Zonadhesin Is Essential for Species Specificity of Sperm Adhesion to the Egg Zona Pellucida

Received for publication, March 12, 2010, and in revised form, May 26, 2010 Published, JBC Papers in Press, June 7, 2010, DOI 10.1074/jbc.M110.123125

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Interaction of rapidly evolving molecules imparts species specificity to sperm-egg recognition in marine invertebrates, but it is unclear whether comparable interactions occur during fertilization in any vertebrate species. In mammals, the sperm acrosomal protein zonadhesin is a rapidly evolving molecule with species-specific binding activity for the egg zona pellucida (ZP). Here we show using null mice produced by targeted disruption of Zan that zonadhesin confers species specificity to sperm-ZP adhesion. Sperm capacitation selectively exposed a partial von Willebrand D domain of mouse zonadhesin on the surface of living, motile cells. Antibodies to the exposed domain inhibited adhesion of wild-type spermatozoa to the mouse ZP but did not inhibit adhesion of spermatozoa lacking zonadhesin. Zan−/− males were fertile, and their spermatozoa readily fertilized mouse eggs in vitro. Remarkably, however, loss of zonadhesin increased adhesion of mouse spermatozoa to pig, cow, and rabbit ZP but not mouse ZP. We conclude that zonadhesin mediates species-specific ZP adhesion, and Zan−/− males are fertile because their spermatozoa retain adhesion capability that is not species-specific. Mammalian sperm-ZP adhesion is therefore more robustly, and species-specific egg recognition by a protein in the sperm acrosome is conserved between invertebrates and vertebrates, even though the adhesion molecules themselves are unrelated.

Sperm adhesion to the egg extracellular coat is a key cell recognition event in animal reproduction. This essential interaction represents the first direct, physical contact between the gametes, and in diverse animals confers species specificity to fertilization (1). Sperm-egg recognition in externally fertilizing marine invertebrates such as sea urchin (phylum Echinodermata, class Echinoidea) and abalone (phylum Mollusca, class Gastropoda) occurs by species-specific binding of single proteins in the acrosome, a secretory organelle on the apical head of spermatozoa, to complementary molecules on the egg (2). Although species-specific gamete recognition in these two taxa is mediated by a conserved subcellular interaction (the sperm acrosome with the egg vitelline layer), the abalone and sea urchin gamete recognition proteins are structurally unrelated to each other (2). Nevertheless, in both taxa rapid molecular evolution by positive selection produced the structural diversity underlying the recognition proteins’ species-specific binding activities (2), and the species specificity of fertilization conferred by these proteins contributes to the reproductive isolation of their constituent species (2).

Decades of effort have not produced a comprehensive understanding of mammalian sperm-ZP4 adhesion. One challenge is that the mammalian fertilization environment in vivo cannot easily be reproduced in vitro. In addition, mammalian sperm populations are functionally heterogeneous because the cells undergo progressive, asynchronous physiological changes during transport and capacitation in the female tract that alter their adhesion activity prior to interaction with the egg at the site of fertilization (1). Despite such complications, however, numerous candidate ZP adhesion molecules have been identified, including β-1,4-galactosyltransferase (3), SED-1(4), proacrosin/acrosin (5), ZP3R/sp56 (6, 7), zonadhesin (8, 9), and others (10). Current evidence collectively suggests that mammalian gamete adhesion occurs by binding of multiple sperm proteins to the glycoprotein components of the ZP (10, 11), but no studies have defined the sequence of those interactions or their relative contributions to the overall specificity of sperm-egg recognition.

Among several suspected ZP adhesion molecules in mammalian spermatozoa (10), zonadhesin is unique in its ability to bind directly and in a species-specific manner to native ZP (8).

The abbreviations used are: ZP, zona pellucida; ES, embryonic stem; GST, glutathione S-transferase; ANOVA, analysis of variance; LSD, least significant difference; VWD, von Willebrand D; MAM, meprin/A5 antigen/mu receptor tyrosine phosphatase; PNA, peanut agglutinin.

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Like species-specific egg recognition proteins of sea urchin and abalone spermatozoa, zonadhesin is an acrosomal protein (12, 13) that becomes exposed by induced exocytosis during fertilization. Zonadhesin is homologous to von Willebrand factor and other adhesion molecules (9, 14) and differs dramatically among species not only as a consequence of rapid evolution by positive selection (2, 15, 16) but also because of domain duplication (14, 17). mRNA splice variation, and processing heterogeneity during the functional maturation of the protein (12, 18, 19). Although the binding activity, localization, and other properties of zonadhesin suggest that it mediates species-specific ZP adhesion at the onset of acrosomal exocytosis (12), its function in fertilization has not been tested directly. Here we report results of in vitro and in vivo loss-of-function experiments showing that zonadhesin is a ZP adhesion molecule that confers species specificity to sperm-egg adhesion.

**EXPERIMENTAL PROCEDURES**

**Zan Targeting**—A null Zan allele was produced by replacing the proximal promoter and first six exons of the gene with a Neo cassette using the vector PGKneolox2DTA and 129S1/Sv-derived R1 embryonic stem (ES) cells. Details of the targeting procedure are provided in the legend for supplemental Fig. S1.

**Null Animal Production and Colony Expansion**—Cleavage stage Crl:(CD-1) ICR embryos (Charles River Laboratory) were aggregated with correctly targeted ES clones (20). Germ line-transmitting chimeras for ES cell clones ZV1-B9 (“B9”) and ZV1-E5 (“E5”) identified by agouti coat color and black eyes of offspring from crosses with CD-1 mice were then bred to 129S2/SvPasCrl (Charles River Laboratory) to establish mouse lines on a relatively uniform genetic background similar to that of the 129S1/Sv ES cells used for Zan targeting. Natural mating studies were performed with 129S2/SvPas mice derived from both ES cell clones and also with 129S2/SvPas lines intercrossed with C57BL/6Crl mice to examine fertility on a more fecund background. Zonadhesin function in vitro was characterized using spermatozoa from littermates bred 5–6 generations to the C57BL/6Crl background.

**Genotyping**—The short arm Southern blot probe (336 bp) was amplified by PCR using Zan_202574F and Zan_202940R (primer sequences listed in supplemental Table S1). The long arm Southern probe (333 bp) was generated using primers Zan_long_F3 and Zan_long_R1. PCR genotyping of DNA from proteinase K-digested ear punches using primers Zan_195666F and Zan_195891 amplified a 225-bp band that is absent in the recombined Zan locus. Zan_199946R and the vector-derived primer ZV1_inloxF amplified a 357-bp band to detect the modified Zan locus.

**Detection of Zonadhesin mRNA**—RNA (2 μg) isolated using TRIzol (Invitrogen) from mouse testes flash-frozen in N2(l) was reverse-transcribed with 200 units of murine-Malone leukemia virus reverse transcriptase (Invitrogen) and 25 ng/ml oligo(dT)15. Two PCR primers sets (11F/13R and D3F/D3R2) amplified a 372-bp product encoded by exons 11–13 (5 kb downstream of the deletion) and a 329-bp product encoded by exons 33–35 (37 kb downstream of the deletion) using the first strand cDNA template (25 μg RNA equivalent). A third primer set (MAMF/R) amplified a 310-bp product in the region deleted by gene targeting and served as a negative control for cDNA from null animals. Positive control primer sets amplified 222-bp β2-microglobulin and 983-bp G3pdh products.

**Zonadhesin Antibodies**—Domain-specific antibodies to mouse zonadhesin and control antibodies to glutathione S-transferase (GST) were affinity-purified from rabbit antisera as for preparation of antibodies to pig zonadhesin (18). Antigenic regions (see Fig. 1A) of von Willebrand D3 (Ile2168→Thr2270) and D3p18 (Cys3602→Lys3621) domains (accession number AAL04416) encoded by nucleotides 6680–6989 and 13,669–14,050, respectively, of the mouse zonadhesin transcript (accession number U97068) were expressed in *Escherichia coli* strain BL21 as recombinant GST fusion proteins using pGEX-4T-1 (Amersham Biosciences).

**Western Blots**—Zonadhesin polypeptides were detected (18) using anti-D3 or anti-D3p18 (80 or 40 ng/ml overnight, respectively) on blots of disulfide-reduced (25 mM dithiothreitol) proteins extracted from cauda epididymal spermatozoa (1×10⁶ sperm cells/lane) (21, 22) and resolved on 4–10% gradient gels. Immunofluorescence—Sperm antigens were detected in methanol-fixed and permeabilized mouse spermatozoa (18) using anti-D3 (2 μg/ml) or anti-D3p18 (1 μg/ml) affinity-purified antibodies or anti-hyaluronidase antiserum (1:400). Bound antibody was visualized with Alexa Fluor 594-conjugated goat anti-rabbit IgG (3 μg/ml; Molecular Probes, Eugene, OR). Acrosomes were then labeled with biotinylated lectin from peanut (*Arachis hypogaea*) agglutinin (PNA; 0.1 mg/ml; Sigma) detected with Alexa Fluor 488-conjugated streptavidin (3 μg/ml; Molecular Probes). Cells were viewed by epifluorescence and phase contrast microscopy at 600× magnification. Zonadhesin on living, motile spermatozoa was detected by incubating cells in suspension with anti-D3 (2 μg/ml) or anti-D3p18 (1 μg/ml) antibodies (37°C, 30 min). After centrifugation (500 × g, 3 min), bound antibody was detected either on cells in suspension or on cells smeared and dried on slides as for immunofluorescence on fixed cells, with simultaneous detection of acrosomes using PNA.

**Sperm Capacitation and Mouse Fertilization in Vitro**—Capacitation was inferred by assessing sperm cells’ exocytotic response to 10 μM calcium ionophore A23187 (Sigma), as only capacitated spermatozoa undergo acrosomal exocytosis induced by the ZP or by subthreshold concentrations of A23187 (23). Spermatozoa were incubated 15–135 min in modified-Tyrode’s medium (24) and then an additional 15 min with or without ionophore. Acrosomes were detected with PNA, and the percentage of capacitation was calculated as the difference in the number of cells lacking acrosomes with and without ionophore addition per 100 cells total (23). Mouse fertilization in vitro was also performed in modified-Tyrode’s medium (24) using 5000 capacitated spermatozoa incubated 3 h with 15–20 cumulus-intact oocytes per 50–μl drop. Fertilization was scored by detection of Hoechst 33258-stained sperm nuclei in the egg cytoplasm and by expulsion of the second polar body. Effects of antibodies on fertilization in vitro were tested by including anti-D3, anti-D3p18, or anti-GST (1.5 μg/ml) during capacitation.

5 T. L. Cheung, M. D. Wassler, S. Tardif, G. A. Cornwall, J. A. Harris, and D. M. Hardy, unpublished data.
and transferring the diluted antibodies with spermatozoa when oocytes were inseminated.

**Sperm-ZP Adhesion Assays**—Mouse sperm-ZP adhesion was assessed by gamete co-incubation as for fertilization *in vitro*, except that cumulus cells were first removed from oocytes with hyaluronidase (0.25 mg/ml, ≤10 min), 10–15 oocytes were incubated 30 min with 2500 spermatozoa per 50-μl drop, and sperm-oocyte complexes were washed twice by pipetting (65–75-mm mouth diameter). Two-cell mouse embryos recovered 28 h after overnight mating were included with oocytes, and oocytes were pipette-washed until all loosely attached spermatozoa were removed from the embryos. Spermatozoa were counted on ZP viewed by differential interference contrast microscopy. Antibody inhibition of mouse sperm-ZP adhesion was determined as for testing antibody effects on fertilization *in vitro*. Pig, rabbit, and bovine oocytes were aspirated from mature follicles of fresh ovaries, cumulus cells were removed with hyaluronidase, and sperm adhesion to their ZP was assessed the same as for mouse oocytes. Pig sperm capacitation was induced by a 3-h incubation in a Krebs-Ringer bicarbonate medium (22). In co-incubination experiments comparing species specificity of *Zan*<sup>+/−</sup> and *Zan*<sup>−/−</sup> sperm adhesion to the ZP simultaneously on the same oocytes, spermatozoa from the two genotypes were separately capacitated 75 min at 1 × 10<sup>7</sup> cells/ml and labeled with Hoechst 33258 or MitoTracker (Invitrogen) for the final 15 min of capacitation. The differentially labeled cells were mixed in defined ratios (25:75 or 50:50% null-wild type), and adhesion of the mixed cells to ZP of mouse, pig, rabbit, or bovine oocytes was tested as for ZP adhesion using unlabeled cells. Neither dye affected sperm motility, and both were used alternately on the two *Zan* genotypes in the ZP adhesion assays. 2500 sperm cells were added to each drop containing 10–15 eggs, representing a 400-fold total dilution of the sperm suspension.

**Statistical Analyses**—Differences in litter sizes, surface exposure of zonadhesin, antibody inhibition of fertilization, and sperm-ZP adhesion were tested for significance by analysis of variance (ANOVA) using general linear model procedures (SAS software). The Fisher’s protected least significant difference (LSD) test was conducted when the main effect was significant (*p* < 0.05). The Z-test was used to compare the ratio of inseminated spermatozoa with the ratio of ZP-adherent cells in co-insemination ZP adhesion assays.

**RESULTS**

**Production of Zonadhesin-null Mice**—To produce spermatozoa lacking zonadhesin, we disrupted the mouse zonadhesin gene by homologous recombination (Figs. 1 and 2 and supplemental Fig. S1). Of seven targeted ES cell clones verified by Southern blotting (Fig. 1), two (B9 and E5) were used to make aggregation chimeras with Crl(CD-1) ICR embryos. Germ line-transmitting, chimeric male founders for both lines were crossed to wild-type mice to produce *Zan*<sup>+/−</sup> and *Zan*<sup>−/−</sup> males and females (Fig. 1). Loss of expression from the targeted allele was confirmed by reverse transcription-PCR (Fig. 2A) to detect zonadhesin mRNA in testes and by Western blotting (Fig. 2B) and indirect immunofluorescence to detect zonadhesin protein in spermatozoa. On permeabilized spermatozoa from *Zan*<sup>+/+</sup> and *Zan*<sup>−/−</sup> males, affinity-purified antibodies specific for the zonadhesin von Willebrand D3 and D3p18 domains detected zonadhesin in 100% of cells by immunofluorescence. Analysis of variance (ANOVA) using general linear model procedures (SAS software). The Fisher’s protected least significant difference (LSD) test was used to compare the ratio of ZP-adherent cells in co-insemination ZP adhesion assays.

**FIGURE 1. Targeted disruption of Zan.** A, mouse zonadhesin is a mosaic protein comprising four extracellular domain types, including meprin/A5 antigen/mu receptor tyrosine phosphatase (WAM) and von Willebrand D (VWD) domains. The tandemly repeated VWD domains of the protein include an expansion of 20 partial domains (D3p1–20) related to the D3 domain that is absent from zonadhesin in spermatozoa of non-rodent species. Affinity-purified antibodies were directed toward 100–120-amino acid segments of the D3 and D3p18 domains (black bar), (SP, signal peptide), aa, amino acids; EGF, epidermal growth factor; TM, transmembrane; C, C terminus. B, homologous recombination of the targeting construct with the Zan locus replaced exons 1–6 and the presumptive promoter of Zan with a PGK-neo cassette and altered BgllI and Nhel restriction fragments. Southern blot probes P1 and P2 flank their respective arms in the wild-type and targeted allelles. Primer locations for PCR genotyping are also shown. B, BgllI site; C, Nhel site. C, Southern blots of BgllI-digested DNA from R1 ES cells (+/+ and targeted ES cell clones (+/−; one of seven shown), hybridization of probe P1 to predicted 15,393-bp (wild-type) and 9924-bp (mutant) fragments confirmed correct recombination of the long arm (left panel). On Southern blots of Nhel-digested DNA from seven expanded ES clones, hybridization of probe P2 to predicted 7047-bp (wild-type) and 5363-bp (mutant) fragments confirmed correct recombination within the short arm (right panel). D, hybridization of probe P1 to the predicted wild-type and mutant BgllI fragments on Southern blots confirmed production of Zan<sup>−/−</sup> offspring from an intercross of Zan<sup>+/−</sup> mice. E, predicted wild-type (357-bp) and mutant (225-bp) bands were amplified in PCR genotyping of offspring from Zan<sup>+/−</sup> intercrosses.
Zonadhesin Function in Mammalian Gamete Adhesion

Progeny from heterozygote crosses showed no deviation ($\chi^2$ (2, $n = 135) = 4.29, p = 0.25$) from the expected 1:2:1 Mendelian ratio. Likewise, male offspring from backcrosses between $\text{Zan}^{+/+}$ males and wild-type females (129S2/SvPasCrl and C57BL/6Crl data combined) revealed no deviation from the expected 1:1 heterozygote to wild-type ratio ($\chi^2 (1, n = 59) = 2.05, p = 0.25$), indicating that the ability of an individual sperm cell to fertilize the egg was not affected by its $\text{Zan}$ allele genotype. This finding could not be attributed to a lack of competition between sperm genotypes because immunofluorescence localization revealed that zonadhesin was present in all spermatozoa from $\text{Zan}^{+/+}$ males, although $\text{Zan}$ is not transcribed until developing male germ cells become haploid spermatids (8, 12, 13).

**Cell Surface Exposure of Zonadhesin during Sperm Capacitation**—Because zonadhesin is an acrosomal protein, we determined whether it is exposed on living, motile cells capable of interacting productively with the ZP. In immunofluorescence, affinity-purified antibodies to the D3p18 or D3 domains of mouse zonadhesin (Fig. 1A) labeled 100% of fixed, permeabilized spermatozoa from wild-type males (Fig. 3, A, B, and D). Neither antibody labeled significant numbers of living, non-capacitated spermatozoa from $\text{Zan}^{+/+}$ males, nor did they label any spermatozoa from $\text{Zan}^{-/-}$ males (Fig. 3, B and C). However, after optimal sperm capacitation in vitro (Fig. 3D), anti-D3p18 labeled the apical heads of some but not all wild-type cells (Fig. 3, A and D). Surface exposure of the D3p18 domain closely tracked the kinetics of sperm capacitation (Fig. 3D). In contrast, anti-D3 immunoreactivity did not increase with sperm capacitation (Fig. 3B). Loss of zonadhesin did not affect PH-20/hyaluronidase immunoreactivity (Fig. 3C and supplemental Fig. S3), nor did it substantially affect the profiles of other gamete recognition proteins of spermatozoa, including SED-1, $\beta$-1,4-galactosyltransferase, proacrosin, ZP3R/sp56, ADAM 2, or ADAM 3, or alter the pattern of sperm phosphoproteins or total protein (supplemental Fig. S3), consistent with the fertility of $\text{Zan}^{-/-}$ males. Exposure of the D3p18 domain on living, motile spermatozoa was dependent on incubation in capacitating medium (Fig. 3E). No comparable capacitation-dependent increase in surface exposure of the D3 domain occurred (Fig. 3E), suggesting that this zonadhesin domain remains masked, while the D3p18 domain becomes exposed.

**Zonadhesin Antibody Inhibits ZP Adhesion**—To examine the gamete adhesion activity of surface-exposed zonadhesin, we tested the effects of the D3 and D3p18 antibodies on fertilization and ZP interaction. The D3p18 antibody partially inhibited fertilization of cumulus intact mouse eggs by wild-type spermatozoa in vitro (Fig. 4A). The partial inhibition was consistent with the surface exposure of zonadhesin on only 25–35% of cells and was significant in comparison with the effect of affinity-purified control antibody to GST (anti-D3p18: 14.3% inhibition; anti-GST: 3.2%; S.E. ± 2.8%; $p < 0.05$). The D3p18 antibody did not inhibit fertilization by spermatozoa from $\text{Zan}^{-/-}$ males (1.9% inhibition cf. 2.7% by anti-GST). The anti-D3p18 antibody also inhibited ZP adhesion. Under conditions where no spermatozoa adhere to the ZP of two-cell embryos (Fig. 4B), adhesion of wild-type spermatozoa to the ZP of cumulus-free mouse eggs averaged 15–16 spermatozoa/oocyte either in the

![FIGURE 2. Males lacking zonadhesin are fertile. A, loss of zonadhesin mRNA was determined by reverse transcription-PCR in testes of littermates from intercrosses of $\text{Zan}^{+/+}$ mice. Primer sets amplified products from three regions of the zonadhesin transcript corresponding to the MAM1 (encoded by exons 3–5 deleted by the targeting strategy) and VWD3 domains (encoded by exons 33–35, 37 kb downstream of the deletion) of the protein. Strong products were amplified from $\text{Zan}^{+/+}$ and $\text{Zan}^{-/-}$ testicular RNA templates in all regions examined (including the MAM3 domain encoded by exons 11–13, not shown). No MAM1 region product (from exons replaced with neo) was amplified from $\text{Zan}^{-/-}$ tests, nor was a MAM3 region product (not shown). A faint VWD3 region product was rarely amplified from testes of 3 out of 11 $\text{Zan}^{-/-}$ males examined, possibly reflecting marginal synthesis of a truncated transcript from a cryptic promoter located among the 82 exons not deleted by the targeting strategy. A G3pdh gene-derived product was amplified from each template RNA. Animals' genotypes are indicated above each lane. (B) The fertility of $\text{Zan}^{+/+}$ males, $\text{Zan}^{-/-}$ males, and wild-type males from the two lines on the 129S2/SvPasCrl background (mean ± S.D. 2.7% by anti-GST). The anti-D3p18 antibody did not inhibit fertilization by spermatozoa from $\text{Zan}^{-/-}$ males, nor did they label any spermatozoa from $\text{Zan}^{-/-}$ males (Fig. 3, B and C). However, after optimal sperm capacitation in vitro (Fig. 3D), anti-D3p18 labeled the apical heads of some but not all wild-type cells (Fig. 3, A and D). Surface exposure of the D3p18 domain closely tracked the kinetics of sperm capacitation (Fig. 3D). In contrast, anti-D3 immunoreactivity did not increase with sperm capacitation (Fig. 3B). Loss of zonadhesin did not affect PH-20/hyaluronidase immunoreactivity (Fig. 3C and supplemental Fig. S3), nor did it substantially affect the profiles of other gamete recognition proteins of spermatozoa, including SED-1, $\beta$-1,4-galactosyltransferase, proacrosin, ZP3R/sp56, ADAM 2, or ADAM 3, or alter the pattern of sperm phosphoproteins or total protein (supplemental Fig. S3), consistent with the fertility of $\text{Zan}^{-/-}$ males. Exposure of the D3p18 domain on living, motile spermatozoa was dependent on incubation in capacitating medium (Fig. 3E). No comparable capacitation-dependent increase in surface exposure of the D3 domain occurred (Fig. 3E), suggesting that this zonadhesin domain remains masked, while the D3p18 domain becomes exposed.

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absence of added antibody or in the presence of anti-GST or anti-D3 (Fig. 4C) but was inhibited by 50% to 7.5 spermatozoa/oocyte by anti-D3p18 (S.E. \( \pm 2.30 \) spermatozoa/oocyte; \( p < 0.05 \)). The D3p18 antibody did not inhibit ZP adhesion by spermatozoa from \( \text{Zan}^{-/-} \) males (20 spermatozoa/oocyte; Fig. 4C). None of the antibodies affected sperm motility (supplemental Fig. S4).

Loss of Zonadhesin Decreases Species Specificity of ZP Adhesion—To determine whether zonadhesin contributes to species specificity of ZP adhesion, we compared the ability of spermatozoa from wild-type or \( \text{Zan}^{-/-} \) males to recognize heterologous ZP. \( \text{Zan}^{-/-} \) genotype did not affect the relatively low adhesion (4–6 cells/ZP) of non-capacitated spermatozoa to pig or rabbit ZP (Fig. 5A). With capacitation, adhesion of wild-type spermatozoa to the pig ZP did not increase, but adhesion of \( \text{Zan}^{-/-} \) spermatozoa increased 3-fold, to more than 15 cells/ZP (Fig. 5A). This capacitation-dependent adhesion of \( \text{Zan}^{-/-} \) mouse spermatozoa to pig ZP was comparable to the adhesion of homologous, capacitated pig spermatozoa (Fig. 5A). Loss of zonadhesin also increased sperm adhesion to rabbit ZP (Fig. 5B); with sperm capacitation, adhesion of \( \text{Zan}^{-/-} \) spermatozoa increased by more than 4-fold, to nearly 25 per rabbit ZP, but adhesion of wild-type spermatozoa did not increase significantly (Fig. 5B). Furthermore, when defined mixtures of sperm cells ranging from 100% wild-type to 100% \( \text{Zan}^{-/-} \) cells were incubated with pig or rabbit oocytes, ZP adhesion progressively increased with increasing proportion of \( \text{Zan}^{-/-} \) spermatozoa in the mixture (not shown).

We also directly compared adhesion of wild-type and \( \text{Zan}^{-/-} \) spermatozoa to ZP by co-inseminating oocytes with sperm cells from the two genotypes. Capacitated spermatozoa from \( \text{Zan}^{-/-} \) and \( \text{Zan}^{+/+} \) males were alternately labeled with differ-

FIGURE 3. Zonadhesin D3p18 domain is exposed during sperm capacitation. A and B, by immunofluorescence, affinity-purified antibody to the zonadhesin D3p18 domain (\( \alpha \)-D3p18; A) labeled the apical head of both living, capacitated spermatozoa (\( \text{Live-labeled} \)) and fixed, permeabilized spermatozoa (MeOH-Fixed), but anti-D3 (\( \alpha \)-D3; B) detected zonadhesin only in fixed cells. The presence of the acrosome was confirmed by labeling with PNA. Bar = 10 \( \mu \)m. C, antibody to sperm hyaluronidase (\( \alpha \)-PH-20) labeled the apical head of fixed, permeabilized spermatozoa from both \( \text{Zan}^{+/+} \) and \( \text{Zan}^{-/-} \) males. D, mouse sperm capacitation assessed by inducing acrosomal exocytosis with 10 \( \mu \)M A23187 (black triangles, \( n = 4 \)) closely tracked exposure of the zonadhesin D3p18 domain (blue squares, \( n = 4 \); correlation = 1.0; \( R^2 = 0.94 \); * and **, significantly different from other time points). The proportion of responsive cells declined after 90 min because of increased spontaneous exocytosis. E, zonadhesin exposure on spermatozoa (determined by immunofluorescence with D3p18 antibody; solid black bars) increased on living, wild-type spermatozoa incubated in capacitating medium (CM) but not on cells incubated in non-capacitating medium (NC). D3 domain immunoreactivity in either capacitated or non-capacitated cells was not different from labeling by control antibody to GST (\( \alpha \)-GST). ANOVA: S.E. \( \pm 2.2 \); LSD A and B; \( p < 0.01 \).
cent dyes (MitoTracker or Hoechst 33258), mixed in defined ratios, and then incubated with cumulus-free oocytes. When mouse oocytes were co-inseminated with equal numbers of mouse spermatozoa from wild-type and Zan-null males, ZP adhesion by the two cell types was identical (Fig. 5C). In contrast, co-insemination of pig, rabbit, or bovine oocytes resulted in preferential adhesion of Zan-null spermatozoa to the heterologous ZP (Fig. 5C). This loss of species specificity was most dramatic in interactions with pig ZP, where adhesion of spermatozoa lacking zonadhesin was nearly 5-fold higher than adhesion of wild-type spermatozoa on co-insemination with equal numbers of the two cell types. Even when pig oocytes were co-inseminated with three times more wild-type than Zan-null spermatozoa, two-thirds of the ZP-adherent cells were Zan-null (Fig. 5D). At all tested ratios, adhesion of Zan-null spermatozoa to heterologous ZP was disproportionately
The robustness that multiple adhesion molecules confer to sperm-ZP recognition (31) should perhaps be expected in a process such as fertilization that is vital to species survival. Furthermore, transient, sequential binding of multiple adhesion molecules during ZP interactions might also be expected because spermatozoa must sustain adhesion as they shed membranes and acrosomal contents during acrosomal exocytosis and then penetrate through the ZP (10). Our results support a model of sperm-ZP interactions (Fig. 6) that includes degenerate/overlapping activities of multiple ZP adhesion molecules, including β-1,4-galactosyltransferase and SED-1 on the sperm cell surface and zonadhesin, proacrosin, and ZP3R/sp56 in the acrosome. In Zan-null cells, the two likely acrosomal mediators of ZP adhesion are proacrosin and ZP3R/sp56. Both of these proteins are prominent components of the sperm acrosomal matrix (32). Proacrosin ZP binding activity is well documented (33) and promiscuous (8). ZP3R/sp56 possesses ZP binding activity (6) and can mediate ZP adhesion (7), although the species specificity of its binding activity has not been reported. Because zonadhesin-null males are fertile, it should be feasible to breed animals with multiple null alleles and thereby identify the other proteins that individually or collectively compensate for its loss.

Reproductive processes are extraordinarily diverse among animal species. Although exocytosis of the acrosome must occur before a sperm cell can penetrate the egg (1), the content of the acrosome varies dramatically between marine invertebrates and mammals. The egg recognition molecules bindin and lysozare predominant components of the sea urchin (34).
Zonadhesin Function in Mammalian Gamete Adhesion

and abalone (35) acrosome, respectively. In these organisms, the primary function of the acrosome is to mediate species-specific gamete recognition when exocytosis is induced by female-derived factors surrounding the egg, and thereby prevent formation of sterile hybrids during external fertilization (2). Mammalian sperm cells also release and expose acrosomal components in response to egg factors (1), but the mammalian acrosome contains hundreds of proteins, including hydrolases (36) and relatively abundant matrix components (32, 37) that presumably support a more complex function of the acrosome during internal fertilization in mammals. Our finding that zonadhesin mediates species-specific ZP recognition shows that at least one function of the acrosome in fertilization is conserved between mammals and marine invertebrates. This conservation of acrosome function seems remarkable considering that the egg recognition proteins (lysin, bindin, and zonadhesin) are evolutionarily unrelated in these diverse species. Because mating barriers limit cross-species fertilization in animals that fertilize internally, potentially relieving the need for species-specific gamete recognition to prevent formation of sterile hybrids (28), the function of zonadhesin in species-specific ZP adhesion may reflect a contribution in ancestral mammals to the speciation process itself (2).

Acknowledgments—We thank Stacy Lew and Raymond Wong for making the aggregation chimeras.

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Zonadhesin Is Essential for Species Specificity of Sperm Adhesion to the Egg Zona Pellucida
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doi: 10.1074/jbc.M110.123125 originally published online June 7, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.123125

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