A critical step during fertilization is the sperm acrosome reaction in which the acrosome releases its contents allowing the spermatozoon to penetrate the egg investments. The sperm acrosomal contents are composed of both soluble material and an insoluble material called the acrosomal matrix (AM). The AM is thought to provide a stable structure from which associated proteins are differentially released during fertilization. Because of its important role during fertilization, efforts have been put toward isolating the AM for biochemical study and to date AM have been isolated from hamster, guinea pig, and bull spermatozoa. However, attempts to isolate AM from mouse spermatozoa, the species in which fertilization is well-studied, have been unsuccessful possibly because of the small size of the mouse sperm acrosome and/or its fusiform shape. Herein we describe a procedure for the isolation of the AM from caput and cauda mouse epididymal spermatozoa. We further carried out a proteomic analysis of the isolated AM from both sperm populations and identified 501 new proteins previously not detected by proteomics in mouse spermatozoa. A comparison of the AM proteome from caput and cauda spermatozoa showed that the AM undergoes maturational changes during epididymal transit similar to other sperm domains. Together, our studies suggest the AM to be a dynamic and functional structure carrying out a variety of biological processes as implied by the presence of a diverse group of proteins including proteases, chaperones, hydrolases, transporters, enzyme modulators, transferases, cytoskeletal proteins, and others. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.020339, 758–774, 2012.

An important step during fertilization is the sperm acrosome reaction in which the acrosome, an exocytotic vesicle overlying the sperm head, releases its contents allowing the spermatozoon to penetrate through the investments surrounding the oocyte. The acrosome is essential for normal fertilization because spermatozoa lacking these structures are infertile (1, 2). The acrosomal contents are compartmentalized into soluble and an insoluble or particulate material termed the acrosomal matrix (AM). The AM has been characterized as an electron dense and membrane-free insoluble material that remains following treatment of spermatozoa with Triton X-100 (3). Functionally it is thought to provide a stable scaffold for interactions between the sperm and oocyte and to allow the controlled and sequential release of matrix associated proteins important for fertilization. The importance of AM function during fertilization is emphasized by its conservation in spermatozoa across species including hamster, guinea pig, bull, stallion, boar, quail, water strider, and human (4–11). Proteases activated by increasing intra-acrosomal pH as a result of the acrosome reaction are thought to contribute to the disassembly of the AM (3). However, the precise mechanism by which this occurs is not known. The mechanism by which the AM forms is also not known but the self-assembly of proteins into a large complex has been proposed (3). Cytoskeletal proteins have also been found associated with the matrix and are thought to contribute to the scaffold structure (12).

Because of its critical role during fertilization, considerable effort has been put toward developing procedures for the isolation of the AM and thus far AM have been isolated from guinea pig, hamster, and bovine cauda epididymal spermatozoa (6, 13, 14). Several AM associated proteins have been identified either by biochemical analyses of the isolated structure or by immunolocalization of proteins to AM that remained associated with spermatozoa following Triton X-100 exposure. These proteins include Acr, proacrosin; Acrbp, proacrosin binding protein; Zpbp, zona pellucida binding protein; Zp3r, zona pellucida 3 receptor; zan, zonadhesin, and others (3). Although collectively these studies have identified a number of AM associated proteins, a full proteomic analysis of the AM has not been carried out. Unfortunately, the AM has also not been successfully isolated from mouse spermatozoa, the species in which fertilization is well-studied and in which gene knockout models are prevalent. Indeed, for some time it was questioned whether mouse sperm acrosomes even possessed an acrosomal matrix structure (15). The difficulty in isolating the mouse sperm AM may stem from the fusiform shape of the sperm head, the small size of its acrosome

1 The abbreviations used are: AM, Acrosomal matrix; RT, Room temperature; HIGS, Heat inactivated goat serum.
compared with that in guinea pig and hamster, and/or the general fragile nature of mouse spermatozoa compared with spermatozoa from other species. Also, to date, isolation of the AM has only been described for mature cauda epididymal spermatozoa and not for immature caput epididymal spermatozoa.

Herein, we describe a procedure for the isolation of the AM from caput and cauda mouse epididymal spermatozoa. Using mass spectrophotometric analyses we then carried out a proteomic characterization of the proteins present in the AM from these two sperm populations. These studies reveal the identity of 501 new proteins not previously found in spermatozoa by a proteomics approach. Furthermore, differences in AM protein composition were observed between the caput and cauda spermatozoa suggesting that the AM may undergo maturational changes during epididymal transit in preparation for downstream functions during fertilization. Together, these studies show the AM as a dynamic functional structure containing a diverse group of proteins including structural proteins, transporters, enzyme modulators, proteases, chaperones, kinases and others.

EXPERIMENTAL PROCEDURES

Isolation of Acrosomal Matrix From Mouse Epididymal Spermatozoa—Male mice (CD1 retired breeders, Charles River Laboratories, Wilmington, MA) were housed under a constant 12L/12D cycle and were allowed free access to food and water. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines for Care and Use of Experimental Animals.

The epididymides were removed and divided into three main regions: caput (regions 1–3); corpus (region 4), and cauda (region 5) (16). Caput and cauda epididymal spermatozoa were collected by puncturing tissues with a needle and allowing the spermatozoa to disperse into phosphate-buffered saline (PBS) (10 mM phosphate, 137 mM NaCl, pH 7.4) containing a protease inhibitor mixture (PIC) (cComplete, Mini, EDTA-free Protease Inhibitor Mixture Tablets, cat.no.11836170001, Roche, San Francisco, CA) for 10 min at 37 °C. Spermatozoa were purified from contaminating epididymal epithelial cells by filtering through a 10 μm diameter nylon mesh (Medifab washed, cat.no.07-10/2, Sefar Inc, Depew, NY) and the collected spermatozoa were washed twice in PBS/PIC by centrifugation at 500 × g for 5 min at room temperature (RT). The number of isolated spermatozoa was calculated using a hemocytometer.

To isolate AM from spermatozoa, a modification of previous methods used to expose but not extract the AM from mouse spermatozoa was followed (15, 17). Because caput and cauda epididymal spermatozoa are in different maturational states, they required different protocols used to expose but not extract the AM fraction from mouse spermatozoa. The sample was then centrifuged at 500 × g for 5 min at 4 °C resulting in a supernatant containing released AM, designated as the AM fraction, and a pellet containing mainly spermatozoa without AM but also some that still had their AM attached. To increase the recovery of AM, the pellet was resuspended in 200 μl 20 mM sodium acetate pH 3, centrifuged at 500 × g for 5 min at 4 °C and the resulting supernatant (AM fraction) pooled with the previous one. The pellet was washed once in 20 ml sodium acetate pH 3 by centrifugation (500 × g, 5 min, 4 °C) and the final pellet containing the extracted and washed spermatozoa without AM was resuspended in 20 ml Tris-HCl, pH 7.4 and designated the extracted sperm (Ext Spz) fraction.

To isolate AM from cauda spermatozoa, 1 × 10⁸ purified spermatozoa were resuspended in 200 μl 20 mM Tris-HCl, pH 7.4 containing PIC and 0.625% Triton X-100 for 2 min on ice. The extraction buffer was supplemented with additional PIC to prevent dispersion of the AM. The remaining purification steps were the same as described for caput spermatozoa. The number of isolated AM and the percent contamination by spermatozoa was calculated by examining an aliquot of AM fraction that was spread on a slide and stained with peanut agglutinin (lectin from Arachis hypogaea, cat.no.L7381, Sigma, Saint Louis, MO) conjugated to FITC that binds to glycoconjugates on the acrosomal matrix (18). The cauda acrosomal matrix preparation ranged from 98–100% pure whereas the caput acrosomal matrix preparation ranged from 89–95% pure. PNA was used to visualize the AM because of the ease of the staining procedure and because its staining correlated well with that of antibodies against known acrosomal matrix proteins such as zonaheins.

Indirect Immunofluorescence Analysis—Isolated AM were spread on a microscope slide (Colorfrost Plus, ThermoScientific, Kalamazoo, MI) and allowed to dry overnight at RT. AM were fixed with 100% methanol (cat.no.A414, ThermoScientific, Fair Lawn, NJ) for 15 min at RT. After washing in TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) for 10 min at RT, slides were incubated in 100% heat inactivated goat serum (HIGS, cat.no.S-1000, Vector Laboratories, Burlingame, CA) for 1 h at 37 °C, then washed in Dulbecco’s PBST (DPBS containing 1 mM CaCl₂, 0.5 mM MgCl₂ (cat.no.21-030, Cellgro, Manassas, VA) for 10 min at 37 °C, then washed in Dulbecco’s PBST for 10 min at 4 °C, then washed in Dulbecco’s PBST for 10 min at 4 °C and the resulting supernatant (AM fraction) pooled with the previous one. The pellet was washed once in 20 ml sodium acetate pH 3 by centrifugation (500 × g, 5 min, 4 °C) and the final pellet containing the extracted and washed spermatozoa without AM was resuspended in 20 ml Tris-HCl, pH 7.4 and designated the extracted sperm (Ext Spz) fraction.

To isolate AM from caput epididymal mouse spermatozoa, 2.6–4 × 10⁶ purified spermatozoa were incubated in 200 μl 20 mM Tris-HCl, pH 7.4 containing 2% Triton X-100 (Surfact-Amps, cat.no.28314, Thermo Scientific, Rockford, IL) for 2 hr on ice. After incubation, the cell suspension was centrifuged at 2000 × g for 5 min at 4 °C to isolate a supernatant containing soluble proteins and membrane that was designated the Triton-soluble fraction and a pellet containing spermatozoa with exposed AM. The sperm pellet was resuspended in 200 μl 20 mM sodium acetate pH 3 and vortexed for 2 min at RT using a Vortex Genie2 (ThermoScientific) set to position 4 to release the AM from spermatozoa. The sample was then centrifuged at 500 × g for 5 min at 4 °C resulting in a supernatant containing released AM, designated as the AM fraction, and a pellet containing mainly spermatozoa without AM but also some that still had their AM attached. To increase the recovery of AM, the pellet was resuspended in 200 μl 20 mM sodium acetate pH 3, centrifuged at 500 × g for 5 min at 4 °C and the resulting supernatant (AM fraction) pooled with the previous one. The pellet was washed once in 20 ml sodium acetate pH 3 by centrifugation (500 × g, 5 min, 4 °C) and the final pellet containing the extracted and washed spermatozoa without AM was resuspended in 20 ml Tris-HCl, pH 7.4 and designated the extracted sperm (Ext Spz) fraction.
(46.8 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol). Protein extracts were separated by SDS-PAGE according to the method of Laemmli (19) on a 15% polyacrylamide gel (Criterion Tris–HCl gel, Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Western blotting was performed using rabbit anti-guinea pig acrosin, rabbit anti-mouse CRES or rabbit anti-mouse ACRBP antibodies following a protocol described previously (20, 21). Briefly, the membranes were blocked in 3% nonfat dry milk in TBS-T (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 0.2% Tween-20) for 1 h with gentle shaking at RT and then incubated with primary antibody (185 ng/ml rabbit anti-CRES (cystatin 8, cst8), 1:4000 for acrosin (ACR), 1:20,000 for ACRBP) in 3% nonfat dry milk in TBS-T overnight at 4 °C with gentle shaking. After washing with TBS-T (3 X 10 min with shaking) the blots were incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase in 3% nonfat dry milk in TBS-T (1:10000, Invitrogen Corporation, Carmaillio, CA) for 2 h at RT. The blots were washed extensively in TBST and the bound enzyme was detected by chemiluminescence (cat.no.34080, Thermo Fisher Scientific, Rockford, IL or cat.no.170-5070, Bio-Rad Laboratories, Hercules, CA) following the manufacturer’s directions.

Sequence Database Search—To get the maximum number of publicly available sequences for analyses, the mouse sequences from Ensembl (http://www.ensembl.org/info/data/ftp/index.html) were merged with those from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/guide/). The database was built using BioPerl modules installed on a MacBook Pro (OS X 10.6.8) using packages from the Fink project (http://www.finkproject.org/). Specifically, from Ensembl two files were downloaded and merged including Mus_musculus.NCBIM37.64.pep.all.fa.gz (containing the superset of all translations resulting from Ensembl known or novel gene predictions) and Mus_musculus.NCBIM37.64.pep.abinitio.fa.gz (containing translations resulting from “ab initio” gene prediction algorithms such as SNAP and GENSCAN). From NCBI, the nonredundant (nr) file (on 11/2011) was downloaded and mouse sequences extracted (Taxonomy ID = 10090). This file was merged with the Ensembl sequences to create a file containing 356,881 sequences of which some were redundant. Duplicates were removed based on identical amino acid sequences to get 203,220 unique sequences used to build our blast database. Command lines used can be obtained from BLAST® Help (http://www.ncbi.nlm.nih.gov/books/NBK1762/) and from the FAS Center for Systems Biology (http://sysbio.harvard.edu/csb/resources/computational/scriptome/).

All searches were run locally on a Dell Optiplex 990 with an Intel® Core™ i7-2600 CPU @ 3.4-GHz processor and 16 gigabytes of RAM.

Protein and Peptide Identification—Spectra obtained from the tryptic digestion products using the LTQ Orbitrap XL mass spectrometer were identified by the Proteome Discoverer™ (version 1.3) program, based on SEQUEST cluster as a search engine (University of Washington, licensed to Thermo Electron Corp., San Jose, CA) against our mouse database (203,220 nonredundant protein sequences). The search engine used the following parameters: precursor ion mass tolerance, 2.5 Da; fragment ion mass tolerance, 0.8 Da; fully tryptic enzyme specificity; two missed cleavages; dynamic modifications of cysteine carbamidomethylation and of methionine oxidation. The proportion of false positive assignments among the tentative peptide identifications, also called false discovery rate (FDR), has been estimated by using decoy databases constructed from the target database (23) and was set at 1%. Spectra and search results may be downloaded from ProteomeCommons.org Tranche using the following hash: kLgCpMG61AQRPwKwA4JScwdjrM0618nWtIWyObpG uQBMMa0r0BuQB77d/bk/yUqCwWrnLPDCu6fIiKRYtev+raB19oPs AAAAAAElw==. Msf files can be visualized using the freely available viewer thermo-msf-parser (24). For bioinformatic analyses, sequence accession numbers of identified proteins were converted to their corresponding gene ID from the Mouse Genome Database (25).

Data Mining and Bioinformatic Analyses—Mouse Sperm Proteome Database—In order to compare our results with the mouse sperm proteome, a database was built by inclusion of genes based on empirical data of a protein’s presence in
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mouse sperm by MS. The data set comprises 11 previously published data sets. After conversion to Mouse Genome Informatics (MGI) ID’s, 820 unique genes from 26 were merged with 324 from 27; 204 from (28); 112 from (29); 101 from (30); 68 from (31); 52 from (32); 49 from (33); 25 from (34); 20 from (35); and 17 from (36). The combined unique genes from all 11 data sets resulted in a total sperm proteome database consisting of 1162 proteins.  

Protein Classification, Gene Ontology Term Enrichment, Biological Network and Pathway Analysis—Script tasks and comparisons of genes list were performed using the R software version 2.11.0 (http://www.R-project.org) and proportioned Venn diagrams were drawn using Venn Diagram Plotter (http://ornics.pnl.gov/software/VennDiagramPlotter.php). Assignment of protein classification categories was carried out using the Panther database (37) after conversion of MGI ID’s to Entrez Gene ID’s. For Gene Ontology (GO) analyses mouse and human orthologs (extracted from MGI HMD_HGNC_Accession.rpt.file (downloaded on 2-20-12) were combined. GO terms that were associated with mouse (Taxon ID 10090) and human (Taxon ID 9606) genes were retrieved from NCBI gene2gofile (downloaded on 2-20-12) using a Perl command line. GO terms that were enriched in the caput or cauda AM compared with whole AM (combination of caput and cauda AM) were identified using the EASE program (38) as described previously (39). A Benjamini and Hochberg correction (40) was performed on the raw p values to correct for multiple tests for each GO term. A term was defined as significantly enriched when it had an adjusted p value p < 0.01. To decipher GO terms that were “specific” to the caput or cauda AM, we looked for the most representative terms of each protein list using two criteria: 1) at least six proteins had to be associated with the GO term; and 2) these proteins represented 70% or more of all proteins associated with the GO term. For example, if a GO term was represented by 10 proteins in the sperm AM proteome, seven of which were detected only in the caput AM, then this GO term was considered as a being caput AM-specific. To compare proteins identified in the isolated AM with those associated with lysosome-related organelles (LRO), proteins associated with endosome (GO:0005768), lysosome (GO:0005764), secretory granule (GO:0003141), synaptosome (GO:0019717), and melanosome (GO:0042470) were retrieved from mouse GO Cellular Component file.  

Sequence alignment of the cystatin 2 family members including the CRES subgroup and lysozyme family members was done using Clustal W (version 2.1, (41)) and amyloid prediction was determined using Watz (42) with parameters set at threshold = best overall performance and pH = 2.6.  

Low Abundant Proteins of Interest—The data generated by shotgun proteomics experiments are highly redundant, i.e. a subset of the peptides present is repeatedly and preferentially selected for fragmentation and thus identified. In contrast, other subsets of peptides, e.g. those derived from low abundance proteins, are more difficult to detect, and a large number of fragment ion spectra have to be acquired to increase the likelihood of their detection (43, 44). To bypass this issue in the identification of type 2 cystatins and CRES subgroup members and since only one CRES member, CST13 was identified with an FDR < 1%, we decided to look for peptides that matched to this protein family without using an FDR filter but which were assigned by Sequest as being of medium and high confidence. For these peptides, the experimental and theoretical MS/MS spectra were confirmed visually (supplemental Data S11).  

RESULTS

Isolation of Mouse Sperm AM—To isolate AM from mouse spermatozoa we began by working with caput epididymal spermatozoa, which are more resistant to extraction than cauda epididymal spermatozoa and therefore provide a more stable structure with which to work. Unlike cauda spermatozoa, caput epididymal spermatozoa have not undergone the maturation process, which includes modifications of the sperm membrane and thus required increased amounts as well as longer incubations in Triton X-100 to remove the sperm membrane. A brief vortexing step allowed the release of the AM from the demembranated spermatozoa. Cauda epididymal spermatozoa required much lower concentrations and extremely short exposure times to Triton X-100, the presence of additional protease inhibitors as well as the brief vortexing step to allow the release of AM from the spermatozoa without full dispersion of the structure. To demonstrate the isolated structures represented AM, AM from both caput and cauda spermatozoa were analyzed by indirect immunofluorescence using antibodies against known AM markers as well as the lectin peanut agglutinin, PNA, which binds to glycoconjugates on the acrosome and acrosomal matrix. As shown in Fig. 1A, both caput and cauda isolated AM were immunostained with zonadhesin (ZAN), acrosin, (ACR), and acrosin binding protein, (ACRBP) antibodies as well as PNA supporting the premise that the isolated structure indeed represents the AM. In contrast to caput AM that appeared to have a full crescent shape characteristic of the acrosome, cauda AM were of a more blunt shape suggesting that the cauda AM may be more fragile and that some dispersion of the AM may occur during the isolation procedure. Indeed, although zonadhesin was present in the majority of the isolated AM from both the caput and cauda spermatozoa, fewer of the isolated AM from both sperm populations contained ACRBP. Because proteins are known to be differentially released from the AM (45), the absence of ACRBP from some of the isolated AM suggests that some proteins are starting to be released during the isolation procedure.  

To confirm the immunofluorescence studies, the isolated AM as well as the Triton-soluble fraction and extracted spermatozoa from both the caput and cauda epididymis were examined for the proform and mature forms of acrosin by Western blot analysis. As shown in Fig. 1B, as expected, the proform of acrosin (53 kDa) was enriched in both the caput and cauda AM with some of the mature acrosin (39 kDa) released into the Triton soluble fraction during the isolation procedure. In addition to the 53 kDa proacrosin, the caput AM also contained a higher molecular weight form (~55 kDa) that may represent a second proform of acrosin. This is similar to the mixture of 53–55 kDa proacrosin forms found in ejaculated porcine spermatozoa (46, 47). A 25 kDa immunoreactive protein was also detected in the Triton soluble fraction, which may represent a processed form of the mature acrosin. Little to no proacrosin/acrosin was detected in the spermatozoa following the removal of the AM confirming its localization within the matrix and the efficiency of the AM extraction procedure. To control for the very small number of spermatozoa that was present in the AM preparation, the same
number of spermatozoa detected in the AM was loaded on the gel for analysis of acrosin. No detectable proacrosin or acrosin was detected in these samples (Spz con) supporting the premise that the acrosin detected in the AM represented that of the isolated AM structure and not contamination by whole spermatozoa.

**MS Analysis of Isolated Sperm AM**—The isolated sperm AM were next analyzed by MS to identify proteins associated with this structure. Both caput and cauda AM were isolated as described and proteins separated by SDS-PAGE followed by Coomassie staining (Fig. 2A). Different electrophoretic patterns were observed between the caput and cauda AM samples, which may reflect differences in the maturational status of the spermatozoa as well as the fact that fewer caput spermatozoa were loaded on the gel compared with cauda spermatozoa because of fewer spermatozoa present in the caput region. The AM lanes including the stacking gel were cut into 8–9 gel slices, protein digested with trypsin, and samples analyzed by MS. From the MS analysis of caput and cauda AM, 1026 proteins that matched to a gene in MGI were identified with a confidence of 99% (p < 0.01) (supplemental Data S1 and S2). Of these, 676 proteins were present in the Panther database and 535 were classified based on protein function (Fig. 2B) (list of proteins in supplemental Data S3). The highest number of proteins were classified as having cytoskeletal, oxidoreductase, and hydrolase functions fol-
lowed by proteins classified as enzyme modulators, proteases, transporters, transferases, nucleic acid binding, chaperones, transfer/carrier proteins, structural proteins, kinases, and others.

We next compared the 1026 proteins identified in our AM preparation against a database we generated, which included all proteins identified during a proteomic analysis of mouse spermatozoa and that matched to a gene in MGI. This database represented the work of 11 different investigators carrying out proteomic analyses of whole spermatozoa or sperm fractions including isolated membranes, lipid rafts, and flagella. A Venn diagram of this comparison showed that of the 1026 proteins in our AM preparation, 525 were also identified in the sperm database but 501 represented new proteins previously not found by MS/MS in mouse spermatozoa (Fig. 2C) (protein list in supplemental Data S4). These new proteins may represent less abundant proteins, which we identified because an enriched AM preparation was analyzed. Of these 501 proteins, 271 were present in the Panther database and 216 of these were classified based on protein function. The remaining 46% of the AM proteins that were not classified based on function may reflect a population of proteins that are unique to the sperm AM and its functions and thus not present in available protein function databases such as Panther. However, of the 216 proteins that were classified based on function, the majority of proteins were similar to that identified in

![Diagram](image-url)
the 1026 AM proteins and included cytoskeletal proteins, nucleic acid binding, enzyme modulators, hydrolases, and others (Fig. 2D) (protein list in supplemental Data S5).

As a confirmation that the structure analyzed by MS represented the AM, we next examined the 1026 proteins identified in the AM to determine if known AM markers were present. Currently there are 22 proteins that have been shown to be associated with the sperm AM including ACR, ACRBP, ZAN, ZP3R, and others. Of these 22 proteins, 17 including those mentioned above, were identified in our AM preparations with the majority of these proteins identified in both the caput and cauda sperm AM (Table I). These observations support our previous immunofluorescence and Western blot studies that AM were successfully isolated from both the caput and cauda spermatozoa.

**Biological Networks and Pathways**—Having validated that the isolated structures represented AM, we next began to examine the 1026 AM proteome in greater detail by using biological network and pathway software to identify pathways enriched in the AM suggestive of AM functions. We also wanted to determine if these pathways differed between the functionally distinct caput and cauda sperm AM. Using the Gene Ontology (GO) database we first examined the 1026 AM proteome for proteins classified by the GO terms fertilization/sperm-egg recognition/acrosome reaction/fusion and identified 23 proteins including Catsper1 and 2, acrosin, Cd46, a regulatory component of the complement system; Park7, which belongs to the C56 family of peptidases with a putative role as a redox-sensitive chaperone and as a sensor of oxidative stress; Spa17, with proposed roles in cell adhesion; Pkdrej, a polycystin that may generate a calcium channel involved in the acrosome reaction; Zbp2, Zp3r, and Izumo1 (Table II). Several of these proteins have previously been shown to be involved in fertilization but not to be associated with the AM.

To determine whether protein composition differed between the caput and cauda AM, we compared the AM proteins present in the caput spermatozoa (664 proteins) to that in the cauda spermatozoa (873 proteins) and found, not surprisingly, that 511 were common as shown in the Venn diagram in Fig. 3A (protein list in supplemental Data S6). However, 153 AM proteins were unique to the caput spermatozoa whereas 362 proteins were unique to cauda spermatozoa suggesting that during epididymal transit some AM proteins may be lost or modified and other proteins may be added to the AM. Although the association of epididymal secretory proteins with the sperm surface including the acrosome is a common occurrence as spermatozoa pass through the epididymis, the addition and incorporation of epididymal secretory proteins into the AM has not been demonstrated. As shown in Table III, analysis of the 1026 AM proteome showed that several known epididymal secretory proteins, that are known not to be expressed in the testis, were found in the isolated sperm AM suggesting they were added during sperm transit in the epididymis. These proteins included Adam7 and Gpx5 which are specifically expressed by the caput epithelium (48, 49) and detected in the caput AM, and defensin beta 30, expressed by all regions of the epididymis and detected in

| MGI Symbol | Gene Name | MGI ID   | Ref. | Sample   |
|------------|-----------|----------|------|----------|
| Acr | acrosin prepropeptide | MGI:87884 | (45)GP | cpt,cda |
| Acrbp | proacrosin binding protein | MGI:1859515 | (45)GP | cpt,cda |
| Zp3r | zona pellucida 3 receptor | MGI:104965 | (15)M | cpt,cda |
| Zp3bp | zona pellucida binding protein | MGI:1855701 | (68)M,(1)M | cpt,cda |
| Zan | zonadhesin | MGI:106656 | (69)P | cpt,cda |
| Acrv1 | acrosomal vesicle protein 1 | MGI:104590 | (70)H | cpt,cda |
| Spaca3 | sperm acrosome associated 3 | MGI:1922872 | (71)H,(72)M | cpt,cda |
| Spaca4 | sperm acrosome associated 4 | MGI:1916613 | (73)H | cpt,cda |
| Spesp1 | sperm equatorial segment protein 1 | MGI:1913962 | (11)H | cpt,cda |
| Spaca1 | sperm acrosome associated 1 | MGI:1914902 | (74)M | cda |
| Spaca4 | sperm acrosome associated 4 | MGI:1916613 | (73)H | cpt,cda |
| Nptx1 | neuronal pentraxin 1 | MGI:1914902 | (76)H | cda |
| Hexb | hexosaminidase B | MGI:96074 | (78)M | none |
| Gsn | gelsolin | MGI:95851 | (12)GP | none |
| Psmd8 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 8 | MGI:1888669 | (8)M | none |
| Cage1 | cancer antigen 1 | MGI:1918463 | (79)M | none |

**Table I**

*AM markers in the mouse sperm AM proteome.* GP, guinea pig; M, mouse; P, porcine; H, human; Ha, hamster; Sample, AM sample in which the protein was detected. cpt, caput; cda, cauda.
Several lipocalin family members including Lcn2 and Lcn5 expressed primarily by the caput region were also detected in the caput and cauda AM, respectively. **Table II: Fertilization proteins in the mouse sperm AM proteome**

| GO Term                        | Gene          | Gene Full Name                  |
|-------------------------------|---------------|---------------------------------|
| fertilization                 | Ins16         | insulin-like 6                  |
|                               | Klhl10        | kelch-like 10 (Drosophila)      |
|                               | Catsper2      | cation channel, sperm associated 2 |
|                               | Acr           | acrosin prepropeptide           |
|                               | Cd46          | CD46 antigen, complement regulatory protein |
|                               | Dnali1        | dynein, axonemal, light intermediate polypeptide 1 |
|                               | Akap4         | A kinase (PRKA) anchor protein 4 |
|                               | Tubgcp3       | tubulin, gamma complex associated protein 3 |
|                               | Akap3         | A kinase (PRKA) anchor protein 3 |
|                               | Park7         | Parkinson disease 7             |
|                               | Spa17         | sperm autoantigenic protein 17  |
|                               | Smcp          | sperm mitochondria-associated cysteine-rich protein |
|                               | Zp3r          | zona pellucida 3 receptor       |
| sperm-egg recognition         | Spaca3        | sperm acrosome associated 3     |
| acrosome reaction             | Acr           | acrosin prepropeptide           |
|                               | Pkdrej        | polycystic kidney disease (polycystin) and REJ (sperm receptor for egg jelly homolog, sea urchin) |
|                               | Akap3         | A kinase (PRKA) anchor protein 3 |
|                               | Spesp1        | sperm equatorial segment protein 1 |
| fusion of sperm to egg plasma | Adam2         | a disintegrin and metallopeptidase domain 2 |
| membrane                      | Catsper1      | cation channel, sperm associated 1 |
|                               | Izumo1        | izumo sperm-egg fusion 1        |
|                               | Spesp1        | sperm equatorial segment protein 1 |
|                               | Rik           | RIKEN cDNA 4930579C15 gene      |

The cauda AM. Several lipocalin family members including Lcn2 and Lcn5 expressed primarily by the caput region were also detected in the caput and cauda AM, respectively. **Protein Functions Enriched in the Caput AM**—Using the Gene Ontology (GO) database and a confidence of 99% ($p < 0.01$), we compared the 664 proteins present in the caput AM with all proteins detected in the AM (1026 proteins) to determine whether there were specific functional classes of proteins that were enriched in the caput AM. As shown in Fig. 3B, four GO terms were enriched in the caput AM including the biological processes of microtubule-based movement and small molecule metabolic processes and the molecular functions of structural molecule activity and structural constituents of the cytoskeleton (supplemental Data S7). Microtubule-based movement included proteins such as dyneins and tubulins involved in the transport processes, whereas small molecule metabolic processes included molecules involved in cell signaling such as calmodulin 3 (Calm), protein kinases (Prkaca, Prkacb), phosphatases (Ppp1ca), and G-binding proteins (Gnb1), proteosome component proteins Psma2, 4, 5, Psmb1, 5, 6, and proteins involved in vesicle trafficking and fusion (Vamp2 and Vapa). Structural molecule activity proteins included desmoplakin (Dsp), vimentin (Vim), and septin7 (Sep7), that are involved in anchoring of proteins to the cytoskeleton or organization of proteins involved in cell signaling, clathrin (Cltc) involved in intracellular trafficking, and catenin (Ctnna1) involved in cell adhesion. The GO term structural constituent of the cytoskeleton included several spectrin family members (Spna2 and Spnb2) that contribute to the formation of scaffolds, plectin (Plec1) that functions as a scaffolding platform for the assembly of signaling complexes, and clycin (Cycl2), which contributes to the formation of the cytoskeletal calyx of the sperm head. The enrichment of these groups of proteins in caput spermatozoa is consistent with an AM that may still be undergoing maturational changes including organization of its infrastructure and establishment of signaling complexes involved in fertilization and the association of these complexes with the AM itself.

Using the same analysis parameters as with the caput AM we examined whether the 873 proteins present in the cauda AM were enriched in distinct functional classes by comparing these proteins to those present in the 1026 AM proteome. However, no enriched groups were identified with $p < 0.01$ or $p < 0.05$. This may be because more proteins were identified in the cauda AM compared with that in the caput and thus the ability to detect enriched groups was decreased. Alternatively, the cauda AM is in a different functional state from that in the caput and many classes of protein are equally important.

**Caput and Cauda AM-specific Protein Functions**—

**Caput AM-specific Proteins**—To examine more closely protein functions that may be distinct to the caput and cauda AM, we carried out a GO analysis of the 153 proteins found only in the caput AM and the 362 proteins detected only in the cauda AM. We looked for the GO terms that were most representative of the proteins in each AM preparation and reflected caput or cauda “specific” AM functions based on the following criteria: 1) at least six proteins had to be associated with
Fig. 3. **Proteomic changes in the AM during epididymal sperm maturation.** A, Venn diagram comparing the caput AM and cauda AM proteins. B, Gene ontology (GO) terms enriched in the caput AM. The 664 caput AM proteins were compared with the 1026 sperm AM proteome to identify protein classes and their corresponding GO terms that were enriched in the caput AM. List hits, number of proteins present in the caput AM that encode for the listed GO term; list size, number of proteins in the caput AM that encode for the GO class. Pop hits, number of proteins present in the whole sperm AM (caput/H11001 cauda) that encode for the listed GO term; Pop size, number of proteins in the whole sperm AM that encode for the GO class. C, GO terms represented by caput AM-specific proteins. The 153 caput AM proteins were examined to identify GO terms specific to the caput AM. To be considered specific, GO terms had to be represented by at least six proteins and these proteins had to represent 70% or more of all proteins associated with this GO term. The bars represent the number of proteins associated with the GO term while the percentage of proteins per term is shown in italics. For example, 11 proteins in the sperm AM (caput + cauda) proteome were classified under the GO term of “synaptic transmission.” Of these, 82% (9 proteins) were present in the caput AM and thus this GO term...
TABLE III

| MGI Symbol | Gene Name                          | MGI.ID   | Epididymal expression | Localization in spermatozoa | Sample | Ref        |
|------------|-----------------------------------|----------|-----------------------|-----------------------------|--------|-----------|
| Adam7      | a disintegrin and metallopeptidase domain 7 | MGI:107247 | Caput                 | Acrosome                    | Caput   | (48)M(80)M |
| Gpx5       | glutathione peroxidase 5           | MGI:104886 | Caput                 | Acrosome                    | Caput   | (49)M(81)M |
| Lyz6       | lysozyme-like 6                   | MGI:1916694 | Unknown               | Acrosome                    | Both    | (82)H     |
| Dci        | enoyl-Coenzyme A delta isomerase 1 | MGI:94871  | Whole tissue          | Acrosome                    | Cauda   | (83)M(82)H |
| Defb30     | defensin beta 30                  | MGI:1920920 | Whole tissue          | Unknown                      | Cauda AM| (84)         |
| Lcn12      | lipocalin 12                      | MGI:1924951 | Whole tissue          | Unknown                      | Both    | (85)M     |
| Lcn2       | lipocalin 2                       | MGI:96757  | Caput                 | Acrosome                    | Caput AM| (86)M     |
| Lcn5       | lipocalin 5                       | MGI:1277241 | Caput                 | Unknown                      | Cauda AM| (85)M     |

Under the GO class cellular compartment, we examined the proteins that were present in the cauda AM but not the caput AM, a GO term representing proteins involved in the biological processes of negative regulation of EGFR signaling pathway and synaptic transmission, associated with cellular compartments such as the endoplasmic reticulum and cortical cytoskeleton or with organelles such as melanosomes, and involved in molecular functions including structural constituent of cytoskeleton and actin binding. We chose to examine in greater detail an example of each GO class and selected synaptic transmission as the example of a biological process based on previous suggestions that spermatozoa have neuronal-like functions (50, 51). The caput AM proteins associated with the GO term synaptic transmission were all involved in cell signaling and protein transport and included carnk4, calcium/calmodulin dependent protein kinase IV (a serine/threonine kinase); akap9, a kinase anchoring protein 9 (binds to regulatory subunit of protein kinase A); aldh2, aldehyde dehydrogenase; ap2a1, ap2a2, ap2b1, adaptor-related protein complex 2 subunits alpha1, alpha 2 and beta 1 (components of the AP-2 adaptor protein complex involved in protein transport via transport vesicles); Glu1, glutamate ammonia ligase which catalyzes synthesis of glutamine from glutamate and ammonia (glutamine is involved in apoptosis and cell signaling); Gnb1, guanidine nucleotide binding protein 1, (G protein, cell signaling); and Myo6, myosin 6, involved in intracellular vesicle and organelle transport.

Under the GO class cellular compartment, we examined proteins that contributed to the GO term melanosome, based on studies suggesting that the sperm acrosome has some functional overlap with lysosome-related organelles including melanosomes (52). Proteins that fell under this GO term included Pmel, premelanosome protein, that forms internal matrix fibers in melanosomes; Anxa2, annexin a2, a calcium-dependent phospholipid binding protein involved in signal transduction; Pdia6 and Pdia3, protein disulfide isomerase family members, which are part of a large chaperone multi-protein complex and inhibit aggregation of misfolded proteins that play a role in the folding of disulfide-bonded proteins; P4hb, prol 4-hydroxylase beta polypeptide, also a member of the protein disulfide isomerase family and at low concentrations facilitates protein aggregation (anti-chaperone) whereas at high concentrations inhibits protein aggregation (chaperone); Hspa9aa1, an inducible chaperone that promotes structural maintenance of proteins involved in signal transduction and which interacts with many different proteins including ion channels; and Canx, calnexin, a calcium binding molecular chaperone that assists in protein assembly and plays a role in quality control in the ER.

Under the GO class cellular function we examined the GO term structural components of the cytoskeleton because the acrosomal matrix has been shown to contain cytoskeletal proteins (12). Proteins that were listed under this term included the spectrins Spna2, Spnb1, Spnb2, scaffold proteins that organize intracellular organelles; Epb4.1, involved in cytoskeleton/plasma membrane interactions; and Plec1, Vim, and Dsp, involved in cell signaling and scaffolding with the cytoskeleton. Together these data suggest that within caput spermatozoa a fine tuning of the assembly and organization of the AM may be occurring allowing key multiprotein complexes to become oriented properly for downstream functions during fertilization.

Cauda AM-specific Proteins—To examine the proteins that were present in the cauda AM but not the caput AM, a GO analysis of the 362 cauda AM proteins was carried out as described above for the caput AM. Consistent with the idea that was classified as being a caput AM-specific term.

D. Example of a GO term for each GO class that is specific to the caput AM. The proteins classified with the GO terms synaptic transmission, melanosome, and structural constituent of cytoskeleton are shown. E. Example of a GO term for each GO class that is specific to the cauda sperm AM. The proteins classified with the GO terms spermatogenesis, proteasome complex, and serine-type peptidase activity are shown. Rik, 1700019N12Rik; Rikb, 1810009J06Rik.
the cauda sperm AM is in a different functional state from that of the caput sperm AM, many GO terms falling under the classes of biological processes, cellular compartments, and molecular functions were identified and thus because of space limitations are shown in supplemental Data S8. Shown in Fig. 3E, however, are examples of a term under each GO class that we examined further including the GO terms spermatogenesis, proteasome complex, and serine-type peptidase activity. Specifically proteins grouped under the term spermatogenesis included Rik, a Riken clone (170009N12Rik) expressed in germ cells; Bat3, a chaperone that regulates the stability of proteins and their degradation by the proteasome; catserper1, catserper2, catserper, components of a voltage gated calcium channel; Ccdc135, coiled coil domain containing protein; Ccin, calcin, basic protein of the sperm head cytoskeleton; Cct6b, member of the chaperonin containing TCP1 complex, assists in folding of proteins upon ATP hydrolysis; Cylc1, cylcin; basic protein of the calyx of the sperm head cytoskeleton, cytoskeletal calyx surrounding the nucleus; H2afx, histone family member; mycbpap, c-myc binding protein associated protein; oaz3, ornithine decarboxylase antizyme, a germ-cell specific regulator of intracellular polyamines by inhibiting od; Prss21, testisin, a serine protease; Spata18, regulator of mitochondrial quality, and spermatogenesis related proteins Spata19, Spata4, and Spata5.

Cauda AM proteins that were grouped under the GO term proteasome complex were components of the 26S proteasome including Psma1, Psmb4, Psmb7 members of the 20S core and Psmd13, Psmd14, Psmd2, Psmd3, nonATPase subunits of the 19S regulator. Proteins that were grouped under the GO term serine-type peptidase activity included Rikb, a Riken clone (181009J06Rik) with predicted serine peptidase activity; Abhd10, adhydrolase domain containing protein 10, Gm2663, predicted to have serine peptidase activity; Gzmn, granzyme H; Htra1, Htra serine peptidase 1, a secretary peptidase that regulates availability of IGFs; Htra2, Htra serine peptidase 2, involved in apoptosis; Immp1, inner mitochondrial peptidase; Prcp, lysosomal prolylcarboxypeptidase involved in activation of cell matrix prekallikrein; Prss21, testisin, a serine protease; Prss52, a chymotrypsin-like serine protease primarily found in Leydig and Sertoli cells; and Tpp2, tripeptidyl peptidase II, a component of the proteolytic cascade acting downstream of the 26S proteasome.

Relationship of the Sperm Acrosome to Lysosomes-related Organelles (LRO)—Several reports have suggested that the sperm acrosome is derived from and has functions similar to that of lysosomes whereas other studies have suggested relationships with endosomes or secretory granules. Indeed, the acidic pH of the sperm acrosome is not unlike that within lysosomes whereas several components of endocytic transport are proposed to be involved in acrosomal biogenesis (53, 54). Also within secretory granules, proteins are known to be compartmentalized and the acrosome reaction has been compared with the secretion process that occurs from secretory granules. Because cumulative studies do not strictly support the acrosome as being solely Golgi-derived or lysosomal in origin, (55) proposed the acrosome as a novel LRO, which are membrane-bound cytoplasmic organelles including melanosomes, endosomes, and synaptosomes that are restricted to specific cell types and that carry out functions unrelated to degradation. LROs use both the synthetic (derived from Golgi) as well as retrograde (endocytic) transport pathways during their biogenesis as seems to also occur during acrosome formation (52). To determine whether the AM showed similarities with LROs, we compared the 1026 AM proteome with those proteins that were classified by the NCBI GO cellular component term as being associated with endosomes, melanosomes, synaptosomes, lysosomes, and secretory granules. Fig. 4A shows the Venn diagram indicating the overlapping proteins between these cellular structures (protein list in supplemental Data S9). Because the GO database considers the sperm acrosome a secretory granule or a lysosome, several proteins that were associated with these organelles were from the acrosome. Although the AM proteome showed only a 2%, 4%, and 4% match to proteins in endosomes, lysosomes, and synaptosomes, respectively, there was a 16 and 21% overlap of proteins with secretory granules and melanosomes suggesting a more similar relationship between these organelles and the sperm AM. Also, although the overlap between the LRO and the AM was not large, it is important to realize that the sperm proteins represented those of the AM only and not the entire acrosome. Thus it is possible that proteins present in the sperm AM may be present within or associated with similar matrix-like structures in the related organelles.

The proteins that were common between the AM and LRO were categorized based on Panther protein classes as shown in Fig. 4B (supplemental Data S10) and included hydrolases (fucosidase, collagenase, Na/K transporting ATPase subunits 1 and 2, galactocerebrosidase, angiotensin converting enzyme, and lysosomal pro-X carboxypeptidase) and chaperones (Hsp90 alpha, Hspa8, 14-3-3 protein, endoplasmin (Grp94), calnexin, and T-complex protein 1) as being represented by the most number of proteins followed by enzyme modulators (Rab2A, Rab14, RhoB, son of sevenless homolog 2, and AKAP3) proteases (pro-X carboxypeptidase, ADAM2, and acrosin), transporters (Na/K transporting ATPases subunits 1,2,3, and Catper4), transfer/carriers (annexin A2, hippocampal cholinergic neurostimulating peptide, and secretory carrier-associated membrane protein 2), isomerases (protein disulfide isomerases A3, A6), membrane trafficking (clathrin heavy chain, vesicle-associated membrane proteins 2,3), and others.

Amyloidogenic Proteins in the AM—The aggregation of proteins is a proposed mechanism by which cells sort proteins to the secretory pathway (56). Also, several recent reports suggest that amyloids, protein aggregates with a specific cross-β sheet structure, may contribute to the formation of stable structures that carry out biological functions within the cell. Specifically, the PMEL protein, known to be associated with a
matrix-like structure within melanosomes, was shown to form amyloid \textit{in vitro} and was associated with an amyloid structure within the lumen of the melanosome (57). \textit{In vitro} this scaffold served as a template for the synthesis of melanin. Recently, several pituitary hormones were shown to be stored as functional amyloid structures within the secretory granules of the pituitary gland (58). Finally, we have previously demonstrated that the cystatin CRES (cystatin-related epididymal spermatogenic) (Cst8), the defining member of a reproductive subgroup within the family 2 cystatins of cysteine protease inhibitors, forms amyloid \textit{in vitro} and \textit{in vivo} within the epididymal lumen (59, 60). Because CRES is also present in developing germ cells and localizes to the sperm acrosome, it, as well as other amyloidogenic proteins, may contribute to the formation of a stable structure within the AM.

With this background in mind, we next used our AM proteome data to determine whether known amyloidogenic proteins were present within the sperm AM. In this approach we either used a cutoff of 99% confidence (\(p < 0.01\)) or no FDR or looked for peptides that fulfilled all the spectral requirements for a confident identification (visual comparison of experimental and theoretical MS/MS spectra). As shown in Table IV, besides PMEL and CRES, several proteins known to form amyloid were found in the AM. These included cystatin C, superoxide dismutase, and lysozyme 1, which typically are associated with amyloids that cause disease. Because CRES was identified by a single peptide match (full spectral data in supplemental Data S11) we carried out Western blot analysis and confirmed its presence in AM isolated from both caput and cauda spermatozoa (supplemental Data S12).

We next expanded our analysis to examine the 1026 AM proteome for proteins related to the amyloidogenic proteins identified in Table IV to determine whether additional putative amyloid-forming proteins were present in the AM. We then used Waltz software to determine whether these related proteins contained domains predicted to form amyloid. Within the type 2 cystatin family, in addition to cystatin C and CRES, several other cystatins were detected in the sperm AM proteome. These included three cystatin proteins, CstR1, CstR2, and CstR1L, which are not well-characterized but which reside on the cystatin locus with the other cystatin family 2 members, as well as two other CRES subgroup family members including Cst13 (cystatin T) and Cst11 (CRES2) (Fig. 5A). The full spectral data for the identification of the cystatin peptides are shown in supplemental Data S11. All cystatins contained several putative amyloid-forming domains with most including one within their signal peptides as well as two to four other domains in the mature proteins, several of which were conserved between family members. Six members of the lysozyme family were also identified within the AM proteome including Lyz1, Lyz11, Lyz4, Lyz5, and SPACA3 and SPACA5 (sperm acrosome associated 3, 5). Except for Lyz1 and Lyz4, the lysozyme family members

| Table IV: Amyloidogenic proteins in the mouse sperm AM proteome |
|------------------|------------------|
| MGI ID | Gene | Gene Name |
| MGI:98301 | Pmel | premelanosome protein |
| MGI:102519 | Cst3 | cystatin C |
| MGI:107161 | Cst8 | cystatin 8 (cystatin-related epididymal spermatogenic) |
| MGI:98351 | Sod1 | superoxide dismutase 1 |
| MGI:96902 | Lyz1 | lysozyme 1 |

Fig. 4. Relationship between the sperm AM and lysosome-related organelles (LRO). A, Venn diagram comparing the mouse sperm AM proteome (1026 proteins) with proteins categorized by NCBI GO cellular component list as being associated with endosome, lysosomes, synaptosomes, melanosomes, or secretory granules. B, The proteins common to the AM and LRO are listed based on protein class. Panther classes containing at least two proteins are shown.
also had putative amyloid-forming domains in their signal sequences and in the mature proteins. Similar to the cystatins, the amyloid domains in the lysozyme family members were fairly well conserved between members (Fig. 5).

**DISCUSSION**

**Isolation of AM From Caput and Cauda Epididymal Spermatozoa**—The studies presented herein provide a protocol for the isolation of AM from mouse caput and cauda epididymal spermatozoa. The isolation process involves several steps, including lysis, centrifugation, and homogenization. The purified AM preparations are then analyzed using proteomic techniques to identify the proteins present in the acrosomal matrix. A high level of conservation is observed among the proteins identified in different species, indicating the evolutionarily conserved nature of the acrosomal matrix proteome.

**A. Type 2 cystatins including CRES subgroups members.**

| Cystatin | Accession Number |
|----------|-----------------|
| CST3     | NP_034106.2     |
| CST8     | AAC35390.1      |
| CST11    | NP_084335.1     |
| CST13    | CAE51410.1      |
| CSTR1L   | XP_485074.3     |

**B. Lysozyme-like family members.**

| Lysozyme | Accession Number |
|----------|-----------------|
| LYZ4     | NP_038618.1     |
| SPAC3    | XP_001481137.1  |
| LYZ6     | NP_080368.1     |
| SPAC5    | NP_081191.1     |
| LYZ1     | NP_083643.1     |

**Fig. 5. Predicted amyloidogenic protein families present in the mouse sperm AM proteome.** A. Alignment of type 2 cystatins including CRES subgroup family members detected by LC-MS/MS in the sperm AM proteome. Accession number of sequences used are: CST3 (NP_034106.2), CST8 (AAC35390.1), CST11 (NP_084335.1), CST13 (NP_0818300.1), CSTR1L (XP_485074.3), CSTR1 (CAE51410.1) and CSTR2 (XP_001481137.1). B. Alignment of lysozyme-like family members identified in the AM. Accession number of sequences used are: LYZ1 (NP_038618.1), LYZ4 (NP_080368.1), LYZ6 (NP_081191.1), SPAC3 (NP_083643.1) and SPAC5 (NP_001078862.1). Underline, signal sequence; solid bar, predicted amyloidogenic region. Hyphens represent gaps introduced for optimal alignment obtained with ClustalW2.
spermatozoa. Because the caput and cauda spermatozoa represent spermatozoa in different functional states, the isolation protocol had to be modified to successfully isolate an “intact” AM from the two different sperm populations. Our studies show that AM are easier to extract from immature caput spermatozoa than mature cauda spermatozoa. This initially was somewhat surprising given that caudal spermatozoa are the mature cells and known to be highly disulfide crosslinked, which provides important structural stability necessary for its functions of progressive motility and the ability to fertilize. However, modifications of the sperm membrane are also known to occur during epididymal transit and as such it appears that this involves an increase in fluidity of the cauda sperm membrane perhaps as a critical step in preparation for fertilization (61). Therefore, extremely low concentrations and short incubation times in Triton X-100 were required to demembranate cauda spermatozoa compared with that used for caput spermatozoa.

A second noticeable difference between the two sperm populations was that the caput sperm acrosome appeared to contain more protease activity at pH 7.4 compared with caput sperm acrosomes and thus additional protease inhibitors were needed during the AM isolation to prevent dispersion of the structure. The combination of appropriate Triton X-100 exposure, protease inhibitors, and a brief vortexing step allowed us to isolate AM from mouse spermatozoa. Using antibodies against known AM markers in immunofluorescence and Western blot analysis as well as LC-MS/MS identification of these markers in the AM preparations demonstrated our successful isolation of the AM from the caput and cauda spermatozoa.

Proteomic Characterization of the AM—Using nano-LC-MS/MS, we carried out a proteomic analysis of the AM isolated from the caput and cauda spermatozoa and generated an AM proteome composed of 1026 proteins, 501 of which have not been previously identified using a proteomic approach in mouse spermatozoa. These proteins may represent less abundant proteins not previously detected in proteomic studies of whole spermatozoa or proteins that are specific to AM functions and thus detectable only in a purified AM fraction. To identify the putative functions of these proteins, the 501 proteins were examined using the Panther database and ~54% of these proteins were present in the database and classified according to putative function. The absence of 46% of the sperm AM proteome from this analysis may be because proteins associated with the AM are rapidly diverging as a part of the process of adaptive evolution (62) and thus were not present in the Panther database generated during a study of evolutionarily related proteins (37). However, an overview of the proteins with assigned functions indicate the AM is a dynamic structure with proteins not only involved in zona and egg interactions but also those involved in quality control including protein folding and turnover, signaling, cell trafficking, proteases and hydrolyases, and those associated with the cytoskeleton. These proteins may carry out their functions either during spermatogenesis when the AM is formed, during AM maturation in the epididymis, and/or during the fertilization process.

The sperm AM proteome also contained what appeared to be some mitochondrial and sperm axonemal proteins. Although it is possible that these proteins may also be present within the AM, we cannot rule contributions from the small percentage of Triton-extracted spermatozoa that remained in the AM preparations. Although protein was not detected by Coomassie staining in the gel lane containing a representative number of spermatozoa that were present in the isolated AM sample, because mitochondrial and axonemal proteins are highly abundant in a single spermatozoon, it is possible that peptides would be detected by MS/MS analysis.

A number of proteins detected in the sperm AM proteome also contained transmembrane domains consistent with their association with the membrane, likely either the inner or outer acrosomal membrane. Although spermatozoa were treated with Triton-X-100 to remove membranes as part of the AM isolation procedure, studies suggest that some matrix proteins may interact with proteins present in the inner or outer acrosomal membrane. For example, zonadhesin, is initially synthesized as an acrosomal membrane protein that becomes part of the AM during sperm maturation and CD46, a membrane protein, may affect dispersion of the AM proteins during the acrosome reaction (63–65). It may be that an intimate association and crossstalk exists between the AM and its associated acrosomal membranes and the detection of these transmembrane domain containing proteins in the AM proteome may represent stable interactions and complexes that were maintained during the AM isolation procedure. Alternatively, it may be that several proteins, like zonadhesin, start out in the membrane but later become associated with the AM.

AM Maturation in the Epididymis—In addition to a global overview of proteins in the sperm AM, studies were also carried out to determine whether there were differences in AM protein composition between caput and cauda epididymal spermatozoa. Region-dependent differences in AM proteins were detected suggesting that, similar to other sperm domains, the sperm AM undergoes maturational changes during epididymal transit. Although it is possible that the inability to detect some proteins in the caput AM could be owing to the fact that fewer caput AM were isolated and analyzed compared with that from cauda spermatozoa, this would not explain the inability to detect some AM proteins in the cauda that were present in the caput suggesting that some AM proteins may be lost or modified during epididymal transit. It is possible that during epididymal transit, some components of the AM are becoming more stabilized as part of the maturation process and thus are more difficult to solubilize during SDS-PAGE preventing them from being analyzed by LC-MS/MS because they do not enter into the electrophoretic...
gel. In addition to the potential loss of proteins from the AM during maturation, we observed that several epididymal secretory proteins, that are not expressed in the testis, were detected in the sperm AM. This suggests that as part of the sperm maturation process, some proteins in the epididymal lumen become associated with the sperm AM.

GO analysis of the proteins found only in the caput or cauda AM revealed functional groups of proteins that were specific to each sperm population. Although the cauda AM contained a large list of proteins categorized under the GO classes of biological process, cellular compartment, and molecular function, the predominant proteins in each class represented those involved in transport, membranes, and hydrolase activity, respectively. These groups of proteins may reflect a mature sperm AM that has established signaling complexes and high enzymatic activity required for downstream fertilization events. In contrast, caput AM contained proteins that were involved in cell trafficking and transport, associations with the cytoskeleton including scaffolding proteins and control of protein folding. These groups of proteins are more consistent with a sperm AM that is immature and undergoing critical organization and establishment of its infrastructure.

AM: A Structural Scaffold—We also used our AM proteome data to begin to address the possibility that the AM consists of a highly stable core scaffold structure that may form as the result of the self-aggregation of specific proteins. Indeed, previous investigators have suggested that the matrix structure may form as a result of protein aggregation (45, 65). Intriguingly, melanosomes have been shown to possess a matrix structure that serves as a functional scaffold for the synthesis of melanin. This scaffold structure was shown to contain self-aggregates of Pmel protein in an amyloid structure providing the first evidence in mammals of an amyloid carrying out a biological function (57). The Pmel protein in an amyloid structure was shown to contain self-aggregates of Pmel protein that serve as a functional scaffold for the synthesis of melanin.

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REFERENCES

1. Lin, Y. N., Roy, A., Yan, W., Burns, K. H., and Matzuk, M. M. (2007) Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. Mol. Cell Biol. 27, 6794–6805
2. Kang-Decker, N., Mantchev, G. T., Juneja, S. C., McNiven, M. A., and van Deursen, J. M. (2001) Lack of acrosome formation in Hrb-deficient mice. Science 294, 1531–1533
3. Buffone, M. G., Foster, J. A., and Gerton, G. L. (2008) The role of the acrosomal matrix in fertilization. Int. J. Dev. Biol. 52, 511–522
4. Olson, G. E., Winfrey, V. P., and Davenport, G. R. (1988) Characterization of matrix domains of the hamster amorph. Biol. Reprod. 39, 1145–1158
5. Noland, T. D., Davis, L. S., and Olson, G. E. (1989) Regulation of proacrosin conversion in isolated guinea pig sperm acrosomal apical segments. J. Biol. Chem. 264, 13586–13590
6. Olson, G. E., Winfrey, V. P., Garbers, D. L., and Noland, T. D. (1985) Isolation and characterization of a macromolecular complex associated with the outer acrosomal membrane of bovine spermatozoa. Biol. Reprod. 33, 761–779
7. Meyers, S. A., and Rosenberger, A. E. (1999) A plasma membrane-associated hyaluronidase is localized to the posterior acrosomal region of stallion sperm and is associated with spermatozoal function. Biol. Reprod. 61, 444–451
8. Yi, Y. J., Manandhar, G., Sutovsky, M., Jonalová, V., Park, C. S., and Sutovsky, P. (2010) Inhibition of 19S proteasomal regulatory complex subunit PSMD8 increases polyspermy during porcine fertilization in vitro. J. Reprod. Immunol. 84, 154–163
9. Sasanami, T., Yoshizaki, N., Dohra, H., and Kubo, H. (2011) Sperm acrosin is responsible for the sperm binding to the egg envelope during fertilization in Japanese quail (Coturnix japonica). Reproduction 142, 267–276
10. Miyata, H., Noda, N., Fairbairn, D. J., Oldenbourg, R., and Cardullo, R. A. (2011) Assembly of the fluorescent acrosomal matrix and its fate in fertilization in the water strider, Aeshna remigis. J. Cell Sci. 226, 999–1006
11. Wolkomirov, M. J., Shetty, J., Westbrook, A., Klotz, K., Jayes, F., Mandal, A., Glickman, S. J., and Herr, J. C. (2003) Equatorial segment protein defines a discrete acrosomal subcompartment persisting throughout acrosomal biogenesis. Biol. Reprod. 69, 735–745
12. Zepeda-Bastida, A., Chiquete-Felix, N., Uribe-Carvajal, S., and Mujica, A. (2011) The acrosomal matrix from Guinea pig sperm contains structural proteins, suggesting the presence of an actin skeleton. J. Androl. 32, 411–419
13. Huang, T. J., Jr., Hardy, D., Yangamichai, H., Teuscher, C., Tung, K., Wild, G., and Yangamichai, R. (1985) pH and protease control of acrosomal content stasis and release during the guinea pig sperm acrosome reaction. Biol. Reprod. 32, 451–462
14. Hyatt, H., and Gwatkin, R. B. (1988) Characterization of isolated acrosomal
molecular matrices from hamster spermatozoa. 

15. Kim, K. S., Cha, M. C., and Gerton, G. L. (2001) Mouse sperm protein sp56 is a component of the acrosomal matrix. 

16. Cornwall, G. A., Orgebin-Crist, M. C., and Hann, S. R. (1992) The CRES gene: a unique testis-regulated gene related to the cystatin family is highly restricted in its expression to the proximal region of the mouse epididymis. Mol. Endocrinol. 6, 1653–1664.

17. Tanii, I., Oh-oka, T., Yoshinaga, K., and Toshimori, K. (2001) A mouse acrosomal cortical matrix protein, MC41, has ZP2-binding activity and forms a complex with a 75-kDa serine protease. Dev. Biol. 238, 332–341.

18. Cross, N. L., and Meizel, S. (1989) Methods for evaluating the acrosomal status of mammalian sperm. 

19. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

20. Tardif, S., Guyonnet, B., Cormier, N., and Cornwall, G. A. (2012) Alteration of the CRES (Cystatin-related epidymal spermatogenic) gene: a unique testis-regulated gene related to the cystatin family is a component of the acrosomal matrix. 

21. Tardif, S., Guyonnet, B., Cormier, N., and Cornwall, G. A. (2012) Immunolocalization of CRES (Cystatin-related epidymal spermatogenic) protein in the acrosomes of mouse spermatozoa. 

22. Syntin, P., and Cornwall, G. A. (1999) Immunolocalization of CRES (Cystatin-related epidymal spermatogenic) protein: a unique testis-regulated gene related to the cystatin family is a component of the acrosomal matrix.

23. Blake, J. A., Bult, C. J., Kadin, J. A., Richardson, J. E., Eppig, J. T., Mouse Genome Database Group (2011) The Mouse Genome Database (MGD): premier model organism resource for mammalian genomics and genetics. Nucleic Acids Res. 39, D842–D848.

24. Berruti, G., Ripolone, M., and Ceriani, M. (2010) USP8, a regulator of endosomal sorting, is involved in mouse acrosome biogenesis through interaction with the spermatid ESCRT-0 complex and microtubules. Mol. Hum. Reprod. 16, 257–266.

25. Blake, J. A., Bult, C. J., Kadin, J. A., Richardson, J. E., Eppig, J. T., Mouse Genome Database Group (2011) The Mouse Genome Database (MGD): premier model organism resource for mammalian genomics and genetics. Nucleic Acids Res. 39, D842–D848.

26. Baker, M. A., Hetherington, L., Reeves, G. M., and Aitken, R. J. (2008) The mouse sperm proteome characterized via IPG strip pre-fractionation and LC-MS/MS/MS identification. Proteomics 8, 1720–1730.

27. Stein, K. K., Go, J. C., Lane, W. S., Primakoff, P., and Myles, D. G. (2006) Protein profiling of protein-abundance distributions and experimental designs. Nat. Biotechnol. 25, 651–655.

28. Reiter, L., Claassen, M., Schirmpf, S. P., Jovanovic, M., Schmidt, A., Buhmann, J. M., Hengartner, M. O., and Aebersold, R. (2009) Protein identification false discovery rates for very large proteome datasets generated by tandem mass spectrometry. Mol. Cell. Proteomics 8, 2405–2417.

29. Schuel, H., and Burkman, L. J. (2005) A tale of two cells: endocannabinoid-signaling regulates functions of neurons and sperm. Biol. Reprod. 73, 1078–1086.

30. Schuel, H., and Burkman, L. J. (2005) A tale of two cells: endocannabinoid-signaling regulates functions of neurons and sperm. Biol. Reprod. 73, 1078–1086.

31. Zitanski, N., Berruti, G., and Hsiia, N. (1997) ADAM7, a member of the ADAM (a disintegrin and metalloproteinase) gene family is specifically expressed in the mouse anterior pituitary and epididymis. Endocrinology 138, 4262–4272.

32. Ghyselinck, N. B., Jimenez, C., Lefrancois, A. M., and Dufaure, J. P. (1990) Molecular cloning of a cDNA for androgen-regulated proteins secreted by the mouse epididymis. Mol. Endocrinol. 4, 5–12.

33. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) The adult boar testicular and epididymal transcriptomes. Mamm. Genome 18, 419–429.

34. Van de Peer, Y., and De Wachter, R. (1995) Use of the sliding window approach to multiple testing. J. Roy. Statist. Soc. B 57, 289–300.
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Reprod. 82, 330–339
56. Stettler, H., Beuret, N., Pescianotto-Baschong, C., Fayard, B., Taunepot, L., and Spiess, M. (2009) Determinants for chromogranin A sorting into the regulated secretory pathway are also sufficient to generate granule-like structures in non-endocrine cells. Biochem. J. 418, 81–91
57. Fowler, D. M., Koutou, A. V., Alory-Jost, C., Marks, M. S., Balch, W. E., and Kelly, J. W. (2006) Functional amyloid formation within mammalian tissue. PLoS Biol. 4, e6
58. Maji, S. K., Perrin, M. H., Sawaya, M. R., Jessberger, S., von, Horsten, H. H., Johnson, S. S., SanFrancisco, S. K., Hastert, M. C., Olson, G. E., Winfrey, V. P., Bi, M., Hardy, D. M., and NagDas, S. K. (2004) Swanson, W. J., and Vacquier, V. D. (2002) The rapid evolution of reproducitive proteins. Nat. Rev. Genet. 3, 137–144
59. von, Horsten, H. H., Johnson, S. S., SanFrancisco, S. K., Hastert, M. C., Olson, G. E., Winfrey, V. P., Bi, M., Hardy, D. M., and NagDas, S. K. (2004) The rapid evolution of reproductive proteins. Nat. Rev. Genet. 3, 137–144
60. Olson, G. E., Winfrey, V. P., Bi, M., Hardy, D. M., and NagDas, S. K. (2004) Zonadhesin assembly into the hamster sperm acrosomal matrix occurs by distinct targeting strategies during spermiogenesis and maturation in the epididymis. Biol. Reprod. 71, 1128–1134
61. Inoue, N., Ikawa, M., Nakanishi, T., Matsumoto, M., Nomura, M., Seya, T., Baba, T., Iwamatsu, A., and Mori, T. (1993) Purification and functional analysis of epididymal secretory proteins. Nat. Rev. Genet. 3, 137–144
62. Cornwall, G. A. (2012) Nonpathological extracellular amyloid is present during normal epididymal sperm maturation. PLoS ONE 7, e36939
63. Cornwall, G. A. (2003) A new subgroup of the family 2 cystatin-related epididymal spermatogenic subgroup of family 2 cystatins. Endocrinology 144, 909–915
64. Mori, E., Baba, T., Iwamatsu, A., and Mori, T. (1993) Purification and characterization of a 38-kDa protein, sp38, with zona pellucida-binding property from porcine epididymal sperm. Biochem. Biophys. Res. Commun. 196, 196–202
65. Bi, M., Hickox, J. R., Winfrey, V. P., Olson, G. E., and Hardy, D. M. (2003) Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pellucida during ecyysisis of the acrosome. Biochem. J. 375, 477–488
66. Foster, J. A., Klitz, K. L., Flickinger, C. J., Thomas, T. S., Wright, R. M., Castillo, J. R., and Herr, J. C. (1994) Human SP-10: acrosomal distribution, processing, and fate after the acrosome reaction. Biol. Reprod. 51, 1222–1231
67. Mandal, A., Klitz, K. L., Shetty, J., Jayes, F. L., Wolkowicz, M. J., Bolling, L. C., Coonrod, S. A., Black, M. B., Diekmann, A. B., Haystead, T. A., Flickinger, C. J., and Herr, J. C. (2003) SLP1, a unique, intra-acrosomal, non-bacterioytic, c lysozyme-like protein of human spermatozoon. Biol. Reprod. 68, 1525–1537
68. Herrero, M. B., Mandal, A., Digilio, L. C., Coonrod, S. A., Maier, B., and Herr, J. C. (2005) Mouse SLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization. Dev. Biol. 284, 126–142
69. Shetty, J., Wolkowicz, M. J., Digilio, L. C., Klitz, K. L., Jayes, F. L., Diekmann, A. B., Westbrook, V. A., Farris, E. M., Hao, Z., Coonrod, S. A., Flickinger, C. J., and Herr, J. C. (2003) SAMP14, a novel, acrosomal membrane-associated, glycosylphosphatidylinositol-anchored member of the Ly-6/urokinase-type plasminogen activator receptor superfamily with a role in sperm-egg interaction. J. Biol. Chem. 278, 30506–30515
70. Mitra, K., Rangaraj, N., and Shivaji, S. (2005) Novelty of the pyruvate metabolic enzyme dihydrolipoamide dehydrogenase in spermatozoa: correlation of its localization, tyrosine phosphorylation, and activity during sperm capacitation. J. Biol. Chem. 280, 25743–25753
71. Atlas-White, M., Murphy, B. F., and Baker, H. W. (2000) Localisation of zonadhesin in normal human sperm by immunogold electron microscopy. Pathology 32, 258–261
72. Hao, Z., Wolkowicz, M. J., Shetty, J., Klitz, K., Bolling, L., Sen, B., Westbrook, V. A., Coonrod, S., Flickinger, C. J., and Herr, J. C. (2002) SAMP32, a testis-specific, isoantigen sperm acrosomal membrane-associated protein. Biol. Reprod. 66, 735–744
73. Noland, T. D., Friday, B. B., Mait, M. T., and Gerton, G. L. (1994) The sperm acrosomal matrix contains a novel member of the pentaxin family of calcium-dependent binding proteins. J. Biol. Chem. 269, 32607–32614
74. Miller, D. J., Gong, X., and Shur, B. D. (1993) Sperm require beta-N-acetylgalactosaminidase to penetrate through the egg zona pellucida. Development 118, 1279–1289
75. Alsheimer, M., Drewes, T., Schütz, W., and Benavente, R. (2005) The cancer/testis antigen CAGE1 is a component of the acrosome of sperm-matids and spermatozoa. Euro. J. Cell Biol. 84, 445–452
76. Oh, J., Woo, J. M., Choi, E., Kim, T., Cho, B. N., Park, Z. Y., Kim, Y. C., Kim, D. H., and Cho, C. (2005) Molecular, biochemical, and cellular characterization of epididymal ADAMs, ADAM7 and ADAM28. Biochem. Biophys. Res. Commun. 331, 1374–1383
77. Jimenez, C., Ghyselinck, N. B., Depegeas, A., and Dufaure, J. P. (1990) Immunoochemical localization and association with spermatozoa of an-drogen-regulated proteins of MR 24000 secreted by the mouse epididymys. Biol. Cell 68, 171–174
78. Li, J., Liu, F., Wang, H., Liu, X., Liu, J., Li, N., Wan, F., Wang, W., Zhang, C., Jin, S., Liu, J., Zhu, P., and Liu, Y. (2010) Systematic Mapping and Functional Analysis of a Family of Human Epididymal Secretory Sperm-Located Proteins. Mol. Cell. Proteomics 9, 2517–2528
79. Johnston, D. S., Jelinsky, S. A., Bang, H. J., DiCandelo, P., Wilson, E., Kopf, G. S., and Turner, T. T. (2005) The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis. Biol. Reprod. 73, 404–413
80. Yenugu, S., Chintalgattu, V., Wingard, C. J., Radhakrishnan, Y., French, F. S., and Hall, S. H. (2006) Identification, cloning and functional character-ization of novel beta-defensins in the rat (Rattus norvegicus). Reprod. Biol. Endocrinol. 4, 7
81. Suzuki, K., Lareyre, J. J., Sánchez, D., Gutierrez, G., Araki, Y., Matusik, R. J., and Orgebin-Crist, M. C. (2004) Molecular evolution of epididymal lipocalin genes localized on mouse chromosome 2. Gene 339, 49–59
82. Chu, S. T., Lee, Y. C., Nein, K. M., and Chen, Y. H. (2000) Expression, immunolocalization and sperm-association of a protein derived from 24p3 gene in mouse epididymis. Mol. Reprod. Dev. 57, 26–36