Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pellucida during exocytosis of the acrosome

Ming Bi*, John R. HICKOX*, Virginia P. WINFREY†, Gary E. OLSON† and Daniel M. HARDY*¹

*Department of Cell Biology & Biochemistry, Texas Tech University Health Sciences Center, 3601 Fourth Street, Lubbock, TX 79430, U.S.A., and †Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.

Zonadhesin is a sperm protein that binds in a species-specific manner to the extracellular matrix ZP (zona pellucida) of the mammalian oocyte. The pig zonadhesin precursor is a 267 000-Da mosaic protein with a Type I membrane topology and a large extracellular region comprising meprin/A5 antigen/mu receptor tyrosine phosphatase, mucin and five tandem von Willebrand D (VWD) domains. Multiple mature forms of zonadhesin in the sperm head differ in their avidities for the ZP. To determine the potential functions of zonadhesin forms in gamete adhesion, we characterized the processing, activation and localization of protein in pig spermatozoa. The predominant polypeptides of processed zonadhesin were M_r 300 000 (p300), 105 000 (p105) and 45 000 (p45). p45 and p105, comprised primarily the D1, D2–D3 domains respectively, and were N-glycosylated. p300 was heavily O-glycosylated, and spanned the meprin/A5 antigen/mu receptor tyrosine phosphatase, mucin and D0 domains. Hydrolysis of the precursor polypeptide occurred in the testis, and N-terminal sequencing of p45 and p105 identified Asp⁸⁰⁶-Pro and Asp¹¹⁹¹-Pro

in the D1 and D2 domains respectively as bonds cleaved in the protein's functional maturation. Testicular zonadhesin was extractable with non-ionic detergents, and localized to the developing outer acrosomal membrane of round and elongating spermatids. As spermatozoa transited the epididymis, most of the protein became incorporated into an extraction-resistant fraction, and the proportions of active and of multimeric zonadhesins in the cells increased. Zonadhesin localized to the perimeter of the acrosome in intact ejaculated spermatozoa and to the leading edge of acrosomal matrix overlying cells with disrupted acrosomal membranes. We conclude that the zonadhesin precursor is specifically proteolysed, glycosylated and assembled into particulate structures in the distal parts of the acrosome where it may mediate specific adhesion to the ZP during the initial stages of acrosomal exocytosis.

Key words: acrosome, adhesion, fertilization, processing, spermatozoa, zona pellucida.

INTRODUCTION

Sperm adhesion to the egg's extracellular matrix is a key recognition event in animal fertilization [1–3]. In spermatozoa of marine invertebrates, adhesion molecules are stored in the acrosome, a unique secretory granule on the cell's anterior head. Egg investments induce exocytosis of the acrosome, and exposed acrosomal proteins then mediate gamete recognition and adhesion by binding to complementary adhesion molecules on the egg [2,4,5]. Species specificity of the interaction derives in part from remarkable variation in the structures of the sperm proteins [2].

Gamete recognition in mammals is more complex than in marine invertebrates. Mammalian fertilization occurs within the female tract, hours or more after mating, rather than externally and seconds after spawning as for marine invertebrates. Testicular spermatozoa of mammals are not fertile, and only acquire the ability to fertilize after sequential post-testicular maturation in the epididymis and post-coital activation in the female's reproductive tract [1]. In addition, mammalian spermatozoa can adhere productively to the oocyte's extracellular matrix ZP (zona pellucida) both before [6] and after [7] acrosomal exocytosis and, probably, at various stages in between [8]. These complexities in the cellular events of mammalian fertilization are reflected in the elaborate structures of the mammalian sperm cell's anterior head [1,9–11], and in correspondingly complex repertoires of sperm surface and acrosomal proteins [12–14].

Zonadhesin is a large, mosaic protein on the anterior head of mammalian spermatozoa that may mediate species-specific adhesion to the ZP [15–17]. Zonadhesin comprises in part three protein domain types that function in cell adhesion, namely MAM (meprin/A5 antigen/mu receptor tyrosine phosphatase), putative mucin and VWD (von Willebrand D) domains [16,18,19]. The cDNAs of pig [16], mouse [18], rabbit [19] and human (T. L. Cheung, M. J. Wassler, J. D. Harris, G. A. Cornwall and D. M. Hardy, unpublished work) zonadhesins encode precursor molecules that exhibit substantial variation in both amino acid identity and in predicted domain structure. In addition, the polypeptide compositions of zonadhesin in mature spermatozoa of various species differ dramatically ([15,16,18,19]; T. L. Cheung, M. J. Wassler, J. D. Harris, G. A. Cornwall and D. M. Hardy, unpublished work; M. Bi, V. P. Winfrey, G. E. Olson and D. M. Hardy, unpublished work). Such inter-species differences in zonadhesin may subserve a function in species-specific adhesion to the ZP.

Post-translational modifications alter the ZP-binding activity of pig zonadhesin. The protein's functional maturation includes both specific proteolysis and the formation of disulphide-bonded oligomers [15,17]. Heterogeneity in this processing generates multiple forms of the protein with differing apparent avidities for the ZP [17]. The p105/45 monomeric form of pig zonadhesin binds preferentially to conspecific ZP, and based on basis peptide sequences and immunochemical results comprises, at a minimum, parts of the precursor's D1–D3 VWD domains

Abbreviations used: DFP, di-isopropyl fluorophosphate; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; HNE, Hepes/NaCl/EDTA solution; MAM, meprin/A5 antigen/mu receptor tyrosine phosphatase; PNGase F, peptide N-glycosidase F; VWD, von Willebrand D; ZP, zona pellucida.

¹ To whom correspondence should be addressed (e-mail Daniel.Hardy@ttuhsc.edu).

[16,17]. Glycosylation may also affect zonadhesin's binding activity in pig, as 19 potential sites for N-glycosylation are present in the precursor's MAM and VWD domains, and the putative mucin domain includes numerous potential sites for O-glycosylation. Despite the potential importance of processing to zonadhesin's function in fertilization, no studies have characterized the glycosylation of the protein or the timing of proteolysis and oligomerization during sperm development.

To understand the function of zonadhesin in adhesion to the ZP, it is essential to determine both the protein's subcellular location and the processes that generate and target the active molecule to that location in mature spermatozoa. We therefore determined zonadhesin's composition, ultrastructural localization and binding activity at different stages of sperm maturation. The results demonstrate that activation of the zonadhesin precursor occurs by a combination of unique proteolysis and assembly of processed forms into a particulate compartment at the periphery of the acrosome, where the activated protein may mediate species-specific adhesion of spermatozoa to the ZP during exocytosis of the acrosome.

EXPERIMENTAL

Animal use

All animal procedures were performed according to methods approved by the institutional committees that oversee care and use of laboratory animals. The procedures conformed to requirements of the Association for Assessment and Accreditation of Laboratory Animal Care.

Antisera and antibodies

Affinity-purified antibodies to the pig zonadhesin D1 and D3 domains, and antisera to the D0–D1 domains and to zonadhesin holoprotein purified from spermatozoa were those described previously [17]. Antisera to the N-terminal MAM domain region were prepared similarly. Briefly, a PCR product (sense primer: 5'-TTGGATCCTGTGAAGAGCTTTC-3'; antisense primer: 5'-GGGCCGGAATTCCTATCTGAAAGACTGG-3'), encoding a predicted antigenic region (Cys¹⁴²-Arg²¹²), was directionally cloned into the *Bam*HI and *Eco*RI sites of pGEX-2T (Amersham Biosciences, Piscataway, NJ, U.S.A.), and recombinant glutathione S-transferase fusion protein was produced in *Escherichia coli* strain BL21 by induction with 0.1 mM isopropyl β -D-thiogalactoside for 2 h at 37 °C. Soluble fusion protein was extracted by sonicating washed bacterial cell pellets in PBS (10 mM NaPO₄/150 mM NaCl, pH 7.4), containing 0.5 mM DFP (di-isopropyl fluorophosphate; Sigma), 1.0 mM EDTA, 10 μ M E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.] and 0.2% Triton X-100, and purified by chromatography on glutathione–Sephadex (Amersham Biosciences). Antisera to the purified fusion protein were raised in two female New Zealand White rabbits [17].

Isolation of sperm membranes

Sperm membranes were isolated by differential centrifugation of cells disrupted by N₂ cavitation at 650 lb/in² (1 lb/in² = 6.9 kPa) as described previously [15,17].

Deglycosylation

Zonadhesin was enzymically deglycosylated using a kit from Prozyme (San Leandro, CA, U.S.A.) according to the supplier's

instructions. Glycosylation patterns of zonadhesin polypeptides in sperm membranes were determined by Western-blot analysis. Briefly, 5 μ l of sperm membrane extract (approx. 35 μ g of protein) was diluted by the addition of 25 μ l of Hepes/EDTA buffer (50 mM Hepes/1 mM EDTA, pH 7.5), 10 μ l of 5 $\times$ reaction buffer (0.25 M sodium phosphate, pH 7.0) and 2.5 μ l of denaturation solution [2% (w/v) SDS/1 M 2-mercaptoethanol]. The mixture was heated to 100 °C for 5 min, cooled to 22 °C and reactions (50 μ l final mixture) were started by adding 2.5 μ l of 2% (v/v) Triton X-100 solution and different combinations of deglycosylation enzymes, including PNGase F (peptide N-glycosidase F), sialidase A, endo-O-glycosidase, β (1-4)-galactosidase and glucosaminidase (1 μ l each). After incubation for 16 h at 37 °C, deglycosylated zonadhesin polypeptides were detected by Western-blot analysis with domain-specific antibodies (anti-D1, anti-D3 and anti-MAM).

To determine the time course of deglycosylation, 19.8 μ g (450 μ l) of affinity-purified zonadhesin [17] was precipitated with trichloroacetic acid, re-dissolved in 2% SDS and incubated with enzymes as described above. At intervals (30 min, 1, 2 and 4 h), 10 μ l aliquots were removed from the reaction solution, and the reaction was terminated by adding an equal volume of 2 $\times$ SDS/PAGE sample buffer and immediate freezing. After SDS/PAGE, separated proteins were first stained with Coomassie Blue, and then re-stained with silver [20].

Collection of epididymal spermatozoa and testis

Testes were removed from two 6-month-old boars and kept on ice for 30 min during transport. Epididymides were removed and caudal spermatozoa were flushed (retrograde) at 22 °C with HNE (Hepes/NaCl/EDTA solution: 20 mM Hepes/130 mM NaCl/1 mM EDTA, pH 7.5). Caput and corpus epididymides were separately minced and shaken gently in HNE for 20 min at 22 °C. Tissue debris was removed by filtration through two layers of cheesecloth, and sperm motility was checked by light microscopy. Testes were cut into pieces and frozen immediately at –80 °C.

Detergent extraction of spermatozoa and testis tissue

Spermatozoa (ejaculated or epididymal) were washed once with HNE. After centrifugation (300 g, 10 min, 22 °C), cell pellets were first extracted with 10 vol. of 1% Triton X-100 solution (1% Triton X-100/150 mM NaCl/1 mM EDTA/10 μ M E-64/0.5 mM DFP/10 mM Tris/HCl, pH 7.5) for 10 min at 22 °C and centrifuged (3000 g, 15 min, 22 °C). The supernatant solution was collected as Triton X-100 extract, and the pellet was re-extracted with 10 vol. of 2% SDS solution (2% SDS/1 mM EDTA/10 μ M E-64/0.5 mM DFP/10 mM Tris/HCl, pH 6.8) by the same procedure. The supernatant solution of the re-extraction was collected as SDS extract. For detergent extraction of denuded spermatozoa, cell pellets obtained after membrane disruption by N₂ cavitation were sequentially extracted with Triton X-100 and SDS as for intact spermatozoa. To extract proteins from testis tissue, 100 mg of frozen testis tissue was ground into powder with a mortar and pestle under liquid N₂. The ground, frozen tissue was then sequentially extracted with 1 ml each of Triton X-100 and SDS solutions as for extraction of spermatozoa.

ZP-binding assay

Direct binding of zonadhesin to particulate ZP was determined as described previously [15–17]. Isolated pig ZP (20 μ g of protein) were mixed with Triton X-100 extract of spermatozoa (200 μ l

containing 200 μ g of protein), and the suspension was rocked for 1 h at 22 °C. ZP with bound sperm protein were rapidly washed at 22 °C with $2 \times 200 \mu$ l of 1 % Triton X-100/HNE and $3 \times 200 \mu$ l of mRIPA (modified radioimmune precipitation assay solution; composition: 1 % Triton X-100/0.5 % sodium deoxycholate/0.1 % SDS/0.5 M NaCl/1 mM EDTA/25 mM Na-Hepes, pH 7.5) by vacuum filtration on a 0.45 μ m hydrophilic membrane (Millipore, Billerica, MA, U.S.A.). The washed ZP-sperm protein complexes were then solubilized in 40 μ l of $1 \times$ SDS/PAGE sample buffer (no reducing agent), separated by one- or two-dimensional SDS/PAGE, and characterized by Western blotting with specific antibodies.

Immunoprecipitation

Proteins in sperm membranes were biotinylated on amino groups as described previously [15,17]. The biotinylated proteins were solubilized at 10 mg/ml in 1 % SDS and then diluted to 1 mg/ml with HNE containing 1 % Triton X-100, 0.5 % (w/v) sodium deoxycholate, 10 μ M E-64 and 100 μ M aprotinin (Roche Molecular Biochemicals). To pre-clear, 1 μ l of irrelevant antiserum (against an insect protein) was added to 400 μ l of diluted extract, and the mixture was incubated for 1 h at 0 °C. Protein A-Sepharose (10 μ l of a 50 % slurry; Amersham Biosciences) was then added, the suspension rocked for 1 h at 4 °C and the beads were collected by centrifugation (10 000 *g*, 15 s, 4 °C). The pre-cleared supernatant solution was then used for immunoprecipitation with anti-D1 antibody (5 μ l), anti-D3 antibody (1 μ l), anti-MAM antiserum (1 μ l) or holoprotein antiserum (1 μ l) and Protein A-Sepharose as for the pre-clearing. The beads from pre-clearing and from immunoprecipitation were each washed by centrifugation with 3×1 ml of HNE containing 1 % Triton X-100, and bead-bound immune complexes were eluted with 50 μ l of $1 \times$ SDS/PAGE sample buffer. Biotinylated proteins were detected on Western blots with streptavidin peroxidase (Biosource International, Camarillo, CA, U.S.A.) and chemiluminescence development.

Electrophoresis and Western blotting

SDS/PAGE and Western blotting were performed as described previously [15,17]. Gradient gels (4–12 % linear) were used for each dimension of two-dimensional gels to resolve both high-molecular-mass zonadhesin isoforms and their constituent polypeptides. Dilutions used to detect zonadhesin on Western blots of pig sperm membranes were: D1 antibody, 1/5000; D3 antibody, 1/50 000; anti-MAM antiserum, 1/50 000; and holoprotein antisera, 1/50 000, each in Tris-buffered saline with 0.1 % Tween 20. In some experiments, a combination of D1 and D3 antibodies was used to detect p45 and p105 simultaneously on one blot. To obtain similar strengths of the signal, the anti-D1 antibody was diluted 1/5000, whereas the anti-D3 antibody was diluted 1/100 000. Bound antibody was detected with horseradish peroxidase-conjugated secondary antibody (Biosource International) diluted 1/50 000 in Tris-buffered saline with Tween, and developed by chemiluminescence.

Localization by light microscopy

Spermatozoa were recovered from pig epididymides and washed as described previously [15]. Washed cells were fixed for 30 min in suspension with freshly prepared 1 % (w/v) paraformaldehyde/PBS [21]. The fixed cells were washed with PBS containing 10 % (v/v) heat-inactivated goat serum, and then

incubated for 1 h in D0–D1 antisera diluted 1/400 in PBS/heat-inactivated goat serum. After washing the cells with PBS, they were smeared on polylysine-coated coverslips [21], and the bound antibody was detected by incubating for 1 h with 15 nm gold-conjugated secondary antibody followed by silver enhancement (Goldmark Biologicals, Phillipsburg, NJ, U.S.A.). Developed coverslips were mounted with Fluoromount G and viewed by differential interference contrast microscopy.

Immunofluorescence

Freshly ejaculated spermatozoa were washed, fixed with 4 % (w/v) paraformaldehyde, and immunofluorescence was performed as described previously [14], except that antiserum to zonadhesin holoprotein (diluted 1/400) was used to detect zonadhesin. Bound antibody was detected with Texas Red-conjugated antibody to rabbit immunoglobulin (Biosource International), and cells were viewed by epifluorescence and phase-contrast microscopy [17].

Immunoelectron microscopy

Post- and pre-embedding immunoelectron microscopy of pig spermatozoa was performed as described previously [22,23], using antisera to the zonadhesin holoprotein to detect zonadhesin and non-immune sera as a negative control. For post-embedding immunolabelling, testes and spermatozoa were fixed with 4 % formaldehyde and 0.25 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and embedded in LR White Resin. Bound zonadhesin antibody on immunolabelled sections was detected with 10 nm gold-conjugated secondary antibody (Amersham Biosciences). For pre-embedding immunolabelling, intact and Triton X-100-permeabilized (0.25 % Triton X-100 in PBS, 20 min, 0 °C) spermatozoa were fixed with 2 % glutaraldehyde in 0.1 M sodium phosphate (pH 7.4), rinsed in PBS containing 50 mM glycine to block free aldehydes, and then immunolabelled in suspension with anti-zonadhesin and gold-conjugated secondary antibody. The immunolabelled spermatozoa were then fixed with glutaraldehyde, post-fixed with OsO_4 and embedded in Epon.

N-terminal amino acid sequencing

Separated polypeptides (4–12 % SDS/PAGE, protein disulphides reduced) of purified zonadhesin (40–50 μ g; [17]) were blotted to polyvinylidene difluoride membranes, stained with Coomassie Blue, and the p105 and p45 bands were excised. N-terminal sequences of both polypeptides were determined by automated Edman degradation in two different laboratories (HHMI/Biochemistry Core Laboratory, UT Southwestern Medical Center, Dallas, TX, U.S.A.; and the Biotechnology Core Laboratory, Texas Tech University Center for Biotechnology & Genomics, TX, U.S.A.), with the same results.

RESULTS

Characterization of zonadhesin forms in mature spermatozoa

We previously identified an M_r 300 000 zonadhesin polypeptide, designated p300 (protein disulphides reduced), that was recognized by antisera to the D0–D1 domains but not by antibodies specific for the D1 domain [17]. Antisera to zonadhesin's N-terminal MAM domains recognized p300 and three additional M_r 40 000–65 000 polypeptides (protein disulphides reduced) on Western blots of sperm membrane proteins (Figure 1). Without

Figure 1 Identification of zonadhesin polypeptides containing MAM domains

Proteins in membrane fractions of pig ejaculated spermatozoa were separated by one-dimensional (**a**) (NR, disulphides not reduced; R, disulphides reduced) or two-dimensional (**b**) SDS/PAGE (4–12% linear gradient gels), and zonadhesin polypeptides were detected on Western blots with anti-MAM antisera. Note that the antisera recognized an M_r 350 000 non-reduced polypeptide (**a**, lane 1) as well as M_r 300 000 and 45 000–60 000 reduced polypeptides (lane 2). In addition, the M_r 300 000 and 45 000–60 000 reduced polypeptides were covalently associated in high M_r (≥ 900 000) disulphide-bonded complexes (**b**). The non-reduced $M_r \geq 900$ 000 complexes were not detected in lane 1 of (**a**) because the complexes are so large that little protein enters the 4% resolving gel, and even less is subsequently transferred out of the gel during blotting.

disulphide reduction, these polypeptides were present in covalent $M_r \sim 350$ 000 and $M_r \geq 900$ 000 complexes (Figure 1). On immunoprecipitation (Figure 2a), anti-D1 and anti-D3 antibodies, which bind p45 and p105 respectively [17], each co-precipitated p300 and p105. The same polypeptides were also precipitated with antiserum to zonadhesin holoprotein, which was raised against zonadhesin purified from pig spermatozoa (including p300, p105 and p45) and recognized disulphide non-reduced zonadhesin on Western blots [17]. In contrast, anti-MAM antisera precipitated little p105 (Figure 2a). The two-dimensional electrophoresis pattern (Figure 2b) of polypeptides precipitated by the anti-MAM antisera was nearly identical with the pattern recognized by the antisera on Western blots (Figure 1b).

To determine whether p300 is a glycoprotein, we deglycosylated sperm membranes and tracked the mobility of p300 on Western blots (Figure 3a). N-deglycosylation with PNGase F for 16 h did not change the mobility of p300. However, the mobility of p300 did increase when O-linked oligosaccharide core structures were removed with endo-O-glycosidase. Maximal deglycosylation of p300, to M_r 160 000–220 000 products, was achieved when sugars on the O-linked core structure that can interfere with the action of endo-O-glycosidase were concomitantly removed with a mixture of sialidase A, β -(1,4)-galactosidase and glucosaminidase. To examine the time course of O-deglycosylation, zonadhesin immunopurified from sperm membranes was incubated for up to 16 h with the full cocktail of enzymes (O + M; Figure 3b). The mobilities of p45 and p105 did not change with 16 h of O-deglycosylation. In contrast, p300 was hydrolysed within 1 h to smaller polypeptides similar to those obtained on O-deglycosylation of crude membranes. No further mobility shift occurred with 15 h of additional incubation, indicating that the digestion had reached an endpoint.

To determine whether other zonadhesin components are also glycoproteins, we deglycosylated sperm membranes and tracked the mobilities of p45 and p105 on Western blots

Figure 2 Immunoprecipitation of zonadhesin polypeptides from sperm membranes

Biotinylated proteins were precipitated with zonadhesin antibodies or antisera, resolved by SDS/PAGE, then detected on Western blots with streptavidin peroxidase. (**a**) Zonadhesin forms were immunoprecipitated with anti-D1, anti-D3, anti-MAM or anti-holoprotein as designated on the top of the blot (4–12% gel, protein disulphides reduced). The control lane shows proteins remaining after pre-clearing with irrelevant antisera. Note that anti-D1, anti-D3 and anti-holoprotein co-precipitated p105 and p300, whereas anti-MAM precipitated relatively little p105. IgG heavy chain (broad $M_r \sim 50$ 000 band in each lane) interfered with the detection of p45. (**b**) Proteins immunoprecipitated with anti-MAM were separated by two-dimensional SDS/PAGE. Note the similarity to the corresponding anti-MAM Western blot (Figure 1b).

(Figure 4a). Digestion with PNGase F increased the mobility of both p45 (Figure 4a, left) and p105 (Figure 4a, right), but digestion with endo-O-glycosidase did not. Removing N-linked oligosaccharides of purified zonadhesin with PNGase F (Figure 4b) for up to 4 h did not affect the mobility of p300, but did increase the mobility of both p105 and p45. N-deglycosylation of p105 and p45 was evident within 1 h and complete by 2 h, as mobilities did not increase with 2 h of additional incubation (Figure 4b). Fully N-deglycosylated p105 and p45 migrated with M_r 90 000 and 40 000 respectively. Oligosaccharide structure usually does not interfere with PNGase F action, so these M_r s probably reflect the actual masses of the bare polypeptide chains of the glycoproteins.

To examine the nature of zonadhesin's association with sperm structures, spermatozoa were partially demembrated by N_2 cavitation at 650 lb/in² (1 lb/in² = 6.9 kPa) and then sequentially extracted with 1% Triton X-100 and 2% SDS solutions. On Western blots, D0–D1 antisera detected p105, p60–90 and p45 in the Triton extract (Figure 5a). This heterogeneous pattern of zonadhesin polypeptides mirrored the pattern in membranes

Figure 3 Enzymic deglycosylation of p300

(a) Proteins in sperm membranes were deglycosylated for 16 h (t_{16}) at 37 °C with combinations of deglycosylation enzymes as indicated (P, PNGase F; O, endo-O-glycosidase; M, mixture of sialidase A, β -(1,4)-galactosidase and glucosaminidase). Deglycosylated p300 was detected on Western blots (4–12 % gel, protein disulphides reduced) with anti-MAM antiserum. Note that the mobility of p300 increased only when endo-O-glycosidase was present, and its mobility increased further with the addition of other enzymes that hydrolyse O-linked sugars. Mobilities of p300 incubated for 16 h with PNGase F or with no enzyme were the same as the mobility of p300 in the starting material (t_0). (b) The time course of O-deglycosylation was determined by incubating purified zonadhesin with a combination of endo-O-glycosidase and a mixture of other O-deglycosylation enzymes (O + M) for 1, 2, 4 or 16 h as indicated. Deglycosylated polypeptides were resolved by SDS/PAGE (4–12 % gel, silver-stained, protein disulphides reduced). Note that deglycosylation of the p300 component was completed within 1 h, with no further changes in mobility apparent in 15 h of additional incubation. Note also that no mobility changes occurred in control reactions incubated without added enzymes.

released by cavitation (Figure 5a and [17]). In contrast, the SDS extract contained primarily p300, p105 and p45 (Figure 5a). Western blots probed with a mixture of anti-D3 and anti-D1 antibodies (Figure 5b) confirmed the presence of authentic p105 and p45 in the three fractions (membranes, Triton extract and SDS extract). Zonadhesin resistant to extraction with Triton X-100 alone was not solubilized with 1 % Triton X-100 solution containing 0.5 M NaCl, but was extracted with 1 % Triton X-100 solution containing 6 M urea (results not shown).

To identify proteolytic processing sites within the zonadhesin precursor, we sequenced the N-termini of purified p45 and p105. The sequence of the first ten amino acids of p45, PHYLTDFGRR, matched Pro⁸⁰⁷-Arg⁸¹⁶ near the beginning of the D1 domain in the pig zonadhesin precursor. The sequence of the first nine amino acids of p105, PHYLTDFGA, was strikingly similar to the N-terminal sequence of p45 and matched Pro¹¹⁹²-Ala¹²⁰⁰ near the beginning of the D2 domain.

Maturation-associated changes in zonadhesin properties

To determine the timing of post-translational processing events, we compared the properties of zonadhesin in spermatozoa from different regions of the epididymis (caput, corpus and cauda), ejaculated semen and testis (Figure 6). Both p45 and p105 (detected with anti-D1 and anti-D3 antibodies respectively) were present in Triton X-100 extracts of spermatozoa at all stages of maturation, from testis to ejaculated cells (Figures 6a and 6b). Most of p105 and p45 in epididymal or ejaculated spermatozoa were resistant to extraction with Triton X-100, and was recovered by subsequent extraction with SDS. The relative amounts of both p45 and p105 in the SDS extract increased as spermatozoa passed through the epididymis. However, neither p105 nor p45 was present in the SDS extract of testis (Figures 6a and 6b). Nearly all

Figure 4 Enzymic deglycosylation of p45 and p105

(a) Proteins in sperm membrane fractions were deglycosylated for 16 h (t_{16}) at 37 °C with combinations of deglycosylation enzymes (O, P and M as in Figure 3). Deglycosylated p105 and p45 were detected on Western blots (SDS/PAGE, 4–12 % gels, protein disulphides reduced) with anti-D1 (left panel) or anti-D3 (right panel) antibodies respectively. Note that the mobilities of p105 and p45 increased only when PNGase F was present; mobilities of p105 or p45 incubated for 16 h with endo-O-glycosidase or with no enzyme were the same as the mobilities of these polypeptides in the starting material (t_0). (b) The time course of N-deglycosylation was determined by incubating purified zonadhesin with PNGase F for 1, 2, or 4 h as indicated. Deglycosylated polypeptides were resolved by SDS/PAGE (4–12 % gel, silver-stained, protein disulphides reduced). Note that deglycosylation of both p105 and p45 was evident within 1 h, and appeared to be complete by 2 h. Note also that no mobility changes occurred in control reactions incubated without added enzymes.

p300 in epididymal spermatozoa required denaturing conditions for extraction (Figure 6c). However, in ejaculated cells, most of the p300 was extractable with Triton X-100.

To determine if oligomers of zonadhesin are formed during epididymal transit, we compared the composition of zonadhesin in caput epididymal and ejaculated spermatozoa. Sperm proteins were resolved by two-dimensional SDS/PAGE, and both p45 and p105 were simultaneously detected on Western blots with a mixture of the anti-D1 and anti-D3 antibodies (as for Figure 5b). The Triton X-100 extract of caput epididymal spermatozoa (Figure 7a, left panel) contained more high- M_r multimer ($M_r \sim 300\,000$ and $900\,000$) than low- M_r p105/45 monomer ($M_r \sim 150\,000$ comprising one molecule each of p45 and p105). Conversely, low- M_r monomer predominated in the SDS extract of these cells (Figure 7a, right panel). In ejaculated spermatozoa, the ratios were reversed with more low- M_r monomer in the Triton-extractable fraction and more multimers in the SDS extract (Figure 7b). Most of the zonadhesin in both sperm populations was associated with the extraction-resistant compartment, as three times more Triton X-100-extracted protein (Figures 7a and 7b, left

Figure 5 Identification of zonadhesin pools in spermatozoa

Shown are Western blots (SDS/PAGE, 4–12% gel, protein disulphides reduced) probed with D0–D1 antisera (**a**) or a mixture of anti-D1 and anti-D3 antibodies (**b**) to detect zonadhesin polypeptides. Sperm membranes (lanes labelled membranes) were isolated by differential centrifugation of cavitated (650 lb/in² N₂) cells. The pellet of partially demembranated cells from membrane isolation was then sequentially extracted first with 1% Triton X-100 (lanes labelled Triton X-100), and then 2% SDS (lanes labelled SDS). Note that the Triton X-100 extract exhibited a heterogeneous pattern of zonadhesin polypeptides similar to sperm membranes, but the SDS extract contained primarily p300, p105 and p45 (**a**). A mixture of anti-D1 and anti-D3 antibodies detected authentic p45 and p105 in each of the three fractions (**b**), indicating that different zonadhesin forms consisting partly of these polypeptides are present in more than one physicochemical compartment of spermatozoa.

panel) was required to obtain signals comparable with those of the SDS extract (Figures 7a and 7b, right panel).

Different zonadhesin forms (protein disulphides not reduced) were evident in Triton X-100 extracts of pig testis and spermatozoa (Figure 8a, right panel), even though the p45 and p105 contents of the extracts were largely the same (Figure 8b, right panel). The amount of p105/45 monomer ($M_r \sim 150\,000$) increased as spermatozoa transited the epididymis (Figure 8a, right panel), and this increase was reflected in a corresponding increase in ZP-binding activity (Figures 8a and 8b, left panel), consistent with the previous finding that p105/45 monomeric zonadhesin from ejaculated cells bound to the ZP more avidly than did other forms [17]. Interestingly, p105/45 monomer in the testis was already able to bind to the ZP, albeit to a lesser extent than that in ejaculated cells.

Ultrastructural localization of zonadhesin

To determine whether zonadhesin is present on the cell surface, we localized it on unpermeabilized cells that were both fixed with paraformaldehyde and incubated with antisera in suspension to minimize cell-membrane disruption. The D0–D1 antisera, which strongly labels the anterior heads of methanol-fixed spermatozoa [17], labelled fewer than half of cells fixed with 1% paraformaldehyde (Figure 9). Fixing spermatozoa with higher concentrations of paraformaldehyde (2–4%) eliminated labelling by the D0–D1 antisera whether or not the cells were permeabilized with detergent after fixation (results not shown), indicating that paraformaldehyde either detrimentally modified the zonadhesin protein, fixed it in a conformation not recognized by antibody, or rendered it physically inaccessible. We therefore localized zonadhesin on aldehyde-fixed spermatozoa using the anti-holoprotein serum, which readily recognized the non-denatured protein

Figure 6 Properties of zonadhesin in spermatozoa at different maturation states

Proteins were extracted sequentially with 1% Triton X-100 and 2% SDS from pig testis and from epididymal (caput, corpus and caudal) or ejaculated spermatozoa. Differentially extracted zonadhesin polypeptides were resolved by SDS/PAGE (4–12% linear gradient, protein disulphides reduced) and detected on Western blots. (**a**) p45 detected with anti-D1 antibody, (**b**) p105 detected with anti-D3 antibody and (**c**) p300 detected with anti-MAM antiserum. Proteins (lanes underlined with SDS). Amounts of protein loaded on to gel lanes were: Triton extract of testis, 10 μ g; Triton extracts of spermatozoa, 5 μ g; SDS extracts of testis, 3 μ g; and SDS extracts of spermatozoa, 1.6 μ g. Note that neither p45 nor p105 was detected in the SDS extract of testis, and that the proportions of these polypeptides in the SDS extract increased as spermatozoa passed from the caput to the caudal epididymis. Note also that the proportion of p300 in the Triton X-100 of ejaculated spermatozoa was dramatically increased in comparison with that in epididymal spermatozoa.

[17]. In immunofluorescence, the holoprotein antiserum specifically labelled the anterior heads of permeabilized cells (results not shown). Post-embedding immunoelectron microscopy revealed zonadhesin at the perimeter of the anterior acrosome, associated with the luminal aspect of the outer acrosomal membrane and adjacent acrosomal matrix (Figure 10a). No labelling was detected in the acrosome's equatorial segment or in other sperm organelles. In pre-embedding immunoelectron microscopy of unpermeabilized cells, zonadhesin was detected on the surface of acrosomal matrix exposed between vesiculated periacrosomal membranes of spermatozoa undergoing spontaneous acrosome release (Figure 10b). No gold particles were evident on cells with intact periacrosomal plasma membranes (Figure 10b). When spermatozoa were permeabilized with Triton X-100 before immunolabelling, zonadhesin was detected on exposed surfaces of acrosomal matrices of all cells (Figure 10c).

In round spermatids, zonadhesin exhibited an unusual, omega-like localization over the nascent acrosomal granule, in close apparent association with the developing outer acrosomal membrane (Figure 11a). Some gold particles were also present in the Golgi apparatus outside the acrosome, consistent with the expected pathway for sorting and transporting proteins to the acrosome. The location of zonadhesin in elongating spermatids was similar to that in mature, ejaculated spermatozoa, except that most immunoreactivity was closely associated with the outer acrosomal

Figure 7 Two-dimensional SDS/PAGE of differential detergent extracts from caput epididymal and ejaculated spermatozoa

Proteins were sequentially extracted with 1% Triton X-100 (left panels, 8 μ g protein each) and 2% SDS (right panels, 1 μ g protein each) from caput epididymal (**a**) or freshly ejaculated (**b**) spermatozoa. Extracted proteins were separated by two-dimensional SDS/PAGE, and zonadhesin was detected on Western blots with a mixture of anti-D1 and anti-D3 antibodies. Note that the multimerization states of zonadhesin in the Triton X-100 and SDS extracts differed, and that the proportions of p105/45 (monomeric) zonadhesin in the Triton X-100 extract and of multimeric zonadhesin in the SDS extract were both increased in ejaculated spermatozoa.

Figure 8 ZP-binding activity of zonadhesin from testis or spermatozoa at different maturation states

ZP-bound proteins from Triton X-100 extracts of pig testis; caput, corpus or cauda epididymal spermatozoa; or ejaculated cells were separated by SDS/PAGE (4–12% linear gradient gels). Bound zonadhesin polypeptides (**a**, NR; **b**, R) were detected on Western blots with a mixture of anti-D1 and anti-D3 antibodies. Note that the proportion of zonadhesin with ZP-binding activity increased as sperm maturation progressed from testis, through the epididymis, to ejaculated cells.

membrane, and relatively little was present in the adjacent acrosomal matrix (Figure 11b).

DISCUSSION

The zonadhesin precursor is processed and activated by a combination of Asp-Pro bond hydrolysis and differential glycosylation of polypeptide products

Collectively, the immunochemical results presented in Figures 1 and 2 indicated that the p300 zonadhesin polypeptide spans the

Figure 9 Immunolocalization of pig zonadhesin by light microscopy

Shown is a differential interference contrast image of localization on pig epididymal spermatozoa fixed with 1% paraformaldehyde and labelled in suspension with D0–D1 antisera. Bound antibody was detected with gold-conjugated secondary antibody and silver enhancement. Less than 50% of the cells were labelled. Note that one cell in this pair was heavily labelled on the apical head, whereas the other had almost no apparent labelling.

MAM–D0 domains, and that whereas some p300 in sperm membranes was covalently associated with p45 and p105, most was not. Deglycosylation with O-glycosidase demonstrated that p300 possesses O-linked oligosaccharides. The deglycosylation time course performed with purified substrate demonstrated that the mobility shift of p300 derives from direct action of the enzymes on zonadhesin. Because endo-O-glycosidase typically fails to remove all O-linked carbohydrate even when used in combination with other glycosidases, the multiple, relatively large products obtained on deglycosylation of p300 probably reflect the presence of O-linked oligosaccharides that were resistant to enzymic digestion. The largest polypeptide that might span MAM–D0 domain of pig zonadhesin (Phe³⁰-Asp⁸⁰⁶; 777 amino acids) would have a deduced mass of 84 535 Da, suggesting that the bulk of p300's apparent mass is O-linked carbohydrate. Thus the region of the p300 polypeptide predicted by sequence analysis to be a mucin domain is indeed extensively O-glycosylated in a manner consistent with its being a mucin.

The N-terminal sequences of p45 and p105 were located near the beginning of the D1 and D2 domains respectively in the pig zonadhesin precursor. These sequences were each preceded by glycine and aspartate residues (Figure 12a), defining Asp⁸⁰⁶-Pro⁸⁰⁷ and Asp¹¹⁹¹-Pro¹¹⁹² within the consensus sequence GDPHYLTFDG as scissile bonds in the processing of the pig zonadhesin precursor. Processing at these sites would produce a 385-amino-acid polypeptide (Pro⁸⁰⁷-Asp¹¹⁹¹) with a predicted molecular mass of 42 286 Da that is close to the observed mass (M_r 40 000) of deglycosylated p45.

Aligning the GDPHYLTFDG consensus sequence with homologous regions in the other D-domains of the precursor identified a nearly identical site in D4 domain (Figure 12a). Processing at this site would produce a 783-amino-acid polypeptide (Pro¹¹⁹²-Asp¹⁹⁷⁵) with a predicted molecular mass of 85 406 Da, which is close to the observed mass (M_r 90 000) of deglycosylated p105. This information suggests a processing model (Figure 12b) in which the 267 000 Da zonadhesin precursor is hydrolysed in the testis at Asp⁸⁰⁶-Pro⁸⁰⁷, Asp¹¹⁹¹-Pro¹¹⁹² and Asp¹⁹⁷⁵-Pro¹⁹⁷⁶

Figure 10 Ultrastructural localization of zonadhesin in pig ejaculated spermatozoa by immunoelectron microscopy with antisera to the pig zonadhesin holoprotein

Bound antibody was detected with 10 nm gold-conjugated secondary antibody. **(a)** Sperm cells were labelled after embedding and sectioning. Note that the outer acrosomal membrane and the perimeter of the acrosomal matrix were labelled: a, acrosome; eq, equatorial segment; n, nucleus; mp, flagellar midpiece; pp, flagellar principal piece. **(b)** Cells were labelled prior to embedding. Note the strong, surface labelling on the exposed acrosomal matrix (am) of a cell with vesiculated membranes (v) overlying its apical head. Cells with an intact plasma membrane (pm) or outer acrosomal membrane (oa) were not labelled. cd, cytoplasmic droplet. **(c)** Spermatozoa permeabilized with Triton X-100 and labelled prior to embedding. Note that the exposed surfaces of acrosomal matrices of all cells (am) were labelled.

Figure 11 Ultrastructural localization of zonadhesin in pig spermatids

(a) View of the developing acrosome overlying the nucleus (n) of a round spermatid. Note the presence of zonadhesin (arrows) at the developing outer acrosomal membrane and in adjacent, granular acrosomal material, but not in the dense core (ag) of acrosomal matrix. (b) Longitudinal section through the anterior head of an elongating spermatid. Note the continued association of zonadhesin with the outer acrosomal membrane and perimeter of the acrosomal matrix: a, acrosome; n, nucleus.

to generate p300, p45, p105 and a C-terminal polypeptide that has not been positively identified in pig zonadhesin. The polypeptides generated by processing at these sites in the pig zonadhesin precursor are completely compatible with the results obtained from tryptic fragment sequencing [16] and reactivity of domain-specific antibodies (Figure 12). Furthermore, applying this processing model to the sequences of the mouse and rabbit zonadhesin precursors yields conceptual products that are consistent with the masses of mouse zonadhesin polypeptides recognized by antibodies to the D3 and D3p18 domains (S. Tardif, M. D. Wilson, G. F. Koop and D. M. Hardy, unpublished work) and with the masses of rabbit zonadhesin polypeptides recognized by various antisera [19].

Eight Asp-Pro bonds are present in the VWD domains of the pig zonadhesin precursor. Nevertheless, processing occurs primarily within the GDPHYLTFDG consensus sequence, suggesting the action of a sequence-specific protease. However, few endoproteases are capable of hydrolysing X-Pro peptide bonds [24–27], and none have been reported to hydrolyse Asp-Pro bonds. Aminopeptidase P can hydrolyse the X-Pro peptide bond [24,25], but no two consecutive amino acids are conserved among the three stretches of the sequence extending 40 positions upstream of the zonadhesin precursor's three GDPHY(L/R)TFDG consensus sequences. It therefore seems unlikely that zonadhesin processing occurs by combined action of an endoprotease and aminopeptidase P. Interestingly, hydrolysis of the Asp-Pro bond within a

Figure 12 Proposed model for the proteolytic activation of zonadhesin

(a) Alignment of partially conserved sequences in the D1, D3 and D4 domains of the pig zonadhesin precursor. The N-terminal sequences of p45 (PHYLTFDGR) and p105 (PHYLTFDGA) are underlined in the D1 and D2 domain sequences respectively. Conserved amino acids are shaded grey. (b) Model for the generation of constituent polypeptides in the functional maturation of zonadhesin. In this model, hydrolysis of the Asp⁸⁰⁶-Pro⁸⁰⁷ peptide bond produces the C-terminus of p300 and the N-terminus of p45. Similarly, hydrolysis of the Asp¹¹⁹¹-Pro¹¹⁹² peptide bonds generates the C-terminus of p45 and the N-terminus of p105. The presence of a similar sequence (D¹⁹⁷⁵PHYRTFDG), 85 000 Da downstream in D4, suggests that hydrolysis of the Asp¹⁹⁷⁵-Pro¹⁹⁷⁶ bond generates the C-terminus of p105. This model is compatible with previous results on the location of tryptic peptides in p45 and p105 and the reactivity of D0-D1 domains and MAM antisera and D1 and D3 antibodies with zonadhesin polypeptides. The locations of segments expressed as immunogens for the preparation of the MAM antisera and D1 and D3 antibodies are marked with horizontal bars. Note that Pro⁸⁸³-Ser¹²²⁴, which was used as the immunogen for the preparation of the D0-D1 antisera, spanned portions of all three processed polypeptides. Although not depicted in the figure, in mature spermatozoa, the processed polypeptides are covalently associated with one another, in heterogeneous stoichiometry, by intermolecular disulphide bonds.

nearly identical sequence (GDPHYVTFDG) of MUC2 may occur autocatalytically in the acidic environment (pH < 6) of the late secretory pathway [28]. The acidic nature of the sperm acrosome could support such a mechanism for the processing and activation of zonadhesin.

Previous functional studies of pig zonadhesin's VWD domains focused on the ZP-binding and disulphide-bond-forming activities of D1-D3 domains because these domains largely comprise p105/45, the predominant active form in sperm membrane preparations [15-17]. However, an *M*_r 56 000 component (p56) of pig sperm membranes also bound in a species-specific manner to native ZP, albeit less avidly than did p105/45 zonadhesin [15]. The apparent mass of p56 is similar to the calculated molecular mass of the C-terminal polypeptide (55 265 Da) that our processing model predicted would include most of the D4 domain (Figure 12b). This predicted D4 polypeptide of pig zonadhesin may correspond to an *M*_r 58 000 polypeptide of rabbit zonadhesin (disulphides reduced) detected with antisera to the C-terminal region [19]. Heat-solubilized rabbit ZP bound to a recombinant protein, ectopically expressed on HeLa cells, that spanned rabbit zonadhesin's D4 domain, transmembrane segment and the cytoplasmic tail [19]. The binding of the recombinant D4 protein to intact rabbit ZP has not been reported, nor has the binding activity of rabbit zonadhesin comprising the polypeptides corresponding to pig p45 and p105. Nevertheless, the results from

studies of both pig and rabbit zonadhesin suggest that, similar to D1-D3 domains, the D4 domain may contribute to the ZP-binding activity of zonadhesin.

Our processing results also illustrate additional species differences in the properties of zonadhesin [16,18,19]. We readily detected both p105 and p45 in Triton X-100 extracts of pig testis, but observed no polypeptide in Triton X-100 or SDS extracts that might correspond to the predicted 267 000 Da pig zonadhesin precursor, suggesting that the precursor is processed very rapidly after translation. In the rabbit, however, a putative precursor was detected in a CHAPS extract of testis, but polypeptides corresponding to those in spermatozoa were not [19]. Thus the kinetics of zonadhesin's proteolytic processing during sperm development seems to vary among species. The timing of oligomerization events in other species has not been reported. Likewise, the timing of glycosylation relative to the proteolytic events is not known for any species, so it remains to be seen whether these processes also vary by species.

Zonadhesin is present in at least two distinct, particulate compartments of pig spermatozoa

Zonadhesin was previously identified and characterized in membranes removed from spermatozoa by N₂ cavitation [15-17]. The differential extraction results shown in Figures 5-7 demonstrate that zonadhesin is present in at least two physicochemically distinct compartments of ejaculated spermatozoa. Zonadhesin forms comprising primarily p105, p60-90 and p45 polypeptides appeared to be membrane-associated and were extractable with mild detergents, whereas forms comprising primarily p300, p105 and p45 polypeptides were more strongly associated with non-membranous, particulate structures and extractable only with denaturants.

Sperm structure and molecular composition change as the cells mature during epididymal transit [1,11,29]. In several species, the shape of the acrosome's apical segment changes, whereas sperm head proteins undergo thiol oxidation, proteolysis and oligosaccharide modifications. Our differential extraction experiments indicated that zonadhesin polypeptides generated in the testis become increasingly associated with an extraction-resistant compartment as spermatozoa transit the epididymis. Indeed, testis contained no extraction-resistant zonadhesin. However, by the time spermatozoa reach the caput epididymis, some of the p300, p105 and p45 are incorporated into the extraction-resistant compartment, with the proportion of extraction-resistant protein being highest in the caudal epididymis. Moreover, in ejaculated cells, most of the p105 and p45 remain extraction-resistant, but most of the p300 becomes extractable.

In ejaculated cells, zonadhesin is present as a mixture of disulphide-bonded oligomers that vary in their apparent avidities for the ZP [17]. Thiol oxidation in the epididymis [1,30] could promote the formation of these oligomers and thereby affect the protein's function in fertilization. However, we observed a paradoxical effect of epididymal maturation on zonadhesin multimers. As spermatozoa transited the epididymis, the extraction-resistant fraction of zonadhesin underwent additional assembly into high-*M*_r multimers, whereas the protein in the Triton-extractable fraction shifted in the opposite direction to a higher proportion of low-*M*_r oligomers. Perhaps, most importantly, the ZP-binding activity of the zonadhesin in the extractable fraction increased with epididymal maturation. This finding is consistent with our previous observation that the p105/45 monomeric form of zonadhesin has the highest apparent avidity for the ZP [17].

Zonadhesin localizes to the outer acrosomal membrane and outer aspect of the acrosomal matrix

Immunolocalization and differential extraction results collectively showed that active zonadhesin is present predominantly in the outer acrosomal membrane and adjacent acrosomal matrix of pig spermatozoa, and not in the plasma membrane. This finding directly demonstrates that most of the zonadhesin is not present on the surface of acrosome-intact spermatozoa, a view that previously could only be inferred from light microscopic studies [17,19] wherein the protein was not reliably detected on living cells or on fixed, unpermeabilized cells. Instead, similar to the sperm proteins that mediate species-specific adhesion in marine invertebrates, zonadhesin is located in or within the boundaries of the acrosomal membrane. Zonadhesin's initial association with the outer acrosomal membrane of elongating spermatids is consistent with the presence of a transmembrane segment as predicted by sequence analysis [16]. The presence of the protein in the adjacent acrosomal matrix of mature spermatozoa indicates that the protein is subsequently redistributed during epididymal maturation. The progressive multimerization of zonadhesin in the epididymis may contribute to this redistribution, resulting in the incorporation of the protein into an extraction-resistant (matrix) fraction of the cell.

Function of zonadhesin in fertilization

The findings reported here suggest that zonadhesin in the periphery of the sperm acrosome mediates high avidity, species-specific adhesion of spermatozoa to the ZP during the acrosome reaction. Acrosomal exocytosis is not an instantaneous event, so there is ample opportunity for proteins in the acrosomal matrix to interact with the ZP [8]. In the early stages of the acrosome reaction, membranes overlying the sperm head vesiculate, and acrosomal matrix becomes exposed between the hybrid vesicles of outer acrosomal and plasma membranes (Figure 10 and [31,32]). Although the vesicle/matrix complex lifts away from the inner acrosomal membrane, it remains attached to the sperm head. Zonadhesin at the leading edge of the complex (Figure 10) is ideally positioned for interaction with the ZP.

Zonadhesin-mediated adhesion of the acrosomal matrix to the ZP could occur whether the acrosome reaction is induced by the ZP or by a soluble agonist (e.g. progesterone). When acrosome reactions occur in response to sperm attachment to the ZP surface, adhesion must be sustained even as membranes and acrosomal contents overlying most of the sperm head are shed from the cell. Indeed, without an adhesive contribution from the acrosomal matrix, spermatozoa undergoing ZP-induced acrosome reactions could detach from the surface of the ZP and fail to fertilize the egg. Once the sperm head has begun to penetrate into the ZP, adhesion of the acrosomal cap remnant may no longer be necessary for successful fertilization. Nevertheless, the released remnant persists at the surface of the ZP as the spermatozoon proceeds through it [1,19], consistent with zonadhesin's high avidity for the ZP.

We conclude that the pig zonadhesin precursor undergoes proteolytic processing in the testis, possibly autocatalytically, at specific Asp-Pro bonds to produce four polypeptides. Also in the testis, the mucin domain of one polypeptide (MAM-D0) is heavily modified predominantly if not exclusively with O-linked oligosaccharides to produce the p300 glycoprotein; the VWD domains of two others (D1- and D2-D3) are less extensively modified with N-linked oligosaccharides to produce the p105 and p45 polypeptides, respectively, of mature zonadhesin; and covalent oligomers of p105/45 begin to form. Oligomerization continues in

the epididymis, with increasing incorporation of p300, p105 and p45 into an extraction-resistant compartment of the cell. The fully mature, active protein is present in the outer aspect of the acrosome where, in a manner similar to gamete recognition proteins in marine invertebrates, it may mediate species-specific adhesion of spermatozoa to the mammalian egg's extracellular matrix.

This work was supported by grants from the NIH to G. E. O. (HD-20419) and D. M. H. (HD-35166), and the Texas Affiliate of the American Heart Association (96G-324) to D. M. H. We thank Dr Steve Sprang, UT Southwestern Medical Center, for amino acid sequencing, and Dr Harry Weilllauf, Dr Gail Cornwall and Dr Charles Faust for a critical review of the paper before submission.

REFERENCES

- Yanagimachi, R. (1994) Mammalian fertilization. In *The Physiology of Reproduction* (Knobil, E. and Neill, J. D., eds.), pp. 189–317, Raven Press, New York
- Vacquier, V. D. (1998) Evolution of gamete recognition proteins. *Science* **281**, 1995–1998
- Bi, M., Wassler, M. J. and Hardy, D. M. (2002) Sperm adhesion to the extracellular matrix of the egg. In *Fertilization* (Hardy, D. M., ed.), pp. 153–180, Academic Press, San Diego
- Glabe, C. G. and Vacquier, V. D. (1977) Species specific agglutination of eggs by bindin isolated from sea urchin sperm. *Nature (London)* **267**, 836–838
- Lopo, A. C., Glabe, C. G., Lennarz, W. J. and Vacquier, V. D. (1982) Sperm-egg binding events during sea urchin fertilization. *Ann. N. Y. Acad. Sci.* **383**, 405–425
- Florman, H. M. and Storey, B. T. (1982) Mouse gamete interactions: the zona pellucida is the site of the acrosome reaction leading to fertilization *in vitro*. *Dev. Biol.* **91**, 121–130
- Huang, T. T., Fleming, A. D. and Yanagimachi, R. (1981) Only acrosome-reacted spermatozoa can bind to and penetrate zona pellucida: a study using the guinea pig. *J. Exp. Zool.* **217**, 287–290
- Gerton, G. L. (2002) Function of the sperm acrosome. In *Fertilization* (Hardy, D. M., ed.), pp. 265–302, Academic Press, San Diego
- Olson, G. E. and Winfrey, V. P. (1985) Structure of membrane domains and matrix components of the bovine acrosome. *J. Ultrastruct. Res.* **90**, 9–25
- Flaherty, S. P. and Olson, G. E. (1991) Ultrastructural analysis of the acrosome reaction in a population of single guinea pig sperm. *Anat. Rec.* **229**, 186–194
- Olson, G. E., Nagdas, S. K. and Winfrey, V. P. (2002) Structural differentiation of spermatozoa during post-testicular maturation. In *The Epididymis* (Robaire, B. and Hinton, B. T., eds.), pp. 371–387, Kluwer Academic/Plenum, New York
- Primakoff, P., Myles, D. G. and Belve, A. R. (1980) Biochemical analysis of the released products of the mammalian acrosome reaction. *Dev. Biol.* **80**, 324–331
- Naaby-Hansen, S., Flickinger, C. J. and Herr, J. C. (1997) Two-dimensional gel electrophoretic analysis of vectorially labeled surface proteins of human spermatozoa. *Biol. Reprod.* **56**, 771–787
- Haden, N. P., Hickox, J. R., Scott, W. C. and Hardy, D. M. (2000) Systematic characterization of sperm-specific membrane proteins in swine. *Biol. Reprod.* **63**, 1839–1847
- Hardy, D. M. and Garbers, D. L. (1994) Species-specific binding of sperm proteins to the extracellular matrix (zona pellucida) of the egg. *J. Biol. Chem.* **269**, 19000–19004
- Hardy, D. M. and Garbers, D. L. (1995) A sperm membrane protein that binds in a species-specific manner to the egg extracellular matrix is homologous to von Willebrand factor. *J. Biol. Chem.* **270**, 26025–26028
- Hickox, J. R., Bi, M. and Hardy, D. M. (2001) Heterogeneous processing and zona pellucida-binding activity of pig zonadhesin. *J. Biol. Chem.* **276**, 41502–41509
- Gao, Z. and Garbers, D. L. (1998) Species diversity in the structure of zonadhesin, a sperm-specific membrane protein containing multiple cell adhesion molecule-like domains. *J. Biol. Chem.* **273**, 3415–3421
- Lea, I. A., Sivashanmugam, P. and O'Rand, M. G. (2001) Zonadhesin: characterization, localization, and zona pellucida binding. *Biol. Reprod.* **65**, 1691–1700
- Morrissey, J. H. (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **117**, 307–310
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Olson, G. E., Winfrey, V. P. and Nagdas, S. K. (1998) Acrosome biogenesis in the hamster: ultrastructurally distinct matrix regions are assembled from a common precursor polypeptide. *Biol. Reprod.* **58**, 361–370
- Olson, G. E., Winfrey, V. P. and Nagdas, S. K. (2003) Structural modification of the hamster sperm acrosome during posttesticular development in the epididymis. *Microsc. Res. Tech.* **61**, 46–55

- 24 Yaron, A. and Naider, F. (1993) Proline-dependent structural and biological properties of peptides and proteins. *Crit. Rev. Biochem. Mol. Biol.* **28**, 31–81
- 25 Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D. and Scharpe, S. (1995) Proline motifs in peptides and their biological processing. *FASEB J.* **9**, 736–744
- 26 Vance, J. E., LeBlanc, D. A. and London, R. E. (1997) Cleavage of the X-Pro peptide bond by pepsin is specific for the *trans* isomer. *Biochemistry* **36**, 13232–13240
- 27 Vance, J. E., LeBlanc, D. A., Wingfield, P. and London, R. E. (1997) Conformational selectivity of HIV-1 protease cleavage of X-Pro peptide bonds and its implications. *J. Biol. Chem.* **272**, 15603–15606
- 28 Lidell, M. E., Johansson, M. E. and Hansson, G. C. (2003) An autocatalytic cleavage in the C-terminus of the human MUC2 mucin occurs at the low pH of the late secretory pathway. *J. Biol. Chem.* **278**, 13944–13951
- 29 Cuasnicu, P. S., Cohen, D. J., Ellerman, D. A., Busso, D., Da Ros, V. G. and Morgenfeld, M. M. (2002) Changes in specific sperm proteins during epididymal maturation. In *The Epididymis* (Robaire, B. and Hinton, B. T., eds.), pp. 389–403, Kluwer Academic/Plenum, New York
- 30 Cornwall, G. A. and Chang, T. S. (1990) Characterization of sulfhydryl proteins involved in the maintenance of flagellar straightness in hamster spermatozoa. *J. Androl.* **11**, 168–181
- 31 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
- 32 Olson, G. E. and Winfrey, V. P. (1994) Structure of acrosomal matrix domains of rabbit sperm. *J. Struct. Biol.* **112**, 41–48

Received 22 May 2003/7 July 2003; accepted 28 July 2003

Published as BJ Immediate Publication 28 July 2003, DOI 10.1042/BJ20030753