Testase 1 (ADAM 24) a plasma membrane-anchored sperm protease implicated in sperm function during epididymal maturation or fertilization

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SUMMARY
Plasma membrane-anchored proteases have key roles in cell signaling, migration and refashioning the cell surface and its surroundings. We report the first example of a plasma membrane-anchored protease on mature sperm, testase 1 (ADAM 24). Unlike other studied sperm ADAMs (fertilin α and β, cyritestin) whose metalloprotease domains are removed during sperm development, we found testase 1 retains an active metalloprotease domain, suggesting it acts as a protease on mature sperm. Testase 1 is a glycoprotein (molecular mass 88 kDa), localized to the equatorial region of the plasma membrane of cauda epididymal sperm. Typically, proteolytic removal of the pro-domain is an initial activation step for ADAM proteases. The pro-domain of the testase 1 precursor (108 kDa) is proteolytically removed as sperm transit the caput epididymis to produce processed (mature) testase 1 (88 kDa). Testase 1 is unique among all studied ADAMs in that its proteolytic processing occurs on the sperm plasma membrane instead of at an intracellular site (the Golgi). Using GST-fusion proteins and a synthetic testase 1 C-terminal peptide, we found that the cytoplasmic tail of testase 1 could be phosphorylated in vitro by protein kinase C (PKC). Thus testase 1 apparently has a cytoplasmic PKC phosphorylation site(s). Protein kinase C is known to stimulate other ADAMs’ protease activity. Because events of the acrosome reaction include PKC activation, we speculate that testase 1 protease function could be important in sperm penetration of the zona pellucida after sperm PKC is activated during the acrosome reaction.

Key words: Testase 1, ADAM, Membrane-anchored protease, Sperm, Fertilization

INTRODUCTION
The rapid pace of gene discovery is posing many questions for developmental and cell biologists. Genes for thousands of proteins of unknown function are being identified and various approaches must be taken to learn about the biology of the gene products. We have been studying the rapidly growing ADAM gene family (a disintegrin and metalloprotease domain). Currently, ~29 distinct ADAM family members from different organisms (Caenorhabditis elegans to humans) have been reported (Primakoff and Myles, 2000). The ADAMs are transmembrane proteins, ~750 amino acids long. Most have the same domain structure that includes: the pro-domain, the metalloprotease, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic tail domains. It is believed that the ADAM proteins have potentially both cell adhesion and protease activity because they have high sequence homology with snake venom disintegrins and metalloproteases. Although all ADAMs have a metalloprotease domain, about one third of the reported ADAMs have an amino acid sequence in the active site region that is incompatible with metalloprotease activity. Thus these ADAMs are believed to lack protease activity.

One of the best-studied cell adhesion molecules of the ADAM family is the heterodimer fertilin α/β (ADAM 1/ADAM 2). Several lines of evidence, including fertilin-β-knockout mice, support the idea that fertilin plays an important role in fertilization, especially in sperm-egg adhesive interactions (Myles et al., 1994; Evans et al., 1995; Cho et al., 1998; Chen et al., 1999). A well-studied example of an ADAM metalloprotease is TACE (ADAM 17), which contains the highly conserved zinc-binding catalytic motif HEXGHXXGXXHD and belongs to the reprolysin subfamily of zinc-dependent metalloproteases. It has been well documented that TACE functions in cell surface shedding of important plasma membrane-anchored proteins, including tumor necrosis factor α (TNF-α) and transforming growth factor α (TGF-α) (Moss et al., 1997; Black et al., 1997; Peschon et al., 1998), and may also be an α-secretase that cleaves the Alzheimer amyloid precursor protein (Buxbaum et al., 1998).

Although 14 of the ADAMs are expressed in a wide variety of somatic tissues, the other 15 are expressed exclusively (12 ADAMs) or predominantly (3 ADAMs) in the testis. It is hard, at this stage, to imagine what 12 testis-specific and 3 testis-predominant ADAMs might do. We have recently described the cloning of three novel, testis-specific ADAMs termed testase 1 (ADAM 24), testase 2 (ADAM 25) and testase 3 (ADAM 26) (Zhu et al., 1999). Based on the amino acid sequence in each metalloprotease domain active site, each of
the three is predicted to be an active protease. As a paradigm for analysis of testis-specific ADAMs, we have chosen testese 1 and posed questions applicable to any testis-specific ADAM: is the ADAM present on spermatozoic cells and/or sperm? Is the ADAM on the cell surface? Is it processed like other sperm ADAMs (ADAMs 1, 2 and 3)? Can we expect that processing regulates activity? If it is on the mature sperm cell surface, what are its possible roles in fertilization?

Our data show that testese 1 is the first identified plasma membrane-anchored protease on sperm. Testese 1 is unique among all studied ADAMs in being proteolytically processed while on the cell surface to release the pro-domain, an activation step that occurs during sperm transit of the epididymis. Our findings allow the study of a sperm surface protease that functions in the complex scheme of fertilization.

MATERIALS AND METHODS

Preparation of antibodies against the cytoplasmic tail domain and the predicted active site of the disintegrin domain of testese 1

A rabbit polyclonal antibody to the cytoplasmic tail of testese 1 was generated by synthesizing a peptide containing the C-terminal 30 amino acids QPAGETVKPYEGAPEYTVKPPDEWNP. This peptide, conjugated to Keyhole Limpet Hemocyanin (KLH), was used as antigen for the immunization of rabbits (Multiple Peptide Systems, San Diego, CA, USA). The crude antisera were purified using a column with bound peptide. The affinity-purified antibody is termed ADAM 24-CT.

To produce antibody to the active site of the disintegrin domain of testese 1, a chimeric peptide PSDDKHEQYKLKASGTLCRARENESLPEWCNGTSH was used. In this peptide, the N-terminal sequence PSDDKHEQYKLK is a T-cell epitope from the malaria circumsporozoite protein that elicits a strong T-cell response and thus obviates the need for conjugating the peptide to a carrier protein (Good et al., 1987). The T-cell epitope is followed by a single A as a spacer and then a C-terminal sequence SGTLCRARENEDLPEW-CNGTSH from the predicted disintegrin domain active site. The chimeric peptide was synthesized and cyclized using two cysteines in the disintegrin domain by the W. M. Keck Biotechnology Resource Center (Yale University, New Haven, CT, USA). The residue after ENE is also a cysteine in the wild-type sequence but substituted with alanine in this peptide to prevent the formation of a disulfide bond with one of the other cysteines. The cyclized peptide was purified by HPLC, and its sequence was confirmed by mass spectroscopy. Female B10.A(4R) mice (The Jackson Immuno-Research Laboratory Inc., Bar Harbor, ME, USA) were immunized on day 0 intraperitoneally with 100 μg of chimeric peptide emulsified in complete Freund’s adjuvant (Sigma Chemical Co., St Louis, MO, USA) followed by two additional intraperitoneal injections (56 day and 84 day) with 100 μg of chimeric peptide emulsified in incomplete Freund’s adjuvant (Sigma). Serum was collected by tail bleeds from the mice 2 weeks after the third injection. This antisera to the disintegrin domain active site region of testese 1 is termed ADAM 24-AS.

Preparations of testicular cells, testicular sperm and epididymal sperm

Testicular cells and testicular sperm were isolated using 52% isotonic Percoll (Sigma) as reported previously (Phelps et al., 1990). Epididymal sperm were isolated from the cauda epididymis and vas deferens of 12-15-week-old male ICR mice (Harlan Sprague Dawley Inc., Indianapolis, IN, USA). Dissected cauda and vas deferens were rinsed, and the sperm were released into Whittingham’s medium (Whittingham, 1971) containing 3% BSA.

In vitro capacitation and in vivo ‘capacitation’

For in vitro capacitation and acrosome reaction, epididymal sperm were collected as described above in Whittingham’s medium containing 3% BSA. Released sperm were incubated at 37°C, 5% CO2 for 3 hours in the same medium or in the same medium containing 10 μM A23187 (Sigma) for capacitation and acrosome reaction.

For in vivo ‘capacitation’, 10-week-old female ICR mice (Harlan Sprague Dawley) were superovulated by the injection of 10 IU of pregnant mare’s serum gonadotropin (PMSG; Sigma) followed 48 hours later by an injection of 5 IU of human chorionic gonadotropin (hCG; Sigma). About 12 hours after hCG injection, each superovulated female mouse was put together with a male ICR mouse in a cage. After 3.5 hours, the females were checked for copulation plugs and the time was recorded as the starting time for in vivo ‘capacitation’. After another 1, 3 or 6 hours, ‘capacitated’ sperm were collected from the female’s uterus in Whittingham’s medium containing 3% BSA.

Immunoblot analysis

Testicular cells, testicular sperm, epididymal sperm and sperm from the uterus were collected as described above, washed twice with PBS, resuspended in 1× SDS sample buffer, heated at 100°C for 4 minutes, and centrifuged at 14,000 rpm for 15 minutes. The supernatants were used for electrophoresis. SDS-PAGE was conducted on 10% resolving gels with 4% stacking gels. About 5×105 cells were loaded in each lane. After electrophoresis, proteins were transferred to nitrocellulose membranes (0.2 μM; Bio-Rad Laboratories, Richmond, CA, USA), which were then blocked with 3% nonfat dry milk and 2.5% BSA in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween, PH 7.5). The affinity-purified antibody ADAM 24-CT at a final concentration of 1 μg/ml, followed by alkaline phosphatase-conjugated secondary antibody (Promega Biotech, Madison, WI, USA), were added in TBST containing 3% nonfat dry milk and 2.5% BSA. Alkaline phosphatase activity was detected by color developed with Western Blue stabilized substrate (Promega Biotech).

Cell surface labeling and immunoprecipitation analysis

Testicular sperm and cauda epididymal sperm were collected as described above, washed twice with PBS, resuspended in PBS containing 2 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA), and incubated at room temperature for 30 minutes. The biotinylated sperm samples were then washed twice with PBS, resuspended in the lysis buffer (PBS containing 30 mM n-octyl-beta-D-glucopyranoside; CALBIOCHEM, La Jolla, CA, USA) and incubated on ice for 45 minutes, and centrifuged at 14,000 rpm at 4°C for 15 minutes. The supernatants were incubated with ADAM24-CT (20 μg/ml) at 4°C for 4 hours, then Protein A beads (Pierce) were added and incubated for another 2 hours with end-to-end rotation. The Protein A beads were pelleted and washed with the lysis buffer 5 times, then resuspended in 1× SDS sample buffer, heated at 100°C for 5 minutes, and centrifuged at 14,000 rpm at 4°C for 15 minutes. The supernatants were collected. Immunoblot analysis was carried out as described above. Alkaline phosphatase-conjugated streptavidin (Promega Biotech) was used to detect the biotinylated proteins.

Sperm immunofluorescence

Cauda epididymal sperm were collected as described above in Whittingham’s medium containing 3% BSA. After incubation at 37°C and 5% CO2 for 15 minutes, sperm were washed once with PBS containing 3% BSA. Preimmune serum or the antisera to the disintegrin active site region of testese 1, at 1:500 final dilution, was mixed with a 100 μl sperm sample (107/ml) in PBS containing 0.3% BSA. After 30 minutes incubation at room temperature, sperm samples were layered onto 0.5 ml of PBS with 3% BSA and pelleted gently. Goat anti-mouse IgG (H+L) conjugated with Alexa Fluor688 (Molecular probes, Eugene, OR, USA) was added to the sperm
sample at a final concentration of 5 μg/ml, incubated for 30 minutes at room temperature in the dark, and sperm were washed through PBS with 3% BSA. Before observation, the sperm were fixed with 1.5% paraformaldehyde for 10 minutes and then washed once with PBS. The fluorescence images from the fixed sperm were acquired with an Axioskop microscope (Carl Zeiss Inc.) connected to a computer with a QED image program.

In vitro phosphorylation by protein kinase C
To test if the cytoplasmic tail of testase 1 can be phosphorylated by protein kinase C (PKC), the 30 C-terminal amino acids of the testase 1 cytoplasmic tail (QPAGETVKKPYYEAQPSYETVKPPDEWANP) was obtained from Multiple Peptide System. In addition, two glutathione-S-transferase (GST)-cytoplasmic tail fusion constructs were made. The first was a GST-cytoplasmic tail of testase 1 (GATKKSRREAAASQPAGETVKKPYYEAQPSYETVKPPDEWANP); the second was a GST-cytoplasmic tail of mouse fertilin α (EVQEVVSPPSSSSESSSSSWSDDSQ). The cytoplasmic tails were constructed by PCR using gene-specific primers with the added restriction enzyme sites BamHI and EcoRI to clone directly into the PGEX-3X vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Each construct was sequenced to rule out any mutations possibly introduced during PCR. The two fusion proteins and GST alone were expressed in E. coli BL21 cells (Amersham Pharmacia Biotech). To purify the fusion proteins or GST, the cells were lysed by mild sonication, and GST-fusion proteins or GST was allowed to bind to a glutathione affinity matrix (Amersham Pharmacia Biotech).

In vitro phosphorylation employed a PKC system (Stratagene, La Jolla, CA, USA) as described in the manufacturer’s instructions. Fifteen μg of each purified GST-fusion protein or GST, or 15 μg of synthesized testase 1 cytoplasmic tail were used as substrates in the reaction that contained 10 μCi [γ-32P]ATP (Amersham Pharmacia Biotech) and 17.5 ng PKC (Stratagene) in a final volume of 30 μl. Reaction mixtures were incubated at 30°C for 10 minutes. After the reaction, an equal volume of 20% (wt/vol) trichloroacetic acid/80% acetone was added to precipitate the proteins, which were then resolved on 16.5% Tris-Tricine SDS-PAGE (Bio-Rad, Hercules, CA). The gel was exposed to X-ray film (Kodak, Rochester, NY) for autoradiography.

Carbohydrate analysis
Testicular cells, testicular sperm and epididymal sperm were collected as described above, washed twice with PBS, resuspended in the lysis buffer which is PBS containing 30 mM n-octyl-beta-D-glucopyranoside (CALBIOCHEM, La Jolla, CA), incubated on ice for 45 minutes, and centrifuged at 14,000 rpm at 4°C for 15 minutes. The supernatants were denatured in the denaturing buffer (0.5% SDS, 1% beta-mercaptoethanol) at 100°C for 10 minutes, then incubated in G5 buffer with Endoglycosidase EndoH (New England Biolabs Inc., Beverly, MA, USA) or in G7 buffer with Endoglycosidase PNGaseF (New England Biolabs) at 37°C for 2 hours. The digested products were used in western blot analysis as described above.

RESULTS
Testase 1 is a sperm surface glycoprotein localized to the equatorial region
The deduced protein sequence of testase 1 contains six potential N-linked glycosylation sites. To determine whether testase 1 is a glycoprotein containing N-linked oligosaccharides, extracts from testicular cells, testicular sperm and cauda sperm were incubated with two endoglycosidases, EndoH and PNGaseF. The digested products were run on SDS-PAGE and then blotted with the antibody ADAM 24-CT. In the case of testicular sperm and cauda sperm, the carbohydrate moieties of testase 1 are resistant to EndoH but sensitive to PNGaseF (Fig. 1), showing that the N-linked oligosaccharides of testase 1 are modified in the Golgi complex prior to its insertion in the plasma membrane. Surprisingly, in the case of testicular cells, the carbohydrate moieties of testase 1 are sensitive to both EndoH and PNGaseF (Fig. 1). This dual sensitivity suggests that either the ‘testicular cell’ population has only a low percentage of cells in which transit of testase 1 through the Golgi is complete, or that testase 1 protein is stored in the endoplasmic reticulum throughout the testicular stage of spermatogenesis.

Individual sperm surface proteins are usually localized to a specific domain of the sperm plasma membrane and each domain has specific functions. To determine the localization of testase 1, live, swimming, acrosome-intact sperm were stained with the antibody ADAM 24-AS, which recognizes the active site region of the testase 1 disintegrin domain. On >90% of sperm we observed bright, fluorescent staining restricted to the equatorial region (as defined in Toshimori et al., 1992; Fig. 2). The pre-immune serum and antisera pre-incubated with 200μg/ml of the immunizing testase 1 peptide did not show any fluorescence (data not shown). Moreover, fluorescence was not diminished when the antisera was pre-incubated with only a T-cell epitope peptide with sequence PSDKHSFQYSLKK (data not shown).

Testase 1 is a monomer on the sperm surface
The ADAM family protein fertilin is a heterodimer of fertilin α (ADAM1) and fertilin β (ADAM2) on the surface of guinea pig (Blobel et al., 1990), bovine (Waters and White, 1997) and mouse sperm (Cho et al., 2000). The fertilin heterodimer can be observed directly on SDS-PAGE if the sperm protein sample is prepared in 0.5% SDS with no reducing agent and is not boiled (Blobel et al., 1990). We asked whether testase 1 associates with another subunit(s) in a multi-subunit protein that can be detected under similar conditions. Cauda epididymal sperm were lysed in PBS buffer containing the non-ionic detergent n-octyl-beta-D-glucopyranoside. When this sperm extract was treated with different final concentration of

![Fig. 1. Testase 1 is a glycoprotein with N-linked oligosaccharides.](image-url)

Protein sample was taken from testicular cells and from sperm cells of different developmental stages. The samples were (+) or were not (−) treated with the endoglycosidases EndoH or PNGaseF at 37°C for 2 hours. The prestained molecular weight markers (GIBCO-BRL) are indicated on the right side. Lanes 1-3, testicular cells; lanes 4-6, testicular sperm; lanes 7-10, cauda epididymal sperm.
SDS without DTT at room temperature (25°C) or at 100°C for 3 minutes before it was subjected to immunoblot analysis, the only detected band was of apparent molecular mass 78 kDa (Fig. 3). When this sperm extract was treated with 2% SDS containing DTT and boiled at 100°C for 3 minutes before it was applied to the western blot, the only detected band was ~88 kDa (Fig. 3). This result suggests that testase 1 does not associate with other subunits. Further evidence from cell surface labeling and immunoprecipitation analysis supports the idea that testase 1 is a monomer on the mature sperm surface (see below).

**Testase 1 is processed during sperm passage through epididymis**

ADAM family metalloproteases are synthesized as precursors (~105 kDa) that include an N-terminal pro-domain (~20 kDa). In some ADAMs it has been shown that the pro-domain maintains the metalloprotease in an inactive state and pro-domain removal, by proteolytic cleavage, is an activation step (Loechel et al., 1998; Lum et al., 1998; Roghani et al., 1999). To determine whether testase 1 undergoes similar proteolytic processing, we immunoblotted testicular cells and sperm cells at different developmental stages with the affinity-purified antibody against the cytoplasmic tail of testase 1. This antibody should be able to recognize both the precursor and processed forms if an N-terminal pro-domain is proteolytically removed. Using the antibody ADAM 24-CT, we detected one major band of 108 kDa in the samples of testicular cells and testicular sperm (Fig. 4). A smaller major band of 88 kDa was found in the samples of corpus sperm, cauda sperm and vas deferens sperm (Fig. 4). There were two bands, 108 kDa and 88 kDa, in the sample of caput sperm (Fig. 4). These results suggest that the pro-domain of testase 1 is removed during sperm passage through the caput epididymis.

For many years it has been suggested that sperm surface proteins are modified during the processes of capacitation or acrosome reaction before they can perform their specific biological functions. To test this possibility of modification for testase 1, we prepared sperm samples after in vitro capacitation and acrosome reaction, and after in vivo 'capacitation' in the uterus. Our results clearly indicate that testase 1, after being processed in the caput epididymis, is not further processed during in vitro or in vivo conditions (data not shown).

**Testase 1 is on the sperm plasma membrane when it is processed**

Removal of the pro-domain is known to be required for protease function for several matrix metalloproteases and ADAM proteases, such as ADAM 9 (Roghani et al., 1999) and ADAM 12 (Loechel et al., 1998). An intracellular furin-like pro-protein convertase appears to be responsible for this processing during the ADAMs’ progress through the secretory pathway. Unlike many other ADAMs, testase 1 does not have a consensus furin cleavage site, RX(K/R)R, as a target for furin-type convertases at the boundary of the pro-domain and metalloprotease domain. This suggests that the mechanism of proteolytic processing and activation of testase 1 is different. As shown above, the pro-domain of testase 1 is not removed...
until sperm pass through the caput epididymis. It is accepted that, after the testicular sperm stage, there is no de novo protein synthesis or protein sorting to the plasma membrane in sperm. This suggests that testase 1 is a novel case, an ADAM protease that is not processed in the secretory pathway, but only after it arrives on the plasma membrane. To test this hypothesis, we first biotinylated surface proteins of testicular sperm and cauda sperm, then performed immunoprecipitation with the ADAM 24-CT antibody. Testase 1 was biotinylated on both testicular and cauda sperm, as shown by a shift to a slightly (~2 kDa) higher molecular mass of the detected bands (Fig. 5, lanes 2,3) which were blotted with streptavidin-conjugated alkaline phosphatase. This result suggests that the testase 1 precursor resides on the plasma membrane of testicular sperm and the testase 1 processed form resides on the plasma membrane of cauda sperm, supporting the idea that testase 1 pro-domain removal occurs on the sperm plasma membrane. That only one biotinylated testase 1 band was detected in this immunoprecipitation also confirms the above result that testase 1 is a monomer (or homomultimer).

**In vitro phosphorylation by protein kinase C**

For reasons detailed in the Discussion, it seems possible that PKC regulates an ADAM protease activity during sperm maturation or fertilization. Examining its sequence, we found the cytoplasmic tail of testase 1 contains two potential PKC phosphorylation sites, both TVK. We synthesized a 30 amino acid peptide representing the C-terminus of the testase 1 cytoplasmic tail and expressed a GST-testase 1 cytoplasmic tail fusion protein. Using purified PKC, we found that both the 30 residue peptide and the testase 1 cytoplasmic tail fusion protein can be phosphorylated in vitro (Fig. 6, lanes 1,3). The control proteins were GST alone (Fig. 6, lane 2) and a GST-fertilin α cytoplasmic tail fusion protein (Fig. 6, lane 4). They both showed relatively limited phosphorylation that might occur at two PKC sites that exist in the GST sequence. (The sequence of the cytoplasmic tail of fertilin α lacks a PKC phosphorylation site). These results indicate that the cytoplasmic tail of testase 1 can function as a PKC substrate in vitro.

**DISCUSSION**

We previously reported that testase 1 is a testis-specific ADAM (Zhu et al., 1999). Using RT-PCR, we found the messenger RNA for testase 1 is first detectable in mouse testes on day 10 after birth, then reaches close to an adult level by day 20 after birth. This result suggests that testase 1 transcripts are made predominantly in round spermatids. The deduced amino acid sequence of testase 1 includes a conserved metalloprotease active site HEXGHXXGXXHD in its metalloprotease domain, a typical disintegrin domain and a typical Arg-Gly-Asp (RGD) motif in its cysteine-rich domain, suggesting that testase 1 has dual functions of protease and adhesion molecule. These general possibilities were derived from analysis of the cDNA sequence.

Data from cloning and sequencing projects must ultimately be expanded and enriched until they become part of our knowledge of how biological processes work. Among the 15 ADAMs expressed exclusively (12) or predominantly (3) in the testis, three of those initially discovered (ADAMs 1-3) have been studied in some detail. ADAMs 2 and 3 have been found to have great importance in fertilization (Evans et al., 1995; Cho et al., 1998; Chen et al., 1999; Yuan et al., 1997; Shamsadin et al., 1999). For example, a gene-knockout of ADAM 2 produces male infertility and sperm defective in multiple steps in fertilization, including migration from uterus to oviduct, binding to the zona pellucida and binding to and fusing with the egg plasma membrane (Cho et al., 1998). For the other 12 ADAMs with exclusive or predominant testis expression, usually the cDNA sequence is all that is known. Thus information is lacking about basic biological features of the testis ADAMs such as what testicular cell type expresses
the processing is different from other ADAMs studied to date. Domain processing suggests that the underlying mechanism of structural feature and the surface location of testase 1 pro-domain. Testase 1 lacks the RX(K/R)R furin site. This boundary region of the pro-domain and metalloprotease site, RX(K/R)R, as a target for a furin-like enzyme in the resulting in activation of the metalloprotease activity. Many metalloproteases' progress through the secretory pathway, to remove the inhibitory free cysteine residue during the pro-domain by an intracellular furin-like convertase is expected in the case of previously studied ADAMs, proteolytic cleavage of the metalloprotease domain (the 'cysteine switch mechanism') (Loechel et al., 1999). In the case of several ADAMs (Izumi et al., 1998; Lammich et al., 1999; Skovronsky et al., 2000; Gutwein et al., 2000). We found the testase 1 cytoplasmic tail is a PKC substrate in vitro and sperm PKC is activated at the time of the acrosome reaction (Breitbart and Naor, 1999). Because testase 1 loses its pro-domain, but is apparently not further processed during capacitation or the acrosome reaction, this ADAM is a plasma membrane-anchored protease on fertilizing sperm. An appealing hypothesis is that testase 1 proteolytic activity on the sperm surface could be functionally important at any point after sperm traverse the caput. One can hypothesize that testase 1 modifies other proteins on the sperm surface during the later stages of epididymal maturation and/or during fertilization.

A second activation step might be phosphorylation of the cytoplasmic tail TKV sites by PKC. PKC activation through phorbol esters results in stimulation of the protease activities of several ADAMs (Izumi et al., 1998; Lammich et al., 1999; Skovronsky et al., 2000; Gutwein et al., 2000). We found the testase 1 cytoplasmic tail is a PKC substrate in vitro and sperm PKC is activated at the time of the acrosome reaction (Breitbart and Naor, 1999). Because testase 1 loses its pro-domain, but is apparently not further processed during capacitation or the acrosome reaction, this ADAM is a plasma membrane-anchored protease on fertilizing sperm. An appealing hypothesis is that testase 1 proteolytic activity, stimulated by PKC phosphorylation at the time of the acrosome reaction, functions in sperm penetration of the zona pellucida. It is known that sperm digest a distinct path through the zona pellucida and an empty slit with sharp borders can be seen in the zona structure where a sperm has penetrated (Yanagimachi, 1994). The morphology of these paths, particularly the sharp borders, suggests the action of a sperm membrane-anchored protease as opposed to soluble proteases, which would diffuse and cause a gradient of zona dissolution. It will be of value to test the role in zona penetration of testase 1 as all the attention until now has been given to soluble, acrosomal proteases. The main candidate among the soluble proteases, acrosin, has been found to have limited importance. Acrosin-null male mice have normal fertility and acrosin-null sperm can penetrate the zona pellucida (Baba et al., 1994), although the kinetics of fertilization by acrosin-null sperm might be altered (Adham et al., 1997). At our current level of knowledge, the role of a plasma membrane-anchored protease should be considered. In
the earlier fertilization step when sperm penetrate the cumulus cell layer surrounding the egg, the plasma membrane-anchored hyaluronidase PH-20 functions to create the path to the zona to the path to the zona (Lin et al., 1994).

Another possibility is that testase 1 has a role in sperm-egg fusion. A potential role for sperm metalloproteases in gamete fusion has been studied by various groups. Meizel and colleagues reported that an ‘early’ metalloprotease activity, acting during the acrosome reaction, is important for the human sperm’s ultimate ability to fuse with zona-free hamster eggs (Diaz-Perez et al., 1988; Diaz-Perez and Meizel, 1992). Lennarz and colleagues demonstrated that inhibition of a ‘late’ sperm metalloprotease activity, acting after sea urchin gamete binding, resulted in a virtually complete block to gamete fusion (Roe et al., 1988). Similarly, our lab recently reported that a TIMP-3-sensitive, Zn^{2+}-dependent metalloprotease plays an important role after mouse gamete plasma membrane binding and before gamete membrane fusion (Correa et al., 2000). It will be of great interest to investigate further whether testase 1 provides one of the metalloprotease activities suggested in these inhibitor studies.

In summary, the experiments presented here tell us that a metalloprotease, testase 1, on the surface of sperm undergoes an initial activation step in the caput epididymis. Thus sperm transiting the corpus and cauda epididymis, and mature sperm have a membrane-anchored metalloprotease on their surface. Testase 1 could act on other sperm surface proteins in preparation for fertilization or on egg coat or surface proteins during fertilization. These studies define a novel type of sperm surface enzyme whose existence has not been previously suspected or posited in models of sperm function.

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