). Mimotope analysis of mouse anti-rRSp17-19K showed that the antibody recognizes the region FRGHLAREDVKKIRTN (aa 121–136) with REDV (aa 127–130) as the dominant epitope (data not shown). This indicates that amino acids downstream of aa 121 are not present in the 17- to 19-kDa triplet. Preimmune serum or secondary antibody only did not recognize either triplet of proteins (lanes 1 and 5). Sp17 therefore appears to be processed from 22–24 to 17–19 kDa by the removal of a C-terminal fragment.

When capacitated, acrosome-reacted spermatozoa were separated into supernatant and pellet fractions by low-speed centrifugation (700*g*, 5 min), both 22- to 24- and 17- to 19-kDa triplets were found in the pellet but not in the supernatant fraction (Fig. 4, lane 1). Ionic detergents (lanes 2 and 4), but not nonionic detergents (lanes 3 and 5), were required to extract both forms of Sp17 from the acrosome-reacted sperm pellet, indicating that Sp17 remains tightly associated with acrosome-reacted spermatozoa.

Processing of rRSp17 by Acrosome-Reacted Rabbit Spermatozoa

To further characterize the processing of Sp17 by the removal of the C-terminal fragment, rRSp17 was added to acrosome-reacted rabbit spermatozoa to investigate whether the recombinant protein was susceptible to proteolytic cleavage. When rRSp17 was added to spermatozoa in CHAPS/Tris buffer (91% acrosome reacted), rRSp17 was

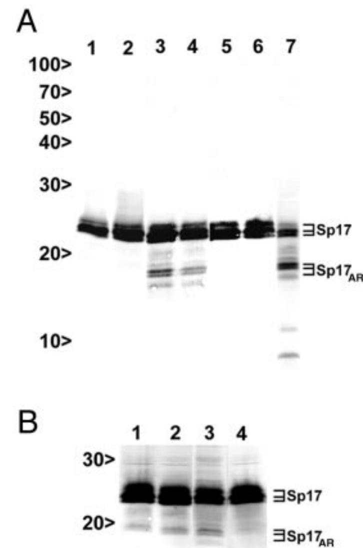

FIG. 2. Western blot of Sp17 in ejaculated, capacitated, and acrosome-reacted rabbit spermatozoa. (A) Lanes 1, 2, 3, 5, and 7 are reduced; lanes 4 and 6 are nonreduced. Lane 1, ejaculated spermatozoa; lane 2, capacitated spermatozoa; lanes 3 and 4, Ca^{2+} ionophore A23187-treated capacitated spermatozoa (37% acrosome reacted); lanes 5 and 6, DMSO-treated capacitated spermatozoa (control for ionophore treatment, 3% acrosome reacted); lane 7, spermatozoa acrosome reacted (99%) by gentle homogenization. (B) Lane 1, 3.3 μg HSRZ-treated capacitated spermatozoa; lane 2, 10 μg HSRZ-treated capacitated spermatozoa; lane 3, Ca^{2+} ionophore A23187-treated capacitated spermatozoa; lane 4, DMSO-treated capacitated spermatozoa. The blots were stained with mouse anti-rRSp17 (1:200). Sp17, 22- to 24-kDa triplet; $Sp17_{AR}$, 17- to 19-kDa triplet. See Fig. 1 for molecular weight markers.

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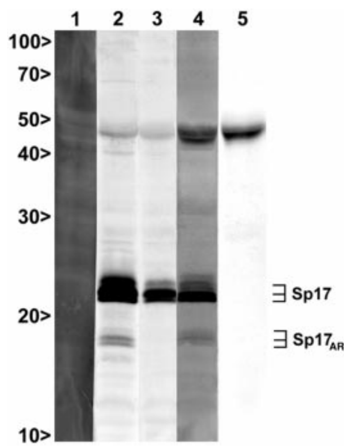

FIG. 3. Analysis of Sp17 22- to 24-kDa and 17- to 19-kDa triplets by immunostaining. Lane 1, stained with mouse preimmune serum (1:100); lane 2, stained with mouse anti-rRSp17 (1:200); lane 3, stained with mouse anti-rRSp17-19K (1:100); lane 4, stained with rabbit anti-R22C (1:100); lane 5, stained with goat anti-rabbit IgG F_c (1:1000) as a secondary Ab control. Rabbit immunoglobulin is seen in all lanes. Only mouse anti-rRSp17 and rabbit anti-R22C stained the 17- to 19-kDa triplet. Sp17, 22- to 24-kDa triplet; $Sp17_{AR}$, 17- to 19-kDa triplet. See Fig. 1 for molecular weight markers.

processed from 26 kDa to fragments of 17–19 and 11 kDa and three fragments less than 10 kDa (Fig. 5A, lanes 1–3). If rRSp17 was added to spermatozoa treated with Tris buffer only, no processing of rRSp17 was found (lane 10), except from spontaneous acrosome reactions (<15%). CHAPS does not cause the proteolytic process, because when rRSp17 was treated with CHAPS/Tris buffer in the absence of spermatozoa, rRSp17 remained intact (lane 11). In addition, the proteolytic processing of rRSp17 could be prevented if increasing amounts of protease inhibitors (lanes 4–6) or benzamidine (lanes 7–9) were added to the CHAPS/Tris buffer. Because rRSp17 has a 6-His tag attached to its N-terminal, mouse monoclonal ^{MRGS}·His Ab was used to examine whether these fragments retained the 6-His tag N-terminal. Fragments of 17–19 and 11 kDa had 6-His tag N-terminals while fragments that were less than 10 kDa were missing the 6-His tag N-terminal (Fig. 5B, lanes 1a and 2a). Thus, similar to native Sp17, rRSp17 must be processed from 26 kDa to fragments of 17–19 kDa by cleavage of the C-terminal. The 17- to 19-kDa fragments were further cleaved to generate the N-terminal 11-kDa fragment and the C-terminal fragments that were less than 10 kDa. Proteolytic enzymes from acrosome-reacted spermatozoa can process rRSp17 into smaller forms, and this process can be prevented by protease inhibitors. The same proteolytic enzymes may be responsible for the processing of native Sp17 during the acrosome reaction.

Calmodulin Binding

One important function of rabbit Sp17 may be to store CaM in its C-terminal binding site until the acrosome reaction begins. Since aa 108–137 of Sp17 contain a predicted CaM binding site (Fig. 6), we examined whether this region was functional in Sp17. To study CaM binding in rRSp17, a truncated recombinant rabbit Sp17 (rRSp17CB) which lacks aa 118–146 was constructed (Fig. 6). After SDS–PAGE, rRSp17CB was 20 kDa (lane 3, Figs. 7A and 7C) under reducing conditions and could only weakly form a dimer under nonreducing conditions (lane 3, Figs. 7B and 7D) in contrast to rRSp17 (lane 4, Figs. 7B and 7D).

The CaM binding activity of rRSp17 and rRSp17CB was analyzed with a biotinylated CaM overlay assay. In the presence of Ca^{2+} (Figs. 7A and 7B) or EDTA (7C and 7D), rRSP17 monomers and dimers (lane 2) bound biotinylated CaM. In contrast, rRSP17CB did not show any binding activity (lane 1, Figs. 7A, 7B, 7C, and 7D). Therefore, rRSp17 has a CaM binding site between aa 118 and 146.

The binding of rRSp17 and CaM was examined by using the crosslinker DSS. As shown in Fig. 8, rRSp17 can bind to CaM in a protein band that corresponds to 42 kDa (lane 6), indicating a 1:1 ratio of rRSp17 (26 kDa) and CaM (16 kDa). Since rRSp17 crosslinked to itself to form dimers and higher molecular weight forms (lane 4), the 42-kDa band was best observed when there was an excess amount of CaM present during the crosslinking incubation (lane 6). The 42-kDa band was not present in the CaM-only control (lanes 1 and 2), in the rRSp17-only controls (lanes 3 and 4), or in samples without DSS (lane 5). When rRSp17CB was incubated with CaM in the presence of DSS (lane 10), no crosslinked

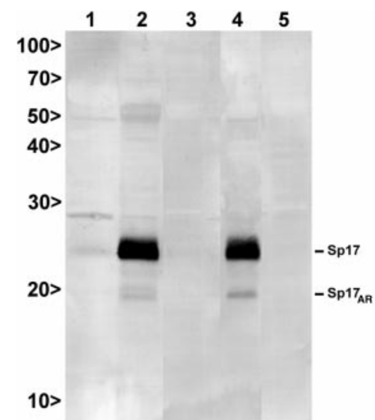

FIG. 4. Identification of Sp17 in capacitated acrosome-reacted spermatozoa. The blot was stained with mouse anti-rRSp17 (1:200). Lane 1, the supernatant fraction of 3×10^8 acrosome-reacted spermatozoa. Lanes 2–5, the pellet fraction of 5×10^7 spermatozoa was loaded per lane. Lane 2, extraction of Sp17 from the pellet with SB. Lane 3, extraction with buffers containing 0.1% NP-40. Lane 4, extraction with buffers containing 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100. Lane 5, extraction with buffers containing 1% Triton X-100. Only buffers containing ionic detergents were able to extract Sp17 from the pellet fraction of acrosome-reacted spermatozoa. Sp17, 22- to 24-kDa triplet; $Sp17_{AR}$, 17- to 19-kDa triplet. See Fig. 1 for molecular weight markers.

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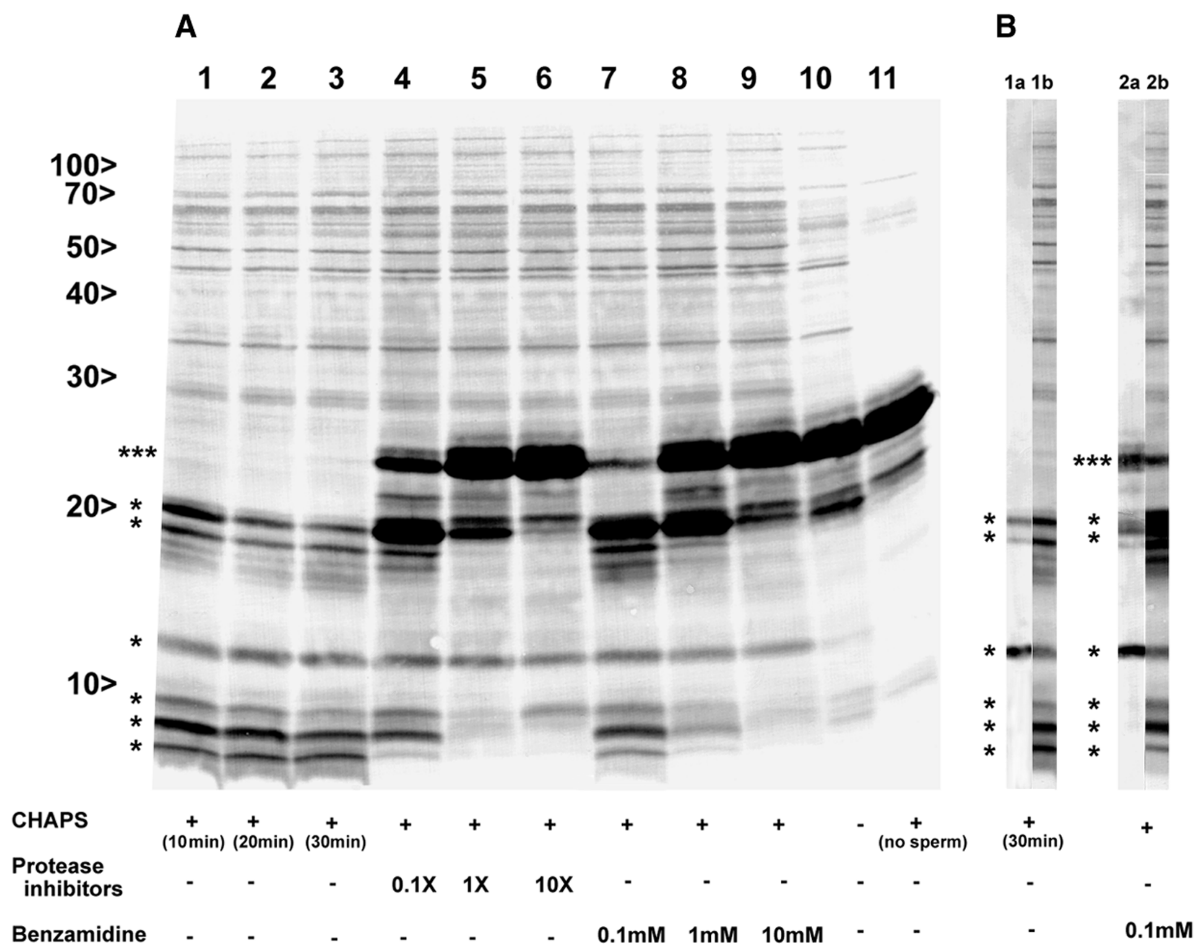

FIG. 5. Western blot of rRSp17 added to CHAPS/Tris buffer- or Tris buffer-treated rabbit spermatozoa. 10 μg rRSp17 was loaded per lane. (A) The blot was stained with amido black. Both rRSp17 and sperm proteins were stained. Lanes 1–3, rRSp17 in CHAPS/Tris buffer-treated spermatozoa for different time intervals. Lanes 4–6, rRSp17 in CHAPS/Tris-treated rabbit spermatozoa with increasing amounts of protease inhibitors. Lanes 7–9, rRSp17 in CHAPS/Tris buffer-treated spermatozoa with increasing amounts of benzamidine. Lane 10, rRSp17 in spermatozoa treated with Tris buffer only. Lane 11, rRSp17 in CHAPS/Tris buffer in the absence of spermatozoa. (B) Lanes 3 and 7 were cut in half and reprobed with mouse monoclonal ^{MRGS}·His Ab (lanes 1a and 2a) and compared to the original amido black staining (lanes 1b and 2b). Fragments that are less than 10 kDa and background sperm protein bands did not stain with the Ab. *** indicates full-length rRSp17; * indicates rRSp17 fragments generated after the acrosome reaction. See Fig. 1 for molecular weight markers.

products were observed compared to rRSp17CB-only control (lanes 7 and 8). Consequently it may be concluded that Sp17 aa 118 to 146 are required for CaM binding activity.

Sp17 Binding to Native CaM

To study the binding activity of Sp17 to native CaM, exogenous biotinylated-rRSp17 was added to sperm lysates and precipitated with streptavidin (Fig. 9). Examination of the precipitate with monoclonal anti-CaM antibodies demonstrated that CaM had been precipitated (lane 2). Addition of streptavidin to sperm lysates containing biotinylated BSA did not result in the precipitation of CaM (data not shown).

DISCUSSION

In this study, we have characterized native rabbit Sp17 during capacitation and the acrosome reaction. Immunoprecipitation of Sp17 demonstrated that it migrates as a 22- to 24-kDa triplet, as previously described by 2-D SDS–PAGE (Richardson *et al.*, 1994). The mechanism for the formation of the triplet is not known. Sp17 remains as a 22- to 24-kDa triplet during *in vitro* capacitation. No disulfide-bonded dimers of native Sp17 were observed under nonreducing conditions or in recombinant rabbit Sp17 expressed in COS cells (Yamasaki *et al.*, 1995). Thus, it would appear that the Cys 82 residue in native Sp17 is prevented from forming disulfide-bonded dimers.

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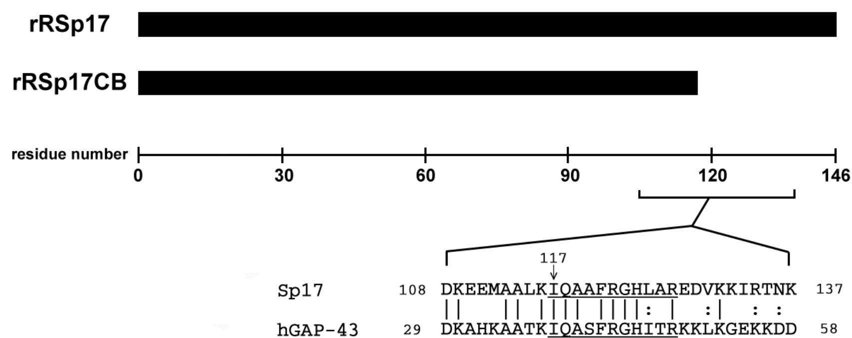

FIG. 6. Diagram of rRSp17 and rRSp17CB. rRSp17 is 146 aa in length; rRSp17CB is 117 aa in length, lacking the predicted CaM binding site, aa 118–146. Comparison of Sp17 and human GAP-43 is also shown. The symbol | represents identical residues and : represents conserved residues. The arrow indicates the C-terminal end of rRSp17CB. The IQ motif is underlined.

During the acrosome reaction Sp17 is processed from a 22- to 24-kDa triplet to a 17- to 19-kDa triplet and both forms appear to be anchored because they are tightly associated with acrosome-reacted spermatozoa and can only be extracted with ionic detergents. As the percentage of acrosome-reacted spermatozoa in the sample increased, the amount of the 17- to 19-kDa triplet also increased. The addition of rRSp17 to acrosome-reacted spermatozoa resulted in its processing from 26 to 17–19 kDa and 11 kDa and three fragments that were less than 10 kDa. Only fragments at 17–19 and 11 kDa retained the 6-His tag N-terminal, whereas fragments that were less than 10 kDa had lost both N- and C-terminals. The similar processing of native and recombinant Sp17 by spermatozoa suggests that the same cleavage sites are available and that the same proteolytic enzymes may be responsible for the processing. The origin of the proteases is unknown and could be either acrosomal, cytoplasmic, or both.

Using anti-peptide antibodies, we have demonstrated that native rabbit Sp17 is processed from 22–24 to 17–19 kDa by removing a C-terminal fragment. It is possible that the N-terminal aa 1–10 were also processed since anti-R22C would not recognize these aa of Sp17. However, if the same protease acted on both native and recombinant Sp17, then the N-terminal of native Sp17 would not be cleaved off because the N-terminal of rRSp17 was not cleaved. In mouse, immunoprecipitation of native Sp17 from testis and sperm lysates without protease inhibitors resulted in a major 24-kDa band and an additional band between 17 and 19 kDa (Kong *et al.*, 1995). The 17- to 19-kDa band was not

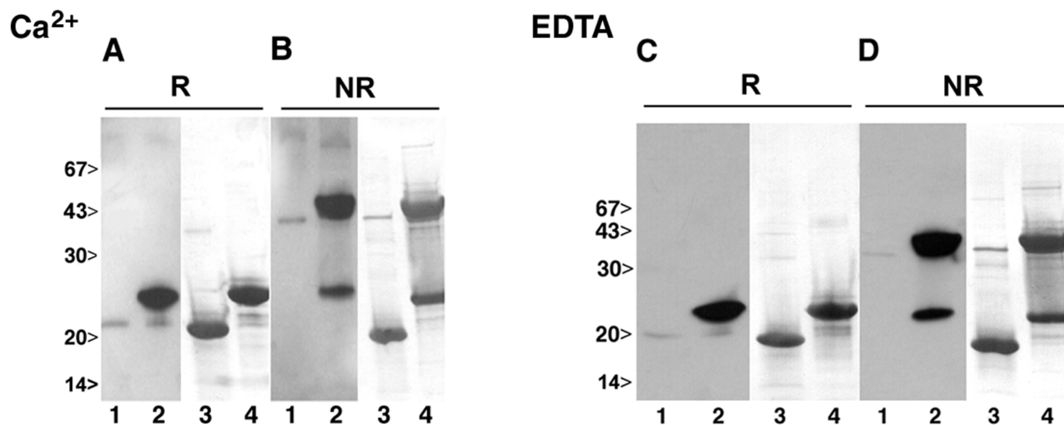

FIG. 7. Biotinylated CaM overlay assays of rRSp17 and rRSp17CB in the presence of 1 mM calcium (A and B) or 2 mM EDTA (C and D). Lanes 1 and 2 were Western blots probed with biotinylated CaM and detected by the ECL system on X-ray films; lanes 3 and 4 were amido black stained. Lanes 1 and 3, 5 μg of rRSp17CB; lanes 2 and 4, 5 μg of rRSp17. A and C, reducing gels; B and D, nonreducing gels. The molecular weight markers indicated are bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa.

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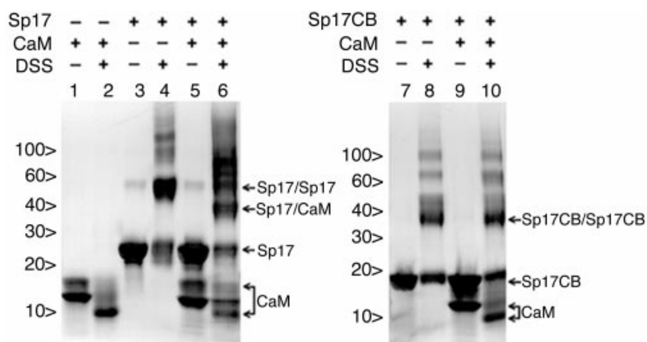

FIG. 8. Coomassie blue-stained SDS–polyacrylamide gel of rRSp17 and rRSp17CB crosslinked to CaM. Recombinant RSp17, rRSp17CB, and CaM were incubated together with or without DSS as indicated above each lane. Arrows indicate positions of CaM, rRSp17, rRSp17CB, and the 42-kDa complex of rRSp17/CaM. Note that CaM migrates faster on the gel in the presence of DSS. See Fig. 1 for molecular weight markers.

observed if protease inhibitors were present. This 17- to 19-kDa band, like that in the rabbit, was generated by removal of a C-terminal fragment as demonstrated by the absence of anti-K18C (aa 116–132) staining.

CaM is expressed abundantly in spermatozoa (Jones *et al.*, 1978) and along with Ca^{2+} mediates many sperm functions including the regulation of spermatogenesis (Sano *et al.*, 1987; Kägi *et al.*, 1988; Moriya *et al.*, 1993), sperm motility (Tash, 1989), capacitation (Adeoya-Osiguwa and Fraser, 1996), the acrosome reaction (Zaneveld *et al.*, 1991), and sperm–egg membrane fusion (Fouquet *et al.*, 1991; Courtot *et al.*, 1994). Several sperm CaM binding proteins have been identified by CaM overlay assays (Noland *et al.*, 1985; Olson *et al.*, 1985; Aitken *et al.*, 1988; Wasco *et al.*, 1989; Manjunath *et al.*, 1993; Trejo *et al.*, 1997); however, only two have been cloned and sequenced, calspermin (Ono *et al.*, 1989) and calcineurin (Mukai *et al.*, 1991; Muramatsu *et al.*, 1992). Sequence analysis has revealed a potential CaM binding site (aa 108–137) at the C-terminal end of rabbit Sp17 (Richardson *et al.*, 1994). This binding site contains the consensus sequence IQ motif found in other CaM binding proteins such as myosins, neuromodulin, and neurogranin (Cheney and Mooseker, 1992). In this study, we have characterized the site by making a truncated rRSp17 called rRSp17CB by deleting the C-terminal aa 118–146. Using biotinylated-CaM overlay assays, we have shown that rRSp17 but not rRSp17CB binds to biotinylated CaM in the presence of Ca^{2+} or EDTA. This indicates that there is a CaM binding site at the C-terminus of Sp17. Both reduced and nonreduced forms of rRSp17 bind to biotinylated CaM. Using the crosslinker DSS, rRSp17, but not rRSp17CB, bound to CaM in a 1:1 ratio. Additionally, biotinylated rRSp17 can bind to native sperm CaM. Therefore, Sp17 may serve as a storage site for CaM before the acrosome reaction.

Sp17 belongs to a family of CaM binding proteins that contain the IQ motif in the CaM binding region (Rhoads and Friedberg, 1997). Proteins in this family such as neuromodulin and neurogranin interact with CaM when Ca^{2+} is absent, while other IQ motif-containing proteins such as scallop myosin bind to light chains in the presence of Ca^{2+}. In both cases, it was demonstrated that CaM stabilizes the α-helical structure of the CaM binding domain (Xie *et al.*, 1994; Gerendasy *et al.*, 1995). Sp17 binds to CaM in the presence of either Ca^{2+} or EDTA, similar to certain unconventional myosins (Wolenski, 1995) and other sperm CaM binding proteins (Noland *et al.*, 1985; Olson *et al.*, 1985; Aitken *et al.*, 1988; Wasco *et al.*, 1989; Manjunath *et al.*, 1993; Trejo *et al.*, 1997). The structural basis of such binding in the presence or absence of Ca^{2+} is not fully understood (Houdusse *et al.*, 1996). It is possible that Sp17 has different CaM binding affinities in the presence or absence of Ca^{2+}, but the overlay assay and crosslinker experiments could not distinguish such differences. The predicted secondary structure of Sp17 indicated that it has an α-helical structure between aa 108 and 137 (O'Rand and Widgren, 1994). CaM could stabilize this α-helix regardless of Ca^{2+} concentration.

A strong PEST sequence (PEST-FIND score = 14.75) is found in Sp17 between aa 87 and 107, and proteins containing PEST sequences are subject to rapid proteolytic degradation. Several CaM binding proteins are known to contain PEST sequences and are substrates of the neutral protease calpain (Wang *et al.*, 1989; Barnes and Gomes, 1995). Calpain has been identified and localized in porcine spermatozoa (Schollmeyer, 1986) and preliminary experiments

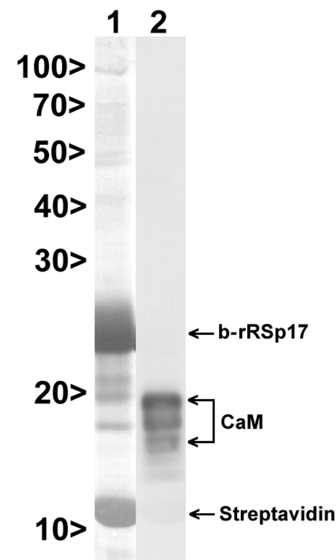

FIG. 9. Western blot of sperm proteins associated with biotinylated rRSp17 that was precipitated by streptavidin. Lane 1, amido black stained; lane 2, stained with mouse monoclonal anti-CaM. Arrows indicate positions of biotinylated rRSp17 (b-rRSp17), CaM, and streptavidin. See Fig. 1 for molecular weight markers.

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have shown that rRSp17 is susceptible to calpain cleavage (data not shown) as found in other CaM binding proteins (Wang *et al.*, 1989). Therefore, interaction of CaM and native Sp17 may not be regulated by Ca^{2+} concentration but rather by the proteolytic processing of Sp17 during the acrosome reaction. Since Sp17 contains a potential PEST sequence between aa 87 and 107 and the C-terminal is Arg and Lys rich, it is likely to be recognized and cleaved by proteases during the acrosome reaction, a process which could remove the C-terminal amino acids and its CaM binding site.

As reported previously (Richardson *et al.*, 1994), Sp17 is a zona binding protein that is located neither in the acrosome nor on the outside surface of the plasma membrane, but rather is found in the cytoplasm of the head and most likely associated with the inner aspect of the plasma membrane of acrosome intact spermatozoa. Sp17 becomes available to bind zona pellucida when the acrosome reaction begins. More recent ultrastructural evidence (Wen and O'Rand, unpublished results) indicates that Sp17 is indeed found in the cytoplasm of the postacrosomal region of the head and throughout the tail regions. This study has demonstrated that upon initiation of the acrosome reaction, Sp17 is processed to smaller forms, losing its CaM binding site. The removal of amino acids downstream of aa 121 would destroy Sp17's CaM binding activity and thus release CaM that would be available to act on downstream kinases and enzymes of the signaling cascade during zona pellucida penetration and sperm–egg fusion.

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