AM67, a Secretory Component of the Guinea Pig Sperm Acrosomal Matrix, Is Related to Mouse Sperm Protein sp56 and the Complement Component 4-binding Proteins*

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James A. Foster‡, Bret B. Friday‡, Maristelle T. Maulit‡, Carl Blobel§, Virginia P. Winfrey¶, Gary E. Olson¶, Kye-Seong Kim‡, and George L. Gerton‡**

From the ‡Center for Research on Reproduction and Women’s Health, Department of Obstetrics and Gynecology, and the §Department of Cell and Developmental Biology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104-6080, the ¶Department of Cellular Biochemistry and Biophysics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, and the ¶Department of Cell Biology, Vanderbilt University, Nashville, Tennessee 37232

The guinea pig sperm acrosomal matrix is the dense core of the acrosome and is likely to be important in acrosome biogenesis and fertilization. Isolated acrosomal matrices are composed of a limited number of major bands when analyzed by SDS-polyacrylamide gel electrophoresis, among which is a Mr 67,000 protein that we have termed AM67. Indirect immunofluorescence demonstrated that AM67 is localized to the apical segment of the cauda epididymal sperm acrosome. Immunoelectron microscopy further refined the localization of AM67 to the M1 (dorsal bulge) domain within the acrosome. Using a polymerase chain reaction product based upon tryptic peptide sequences from AM67, a Agt11 guinea pig testis cDNA library was screened to yield two cDNA clones that encode the AM67 peptides. Northern analysis revealed that AM67 is transcribed as a 1.9-kilobase mRNA. The complete AM67 sequence encodes a prepropeptide of 533 amino acids with a calculated Mr of 59,768. Following cleavage of a probable signal sequence, the polypeptide was predicted to have a Mr of 56,851 and seven consensus sites for asparagine-linked glycosylation. The deduced amino acid sequence of AM67 is most similar to those of the mouse sperm protein sp56 and the α-subunits of complement component 4-binding proteins from various mammalian species. Although mouse sp56 has been reported to be a cell-surface receptor for the murine zona pellucida glycoprotein ZP3, standard immunoelectron microscopy using the anti-sp56 monoclonal antibody 7C5 detected sp56 within the mouse sperm acrosome, but failed to detect sp56 on the surface of acrosome-intact mouse sperm. Furthermore, acrosomal labeling was detected in mouse sperm prepared for immunofluorescence using paraformaldehyde fixation, but was not observed with live unfixed sperm. Thus, the finding that sp56 is present within the acrosome provides further support that sp56 and AM67 are orthologues and suggests that sp56 may function in acrosomal matrix-zona pellucida interactions during and immediately following the acrosome reaction in the mouse.

The sperm acrosome reaction has the characteristic hallmarks of regulated secretion: 1) secretory products are concentrated and condensed; 2) secretory granules are stored for long periods of time; and 3) secretion is coupled to an extracellular stimulus (in this case, the zona pellucida) (1). Regulated secretion from sperm (i.e. the acrosome reaction) is obligatory for fertilization (2). It is presumed that the release of specific secretory components from the acrosome is involved in assisting the sperm in the penetration of the investments surrounding the egg. Furthermore, although sperm contain only one secretory vesicle, different enzymatic activities are released from sperm at various times following the acrosome reaction (3–8). The differential temporal release of secretory components occurs in other regulated secretory tissues such as the pancreas, but since cells of such tissues contain multiple secretory granules, it has been proposed that this “non-parallel secretion” is due to exocytosis from heterogeneous sources within the tissue or cell of study (9). In guinea pig sperm, the acrosome reaction results in the differential release of acrosomal components possibly due to their compartmentalization within soluble or insoluble phases of the acrosome (5, 8). Following the exocytotic acrosome reaction, certain acrosomal proteins, such as proacrosin, remain associated with the matrix that establishes the “dense core” of the acrosome and are released at a slower rate than components, such as dipetidyl peptidase, that are found in a more readily solubilized compartment.

The results presented here and in recent papers (10–14) demonstrate that two of the components of the dense core (matrix) of the acrosome, AM50 and AM67, represent monomers of large homopolymeric proteins. AM50 has been shown to be a novel, testis-specific member of the pentraxin family of proteins (11, 13). Preliminary tryptic peptide sequencing indicated that AM67 is related to members of the complement-binding protein family (11). In this paper, we show that AM67 is, indeed, a member of this class of proteins. Furthermore, the amino acid sequence of AM67 is most closely related to that of the mouse sperm protein sp56. This protein is a candidate for the cell-surface receptor of ZP3, a glycoprotein of the egg’s extracellular matrix, the zona pellucida (15). A standard postembedding immunoelectron microscopic technique demonstrated that AM67 is restricted to a specific dorsal compartment (M1 domain of the apical segment) within the acrosome of...
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EXPERIMENTAL PROCEDURES

Materials—Guinea pigs (male retired breeders) were purchased from Charles River Laboratories (Wilmington, MA). Reagents used for electrophoresis were obtained from Bio-Rad. Percoll, leupeptin, pepstatin A, and benzamidine HCl were from the Sigma, Nitrocellulose and Immobilon-P membranes were from Schleicher & Schuell and Millipore Corp. (Bedford, MA), respectively. An enhanced chemiluminescence kit (ECL, Amersham Corp.) was used for immunoblot analyses. Digoxigenin labeling reagents, detection kits, and Genius buffers were from Boehringer Mannheim. Plasmid pGEM-1ZFH(+) was from Promega (Madison, WI). Tyu DyeDeoxy terminator Cycle sequencing kits were from Applied Biosystems, Inc. (Foster City, CA). Fluorescin isothiocyanate-conjugated goat anti-rabbit IgG was purchased from Zymed Laboratories, Inc. (South San Francisco, CA), and colloidal gold-labeled goat anti-mouse IgG antibody and Texas Red-conjugated horse anti-mouse IgG were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). LR Gold was obtained from Polysciences (Fort Washington, PA). Monoclonal antibody 7C5, specific to mouse sperm sp56, was purchased from QED Biologicals (Lisbon, CA).

Preparation of Acrosomal Matrices—Acrosomal matrices were prepared by a modification of the procedure of Hardy et al. (5). Sperm were extruded from the cauda epididymides and washed three times in Tyrode’s buffer with 1 mM CaCl2 and 10 mM HEPES, pH 7.4. After washing three times with 20 mM sodium acetate, pH 5.2, containing 0.15 M NaCl and 0.5 mM EDTA, the acrosomal matrices eluting from the column were collected by centrifugation. After washing once with 20 mM sodium acetate, pH 5.2, containing 0.15 M NaCl, 5% sucrose, and 0.5 mM EDTA, the acrosomal matrices were dislodged from the remainder of the detergent-extracted sperm by trypsinization with 0.25% trypsin in a solution of 20 mM sodium acetate, pH 5.2, containing 0.15 M NaCl and 0.5 mM EDTA. The gels were stained with a solution of 0.1% Coomassie blue, 20% methanol, and 10% acetic acid.

Protein Analysis and Sequencing of Tryptic Peptides—Protein concentrations were determined by the Pierce BCA method according to the manufacturer’s specifications and employing bovine serum albumin as a standard (10). SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (17). Follow ing electrophoresis, gels were stained with silver by the method of Gerton and Millette (18).

For the partial amino acid sequencing of AM67, an acid extract of guinea pig sperm (consisting mostly of solubilized acrosomal components) was separated on several gels by non-equilibrium pH gradient electrofocusing followed by SDS-PAGE and transfer to Immobilon-P (19, 20). The transfer membranes were stained with Ponceau S, and the spots corresponding to AM67 (as determined by probing a stained replicate blot with anti-AM67) were excised. Tryptic peptides were generated from the transfer membranes, purified by high-performance liquid chromatography, and sequenced by the Edman degradation procedure by Dr. John Leszynski (Worcester Foundation for Experimental Biology).

AM67 was also affinity-purified using the monoclonal antibody PH1, which binds the guinea pig sperm protein fertin (21) and apparently cross-reacts with AM67. Affinity-purified AM67 was reduced and alkylated, fractionated by SDSPAGE, and electrophrothised onto nitrocellulose membranes. The Ponceau S-stained Mf, 67,000 band was excised and processed for internal amino acid analysis by Drs. P. Tempst, H. Blobel, and colleagues at Cold Spring Harbor Laboratory. The immobilized protein samples were subjected to trypsinization, and trypptic fragments were separated by reverse-phase HPLC. HPLC peak fractions (over trypsin background) were analyzed by automated Edman degradation using an Applied Biosystems Model 477A Sequencer, with instrument and procedure optimized for femtomole level analysis as described (23).

Immunological Analysis of AM67—Acrosomal matrices were separated by preparative SDS-PAGE and electrotransferred to nitrocellulose paper by the method of Towbin et al. (24). The protein bands corresponding to AM67 were excised, eluted from nitrocellulose, and either run on SDS-PAGE and transferred commercially from the nitrocellulose strips (Cocalico, Reamstown, PA). For immunoblot analysis, proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and processed as described previously using ECL procedures (20).

Indirect Immunofluorescence of Guinea Pig and Mouse Sperm—Indirect immunofluorescence of guinea pig sperm was performed on unfixed coverslips coated with 4% paraformaldehyde and placed onto a microscope stage. After washing twice with PBS containing 1% Triton X-100, the coverslips were incubated with Texas Red-conjugated horse anti-mouse IgG (diluted 1:200) for 1 h at room temperature. After washing, the coverslips were incubated with PBS containing 1% Triton X-100 and 1% bovine serum albumin for 1 h, washed again, and mounted using Fluoromount G. Slides were examined using a Zeiss Photomicroscope III equipped with epifluorescence and photographed with Kodak T-Max P3200.

Immunoelectron Microscopy—Immunoelectron microscopy of guinea pig sperm from cauda epididymides was performed on LR White-embedded sections using 10-nm gold particles as described previously (18). Guinea pig sperm were obtained from the cauda epididymides and were washed twice by centrifugation for 5 min at 300 g and resuspended in PBS. For the post-embedding immunostaining procedure, sperm were fixed for 30 min in PBS containing 1% formaldehyde, 1% glutaraldehyde, and 0.1% sodium azide at 4 °C for 30 min. Following a washing step (3 min in PBS), all grids were fixed and counterstained with 5% aqueous uranyl acetate and embedded in LR White (25). After washing three times in Tris-buffered saline, the grids were incubated in blocking buffer containing 12-nm colloidal gold conjugated to a goat anti-mouse IgG antibody diluted 1:40. The same procedure was used for AM67 immunostaining, except that the grids were treated with either an anti-AM67 rabbit antiserum or a 1:100 control serum containing 1% BSA, 1% normal mouse IgG, 1% goat serum, and 1% mouse serum. The grids were incubated in blocking buffer containing 12-nm colloidal gold conjugated goat anti-rabbit antibody diluted 1:50. After washing three times in Tris-buffered saline, all grids were fixed and counterstained with 1% osmium tetroxide followed by aqueous uranyl acetate.

Isolation of cDNA Clones—To screen for cDNA clones encoding AM67, a partial DNA probe was prepared by PCR of a guinea pig testis cDNA library with degenerate primers based upon conserved regions in cDNAs encoding C4BP from various mammals and the continuous peptide sequence (MAILVEYKLSLEIEQKELEY) previously reported for AM67 (11). The primer pair used was 5’-TTGGATCATGAAAGATAAATTGATGCAAG-3’ and 5’-CTAATGTTTGGCAATCTT/TCCAGACAGAC-3’. Cycling conditions were 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for a final extension at 72 °C for 6 min. Preliminary DNA sequencing of the ∼1200-base pair PCR product confirmed that the product corresponded to a fragment of the AM67 mRNA.

After labeling by the random priming method with digoxigenin-labeled dUTP as one of the nucleotides, the probe was used to screen the library for AM67 according to standard procedures (26, 27). Briefly, plates of Y1090 cells infected with phage particles were grown at 42 °C for 5–7 h. Phage plaques were lifted onto Hybond-N (Amersham Corp.) filters, and phage DNA was denatured by sequentially placing filters onto filter paper saturated with 0.5 mM NaOH, 1.5 mM NaCl, 0.5 mM Tris-HCl, pH 7.5, 1.5 mM NaCl; and finally, 2 x SSC. Filters were dried and fixed with UV light. Following prehybridization for 1 h at 65 °C in Church buffer (0.25 M sodium phosphate, pH 7.4, 1 mM EDTA, 7% SDS, and 1% bovine serum albumin), hybridization was performed as described previously with a 10-ng/ml digoxigenin-labeled AM67 PCR product as a probe overnight at 62 °C. Filters were washed in Genius Buffer 1 (100 mM NaCl, 100 mM Tris-HCl, pH 7.5) for 10 min at room temperature and then again at 65 °C. Filters were blocked in Genius Buffer 2 (100 mM NaCl, 100 mM Tris-HCl, pH 7.5, and 2% blocking reagent) for 30 min, followed by incubation for 1 h at room temperature with a probe solution of 10 ng/ml digoxigenin-labeled AM67 PCR product. After a washing step (3 min in Genius Buffer 2), the filters were hybridized with a probe solution of 10 ng/ml digoxigenin-labeled AM67 PCR product overnight at 42 °C. Following a washing step (3 min in Genius Buffer 1), the filters were hybridized with a probe solution of 10 ng/ml digoxigenin-labeled AM67 PCR product overnight at 62 °C. Following a washing step (3 min in Genius Buffer 1), the filters were hybridized with a probe solution of 10 ng/ml digoxigenin-labeled AM67 PCR product overnight at 62 °C.
Acrosomal Matrix C4-binding Protein Homologue

Fig. 1. Composition of acrosomal matrices from guinea pig cauda epididymal sperm. Acrosomal matrices (right lane) were analyzed by SDS-PAGE (10 μg of protein with 40 mM DTT) on a gel containing 7% polyacrylamide. The major proteins detected by silver staining are proacrosin, AM50, and AM67. Molecular weight standards are shown in the left lane.

Fig. 2. Immunoblot analysis of AM67 from isolated guinea pig acrosomal matrices treated under nonreducing and reducing conditions. Acrosomal matrices were extracted with 1% acetic acid containing 50 mM benzamidine. The extracts were then diluted in sample buffers containing increasing amounts of DTT, and 5 μg of protein were analyzed per lane by SDS-PAGE (7% polyacrylamide in the separating gel). The numbers listed above each lane represent the amount of DTT (mM) added to each sample. After transfer to nitrocellulose, the membrane was probed with anti-AM67 and developed by enhanced chemiluminescence. In the absence of reducing agent, AM67 barely migrated into the running gel, demonstrating that AM67 is part of a high molecular weight complex. With increasing amounts of DTT, this complex began to be reduced to smaller complexes and, eventually, to monomers.

followed by incubation for 1 h in anti-digoxigenin antibody conjugated to peroxidase (1:1000 in Genius Buffer 2) at room temperature. Filters were washed in Genius Buffer 1 and detected using ECL reagents. Positive clones were plaque-purified, and the cDNA inserts were subcloned into pGEM-11Zf(+) and sequenced using the T3q DyeDeoxy™ Terminator Cycle sequencing kit chemistry and the appropriate primers. The products were separated by electrophoresis and analyzed with an Applied Biosystems Model 373A Automated Sequencer. Both strands of each insert were sequenced.

The deduced amino acid sequence of AM67 was analyzed with the MacVector™ molecular biology program (Kodak Scientific Imaging Systems) or the MacPattern program using the Prosite and Blocks data bases (28). Homology searches of GenBank™ and other sequence data bases were performed using the BLAST program of the National Center for Biotechnology Information (29). Alignments were created using the CLUSTAL V program (30) and were displayed using SeqVu Version 1.0.1.3. The N-terminal amino acid of mature AM67 was deduced with the AnalyseSignalase2.03 program, which predicts signal peptidase cleavages based upon the method of von Heijne (31).

Northern Blot Analysis—Total RNAs (20 μg) from various guinea pig tissues were denatured by glyoxal, separated on 1% agarose gels, and transferred onto Hybond-N membranes. The blots were probed with an AM67 PCR probe 32P-labeled by the random priming method, and the hybridized bands were detected by autoradiography as described previously (20).

RESULTS

Protein Composition of Acrosomal Matrices—The protein composition of acrosomal matrices from guinea pig sperm was relatively simple (Fig. 1). The protein profile was dominated by a major protein band of M_\text{r} \sim 50,000. As determined by non-equilibrium pH gradient electrophoresis/SDS-PAGE followed by immunoblotting, this band actually comprised two proteins of similar molecular weights, proacrosin and AM50 (also called p50 and apexin in previous publications (11, 13)). A less prominent protein was AM67, represented by a protein band of M_\text{r} \sim 67,000. Both AM50 and AM67 form high molecular weight homomeric complexes in the absence of reducing agents (11–14). A more variable constituent of these preparations was a M_\text{r} \sim 32,000 protein, believed to be proacrosin-binding protein.

Fig. 3. Immunoblot analysis of AM67 in various extracts of sperm and germ cells. The soluble fraction (SF) and acrosomal matrices (AM) were prepared as described under "Experimental Procedures." Whole cauda epididymal sperm (wS) were extracted in Laemmli sample buffer. Mixed germ cells (GC) were prepared as described (25) and extracted with 1% Triton X-100 and 1% sodium deoxycholate in PBS containing a protease inhibitor mixture (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM aminobenzamidine). Cauda epididymal sperm (eS) were also extracted with 1% acetic acid containing 5 mM acetic acid. Each lane contained 5 μg of protein separated by SDS-PAGE following reduction with 40 mM DTT. After electrophoresis, the proteins were transferred to Immobilon-P, and the membrane was probed with anti-AM67 and developed by enhanced chemiluminescence methods.

Fig. 4. Indirect immunofluorescence demonstrating localization of AM67 to the apical segment of the acrosome. Sperm were attached to polylysine-coated coverslips. Following fixation with 4% paraformaldehyde, the sperm were permeabilized with methanol (−20 °C). The coverslips were treated with anti-AM67 (1:100 dilution) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and viewed by fluorescence microscopy to localize AM67 protein. AM67 protein was exclusively restricted to the apical segment of the acrosome. No staining was observed in the principal or equatorial segment of the acrosome. In addition, staining was not observed in sperm treated with preimmune serum instead of anti-AM67 or in sperm that had not been permeabilized prior to antibody treatment. A and B, paired phase-contrast and fluorescence micrographs of anti-AM67-treated sperm; C and D, paired phase-contrast and fluorescence micrographs of sperm treated with preimmune serum. Arrows indicate the apical segment region of the sperm acrosome.
AM67 Forms a High Molecular Weight, Disulfide-bonded Complex—An antiserum to AM67 was produced by immunization of rabbits with nitrocellulose blot-purified AM67. The antiserum was specific to AM67 as shown by immunoblot analysis (Fig. 2). As demonstrated previously by the electrophoresis of AM67 under nonreducing conditions followed by separation after reduction, AM67 formed a \( M_r \approx 200,000 \) disulfide-bonded complex. Although initially reported to have a molecular weight approximating 520,000 (14), a reanalysis of these data provided an estimate of \( M_r \approx 380,000 \) for the AM67 multimer (data not shown). In the absence of reducing agent, AM67 barely migrated into a 7.0% running gel. With increasing amounts of DTT, this complex was reduced to smaller complexes and eventually to monomers (Fig. 2).

Sperm prepared in various ways were examined to determine whether all of the AM67 remained associated with the acrosomal matrix during fractionation of sperm and to ascertain whether there were any gross alterations in the size of AM67 as a result of germ cell maturation. As can be seen in Fig. 3, AM67 can be detected in the Triton X-100-soluble fraction of sperm (solubilized during the course of acrosomal matrix preparation) as well as in the acrosomal matrix (pellet). This suggested that there was a subpopulation of AM67 that was not tightly bound to acrosomal matrix and that could be released with gentle non-ionic detergents. This result was in contrast to AM50, which remains firmly associated with the matrix during isolation (11). In addition, the size of AM67 acid-extracted from testicular germ cells approximated that from epididymal sperm, indicating that there were no gross structural alterations in the AM67 monomer as a result of sperm maturation. However, if sperm were extracted with Laemmli sample buffer without reducing agents, AM67 was partially degraded, presumably by proteases, such as acrosin, that remain active in SDS in the absence of reducing agent (25, 33).

**Intra-acrosomal Localization of AM67 in the Apical Segments of Guinea Pig Sperm Acrosomes**—The biochemical studies presented above indicate that AM67 is part of the acrosomal matrix. However, the morphology of the acrosome is somewhat complex and can be differentiated into three segments: the apical segment (the region that extends beyond the anterior tip of the sperm nucleus), the principal segment (the region that encases the anterior half of the nucleus), and the equatorial segment (the region that forms a narrow band around the posterior region of the acrosome and where the outer acrosomal membrane makes the transition to the inner acrosomal membrane). Furthermore, individual acrosomal proteins have been localized to one or more segments of the guinea pig sperm acrosome (5, 12, 34). To determine whether AM67 was restricted to a particular segment of the acrosome, sperm were examined by indirect immunofluorescence. AM67 protein was found to be restricted to the apical segment of the acrosome (Fig. 4B). Staining was also not observed in sperm treated with preimmune serum instead of anti-AM67 (Fig. 4, C and D) or in cells that had not been permeabilized prior to antibody treatment (data not shown).

Ultrastructurally, the apical segment can be morphologically differentiated into three domains: M1 ("dorsal bulge"), M2 (intermediate zone), and M3 (ventral zone). Immunoelectron microscopic localization using thin sections of guinea pig sperm provided the resolution demonstrating that AM67 is restricted to the M1 domain of the apical segment (Fig. 5). No staining was observed in the principal and equatorial segments of the acrosome. Few gold particles were seen in the M2 and M3 domains of the acrosome, and none were detected on the sperm plasma membrane. Within the M1 domain, the gold particles were generally excluded from apparently spherical, denser regions. Thus, AM50 and AM67, which are both high molecular weight homomeric complexes of the acrosomal matrix and are

![Fig. 5. Immunoelectron microscopic localization of AM67 in the M1 (dorsal bulge) domain of guinea pig sperm. Guinea pig sperm were fixed and embedded in LR White resin. Thin sections were processed with anti-AM67 as described under "Experimental Procedures." The immunogold particles (arrows) were found solely within the M1 (dorsal bulge) domain of the lumen of the acrosome. In general, the gold particles were excluded from spherical bodies within this region. No staining was observed in the M2 (intermediate zone) or M3 (ventral zone) region.](http://www.jbc.org/)

**FIG. 5.** Immunoelectron microscopic localization of AM67 in the M1 (dorsal bulge) domain of guinea pig sperm. Guinea pig sperm were fixed and embedded in LR White resin. Thin sections were processed with anti-AM67 as described under "Experimental Procedures." The immunogold particles (arrows) were found solely within the M1 (dorsal bulge) domain of the lumen of the acrosome. In general, the gold particles were excluded from spherical bodies within this region. No staining was observed in the M2 (intermediate zone) or M3 (ventral zone) region. Magnification \( \times 51,000 \).
Potential sites of asparagine-linked glycosylation are indicated by as- amino-terminal amino acids of AM67 tryptic peptides are underlined. Testis (\text{testis}), brain (\text{brain}), liver (\text{liver}), but not in heart (\text{heart}), or kidney (\text{kidney}); a testis-specific gene product. A message of at amino acids 68, 77, 185, 191, 200, 433, and 455.\footnote{\text{12718}} As reported earlier, two tryptic peptides of AM67—\text{ponent 4-binding Proteins—}\text{were sequenced and were found to be highly homologous to contig-}

Using the tryptic peptide sequencing information, degener-

The cDNA sequence of AM67 encoded a 533-amino acid M, 59,768 protein (Fig. 6A). Following removal of a predicted 28-

The DNA sequence of AM67 encoded a fragment homologous to C4BP\text{a}.

Comparison of the sequence of AM67 with GenBank data using the BLAST algorithm demonstrated that AM67 is most closely related to mouse sperm protein sp56 and other members of the C4BP\text{a} family of proteins (Fig. 7A). Although guinea pig C4BP\text{a} has yet to be cloned and sequenced from the liver (the tissue that synthesizes bona fide C4BP\text{a}), the conclusion that AM67 is different from C4BP\text{a} was confirmed by partial DNA sequence analysis of a reverse transcription-PCR product from guinea pig liver using degenerate primers for C4BP\text{a} (data not shown). The highest homology of AM67 to C4BP\text{a} proteins from different species was to the human protein, followed by bovine, rat, and mouse. Based upon the molecular weight of the unreduced form of AM67 and its homology to C4BP\text{a}, we hypothesize that six to eight monomers oligomer-

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fig. 6B). The PCR product was then labeled by random priming using

digoxigenin-labeled dUTP. The digoxigenin-labeled probe was then used to screen the cDNA library using an anti-digoxigenin antibody and the ECL detection system. From a screen of 120,000 plaques, six positives were obtained after three rounds of screening and were subcloned into the \text{No}l site of pGEM-11Zf(\text{+}). Preliminary DNA sequencing of these plasmids indicated that all six of the clones were true positives, encoding a C4BP\text{a}-like protein. Two of the plasmids containing the largest inserts, p67-9 and p69-10, were chosen for complete sequencing of the insert and found to encode the complete open reading frame of the protein (Fig. 6A). Plasmid p67-9 represented bases 1–1735, and p67–10 represented bases 83–1864 in the composite sequence. Overall, the composite sequence encoded 83 nucleotides of 5’untranslated region and 82 nucleotides of 3’untranslated region. Although no poly(A) tail was observed in these clones, a possible poly(A) signal (TATAAA) was found at positions 1851–1856. An in-frame stop codon was found 9 bases upstream from the predicted translation start codon, demon-

Fig. 6. Cloning of cDNAs encoding AM67 and testis-specific expression of AM67 mRNA A, the deduced amino acid is shown below the nucleotide sequence numbered in the 5’ to 3’ direction. The amino-terminal amino acids of AM67 tryptic peptides are underlined. Potential sites of asparagine-linked glycosylation are indicated by as- asterisks at amino acids 68, 77, 185, 191, 200, 433, and 455. B, AM67 is a testis-specific gene product. A message of ~19 kilobases was found in testis (\text{T}), but not in heart (\text{H}), brain (\text{Br}), liver (\text{L}), or kidney (\text{K}).

found in the M3 and M1 domains, respectively, do not overlap in their subcellular localization (10, 14). AM67 is Homologous to Mouse sp56 and Complement Component 4-binding Proteins—

As reported earlier, two tryptic peptides of AM67—MALVEYK and LSLEIQLKEKEYK—were sequenced and found to be highly homologous to contiguous regions in human C4BP\text{a}, diverging only at the last four amino acids (KEKY in AM67 and LQRD in human C4BP\text{a}) (11). Four additional tryptic peptides were subsequently sequenced and also found to be homologous to C4BP\text{a} (Figs. 6 and 7).

Using the tryptic peptide sequencing information, degener-

ate primers were designed for PCR using DNA from the \text{Agt11}
guinea pig testis cDNA library as the template. A product near the expected size (1197 base pairs) was obtained and found by DNA sequencing to encode a fragment homologous to C4BP\text{a}. The PCR product was then labeled by random priming using digoxigenin-labeled dUTP. The digoxigenin-labeled probe was then used to screen the cDNA library using an anti-digoxigenin antibody and the ECL detection system. From a screen of 120,000 plaques, six positives were obtained after three rounds of screening and were subcloned into the \text{No}l site of pGEM-11Zf(\text{+}). Preliminary DNA sequencing of these plasmids indicated that all six of the clones were true positives, encoding a C4BP\text{a}-like protein. Two of the plasmids containing the largest inserts, p67-9 and p69-10, were chosen for complete sequencing of the insert and found to encode the complete open reading frame of the protein (Fig. 6A). Plasmid p67-9 represented bases 1–1735, and p67–10 represented bases 83–1864 in the composite sequence. Overall, the composite sequence encoded 83 nucleotides of 5’untranslated region and 82 nucleotides of 3’untranslated region. Although no poly(A) tail was observed in these clones, a possible poly(A) signal (TATAAA) was found at positions 1851–1856. An in-frame stop codon was found 9 bases upstream from the predicted translation start codon, demon-

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three-dimensional structure of each SCR in human C4BPα (35).

The observation that the amino acid sequence of AM67 was most similar to the sequence of mouse sperm protein sp56 was of great interest since this protein has been identified as a candidate cell-surface receptor for the ZP3 glycoprotein of the egg zona pellucida (15). There are large stretches of identical residues and conservative substitutions throughout the sequence (Fig. 7A). For example, following removal of the signal peptides (AM67, residues 1–28; and sp56, residues 1–32), 12 out of the first 13 amino acids of mature mouse sp56 were identical to mature AM67, with the one differing residue being a conservative substitution. A 50-amino acid stretch conserved in guinea pig AM67 (residues 346–395) and mouse sp56 (residues 351–400) appeared to have diverged extensively from the C4BPα sequences (Fig. 7A, bar). However, another region in mouse sp56 (residues 414–453) had no corresponding counterpart in guinea pig AM67 and was divergent from the corresponding regions in human and mouse C4BPα proteins. More important, mouse sp56 was more homologous to guinea pig AM67 than to mouse C4BPα.

Given the similarity of amino acid sequences, one would predict that AM67 and sp56 would be localized to the same cellular compartment. Using standard immunofluorescence and immunoelectron microscopic procedures, AM67 was localized within the acrosome (Figs. 4 and 5). To the contrary, sp56 was previously reported to be localized to the sperm surface over the acrosome using the 7C5 monoclonal antibody and an unconventional immunogold surface replica approach (15, 36).

Thus, we re-examined the subcellular localization of sp56 in mouse sperm using monoclonal antibody 7C5 and a standard post-embedding immunoelectron microscopic approach that has been used extensively in previous studies (5, 12, 37). Under these conditions, sp56 was not detected on the plasma membrane of mouse sperm as was previously reported. Instead, it was detected abundantly within the acrosome and did not appear to associate preferentially with the acrosomal membranes (Fig. 8, A–C). The acrosomal localization of sp56 by this method was consistently reproducible and was identical both in mature epididymal sperm and in sperm incubated under capacitating conditions (data not shown). Control sperm incubated with an equal concentration of nonspecific whole IgG showed no cross-reactivity (data not shown). To enhance the possibility of detecting sperm-surface sp56, we also used a pre-embedding immunoelectron microscopic approach. Under these conditions, sp56 was not detected on the surface of acrosome-intact sperm, but was associated with the acrosomal contents in sperm with damaged acrosomes (data not shown). Furthermore, using indirect immunofluorescence for localizing sp56 in mature mouse sperm with the 7C5 monoclonal antibody, bright acrosomal staining was observed in paraformaldehyde-fixed sperm (Fig. 9, A and B), but no acrosomal staining was observed in unfixed sperm (Fig. 9, C and D).
DISCUSSION

This study demonstrates that the guinea pig sperm acrosome contains a testis-specific protein related to mouse sperm sp56 and other members of the complement regulatory protein superfamily. This result is reminiscent of our previous reports concerning the acrosomal matrix protein AM50, which was found to be a member of the pentraxin superfamily (11, 13). Like AM50, AM67 was found to be a testis-specific member of a protein superfamily, was localized to a specific domain within the acrosome, and also formed high molecular weight homopolymers. Similar to C4BPα, we estimate that six to eight monomers of AM67 oligomerize to constitute the unreduced form of the protein ($M_r \sim 380,000$). Based upon the C4BPα sequence homology, AM67 might be predicted to form calcium-dependent complexes with other ligands within the acrosome or, after exocytosis, with extracellular ligands. Comparable calcium-dependent binding of AM50 to the apical segment complex (containing the acrosomal matrix) has already been demonstrated (11).

Of all the sequences found to be homologous to AM67, mouse sperm protein sp56 and the serum complement component 4-binding proteins within the complement regulatory protein superfamily ranked the highest in a BLAST search of GenBank™ sequences (29). The significance of this is not clear at the present time; but it is noteworthy that serum C4BP has been shown to interact with serum pentraxins (C-reactive protein and serum amyloid P component) (38–40), and thus, AM67 might be predicted to interact with AM50. Although immunoelectron microscopy demonstrated that AM67 and AM50 are localized in distinct regions of the acrosomal matrix of acrosome-intact sperm, it is possible that these components could interact following the acrosome reaction. C4BPα and the pentraxins are known to bind to plasma membranes, and based upon the homologies to these proteins, AM67 and AM50 might also be expected to interact with components of the acrosomal membranes. If AM67 and AM50 bind to different ligands of the acrosomal membrane, aggregation of these proteins with their ligands during acrosome biogenesis may help to establish the M1 and M3 domains. Additionally, AM50 and AM67 may interact with membranes or react with each other upon their release following the acrosome reaction and the dissolution of the acrosomal matrix.

AM67 is not the first member of the complement regulatory protein superfamily to be found in sperm. Additional members recently identified include another guinea pig sperm C4BP distinct from AM67 (13) as well as decay-accelerating factor.

**FIG. 8.** Ultrastructural localization of sp56 in mature mouse sperm by post-embedding immunoelectron microscopy. Shown is the localization of mouse sp56 within the dorsal region of the acrosome (asterisks) overlying the nucleus (n) in longitudinal (A; magnification × 30,000), cross (B; magnification × 31,000), and oblique (C; magnification × 26,000) thin sections through mature mouse sperm treated with sp56-specific monoclonal antibody 7C5. Control incubations with an equal concentration of nonspecific whole IgG demonstrated the lack of nonspecific antibody interactions with sperm (not shown).

**FIG. 9.** Indirect immunofluorescence of paraformaldehyde-fixed and live mouse sperm. Shown are corresponding phase-contrast (A and C) and immunofluorescence (B and D) micrographs of mature mouse sperm immunostained with anti-sp56 monoclonal antibody 7C5. Paraformaldehyde-fixed sperm (A and B) showed bright acrosomal fluorescence localization of sp56 (B), whereas live unfixed sperm (C and D) showed no immunofluorescence (D). Magnification × 1400.
present in sperm-specific forms. The testis-specific expression are debatable at this point, but several of them appear to be regulatory proteins in fertilization and reproductive immunology. The roles of these complement regulator of complement, is secreted by the Sertoli cells and the epididymal epithelium and has also been shown to be associated with sperm. The very remarkable similarity of the amino acid sequence of guinea pig AM67 to that of mouse sp56 suggests that these proteins are orthologues. Although the apparent molecular weights of the native AM67 and sp56 multimeric proteins were ~380,000 and 110,000, respectively (14, 15), these sizes are similar to the differences in the properties of the C4BPs from other mammalian species compared with mouse (44). Furthermore, AM67 and sp56 are soluble at low pH, a commonly used condition for the extraction of acrosomal proteins (45). However, our finding that AM67 is intracellular contrasts with previous reports characterizing mouse sp56 as a cell-surface protein (15, 46).

To examine the discrepancy in the localizations of AM67 and sp56, we used the post-embedding immunoelectron microscopic method, but found that both AM67 and sp56 were localized to the acrosomal contents in mature guinea pig and mouse sperm, respectively, while neither was observed on the plasma membrane. It seems unlikely that there is a pool of sp56 on the mouse sperm surface that was not detected by this method since both surface and intracellular antigens should be detected equally well. This conclusion was further substantiated by results from pre-embedding immunoelectron microscopy, a standard method used to localize cell-surface antigens, showing that sp56 was associated with acrosomal material in damaged sperm, but not on the plasma membrane (data not shown). It is possible that the colloidal gold labeling of sp56 interpreted as surface labeling by the immunogold surface replica method (15, 36) could, in fact, be due to the exposure of acrosomal sp56 on the surface by permeabilization of the outer acrosomal and plasma membranes. The outer acrosomal membrane and the plasma membrane over the acrosome are known to be susceptible to mechanical and chemical disruption, and these membranes are also destabilized during capacitation (47).

Furthermore, the mouse sperm utilized for the localization of sp56 by the immunogold surface replica technique were capacitated and lightly fixed with 1% paraformaldehyde, but not embedded prior to immunolabeling (36). Since formaldehyde fixation is reversible (48), it appears that the "surface labeling" of sp56 may actually be attributable to the exposure of intra-acrosomal sp56 caused by the permeabilization, rupture, or partial fusion of the destabilized outer acrosomal and plasma membranes during the course of the sample manipulations.

These results do not negate the results showing the ability of mouse sp56 to bind to the zona pellucida, but should cause us to reassess our concepts regarding sperm capacitation, sperm-egg recognition, and the acrosome reaction. Evidence from a variety of systems indicates that small dynamic fusion pores form in a common bilayer created by the apposing leaflets of the vesicular and plasmalemmal membranes (reviewed by Monck and Fernandez (49)). These small pores are thought to occasionally close after the release of small non-quantal amounts of secretory products. In an application of this "dynamic fusion pore" hypothesis to sperm capacitation, capacitation may represent a state where the small fusion pores are dynamically opening and closing between the outer acrosomal and plasma membranes, thereby exposing acrosomal components. Thus, it might be possible that a capacitated sperm encountering an egg may actually bind to the zona via the transiently exposed acrosomal proteins, not a cell-surface molecule. Binding to the zona in this manner might "lock" the fusion pore into the static open state, driving exocytosis to completion.

In consideration of the resistance of the acrosomal matrix to Triton X-100 solubilization, we propose that acrosomal matrix components such as AM67 and AM50 help form the dense core of the acrosome and establish the different compartments (M1, M2, and M3 domains) within this organelle. As proposed for the dynamic fusion pore hypothesis, the relative distribution of these proteins may position them for binding to other molecules such as the zona pellucida glycoproteins, and the affinity of AM67 and AM50 for other acrosomal components may regulate the differential release of certain secretory products following the acrosome reaction. Other acrosomal proteins such as dipeptidyl peptidase, autoantigen 1, and soluble hyaluronidase may not associate tightly with the matrix, and thus, they would be freely released following the acrosome reaction (5, 6, 8, 34). Proteins such as proacrosin would be more firmly associated with certain components of the matrix (e.g. AM67, AM50, and proacrosin-binding protein) (50–52). The gradual dissolution of the matrix may result from the dissociation of matrix components resulting from partial proteolysis following the progressive conversion of the zymogen proacrosin to enzymatically active acrosin. With the activation of proteolysis, AM50 would be processed to AM50 AR by proteolysis, leading to the destabilization of the AM50 oligomers and continued dissolution of the matrix (12). This working hypothesis opens up new avenues for examining the release of materials following acrosomal exocytosis.

In studies in progress, the binding properties of AM67 and AM50 are being examined to identify ligands for these large oligomers and to determine their roles in acrosomal bio-genesis and fertilization.

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AM67, a Secretory Component of the Guinea Pig Sperm Acrosomal Matrix, Is Related to Mouse Sperm Protein sp56 and the Complement Component 4-binding Proteins

James A. Foster, Bret B. Friday, Maristelle T. Maulit, Carl Blobel, Virginia P. Winfrey, Gary E. Olson, Kye-Seong Kim and George L. Gerton

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