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Mapping the mouse ZP3 combining site for sperm by exon swapping and site-directed mutagenesis

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ABSTRACT **During fertilization in mice, sperm bind to mouse ZP3 (mZP3), a $M_r \approx 83{,}000$ glycoprotein present in the ovulated egg extracellular coat, or zona pellucida. Sperm recognize and bind to specific serine/threonine-linked (O-linked) oligosaccharides present at the mZP3 combining site for sperm. Binding to mZP3 induces sperm to undergo a form of exocytosis, the acrosome reaction. To map the mZP3 combining site for sperm, we examined the effect of exon swapping and site-directed mutagenesis on the glycoprotein's two activities, sperm binding and induction of the acrosome reaction. Stably transfected embryonal carcinoma cell lines were established that synthesized recombinant glycoproteins and secreted them into the culture medium. The glycoproteins were partially purified from culture medium and assayed for sperm-binding and acrosome reaction-inducing activities. Results of these assays suggest that glycosylation of one or more of five serine residues, clustered together in a polypeptide region encoded by mZP3 gene exon 7, is required for activity. Interestingly, this polypeptide region exhibits considerable sequence divergence during evolution and may be related to the proposed role for oligosaccharides in species-specific gamete adhesion during mammalian fertilization.**

To initiate fertilization in mammals, sperm bind in a relatively species-specific manner to the ovulated egg extracellular coat, or zona pellucida (1–3). Free-swimming sperm recognize and bind tightly to ZP3, a zona pellucida glycoprotein (4–6). ZP3 from different mammals consists of a well-conserved polypeptide that is differentially glycosylated (6, 7). The latter point is particularly relevant since the biological activities of mouse ZP3 depend upon certain of its serine/threonine-linked (O-linked) oligosaccharides (4–9).

Mouse and hamster ZP3 (mZP3, $M_r \approx 83{,}000$, and hZP3, $M_r \approx 56{,}000$, respectively) consist of a $M_r \approx 44{,}000$ polypeptide (82% identical) that is glycosylated to different extents in the two species (6, 10–12). Embryonal carcinoma (EC) cells transfected with mZP3 and hZP3 genes synthesize and secrete glycoproteins (EC-mZP3 and EC-hZP3, respectively) that resemble their egg counterparts (13). However, while EC-mZP3 exhibits both sperm-binding and acrosome reaction-inducing activities *in vitro*, EC-hZP3 is inactive. The latter observation has been attributed to the failure of EC cells to properly glycosylate serine/threonine residues at the hZP3 combining site for sperm. Apparently, this reflects cell-type rather than species differences, since oocytes of mice carrying hZP3 as a transgene secrete active hZP3 (14).

Recently, we found that limited proteolysis of purified mZP3 produces a $M_r \approx 55{,}000$ glycopeptide that originates from the polypeptide's C-terminal third and exhibits both sperm-binding (15) and acrosome reaction-inducing (E. Litscher and P.M.W., unpublished results) activities *in vitro*. Results of these experiments also suggest that a specific portion of the glycopeptide (amino acids 328–343 of the mZP3 polypeptide) is directly involved in these activities. Here, we took advantage of these findings, together with those made by using EC cells transfected with recombinant ZP3 genes, to map, by exon swapping and site-directed mutagenesis, a region of mZP3 that may account for its activities during fertilization in mice.

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MATERIALS AND METHODS

Plasmid Construction for Exon Swapping. Plasmid pPGK/ZP3[h/m] was constructed as follows: pPGK/mZP3 (13) was digested with *Bam*HI and *Xba* I and the 2.7-kb fragment, containing mZP3 exons 6–8, was inserted into *Bam*HI- and *Xba* I-cleaved pGEM-7Zf(+) (Promega), generating plasmid pGEM-7/ZP3[m]. pPGK-hZP3 (13) was digested with *Nsi* I and the ≈6-kb fragment, containing the mouse *pgk-1* promoter (16, 17) and hZP3 exons 1–5, was ligated to *Nsi* I-cleaved pGEM-7/ZP3[m], generating plasmid pPGK/ZP3[h/m]. Plasmid pPGK/ZP3[m/h] was constructed as follows: pGEM-hRa (12) was digested with *Nsi* I and the 2.7-kb fragment, containing hZP3 exons 6–8, was ligated to *Pst* I-cleaved pHSG399 (Takara Shuzo, Kyoto), generating plasmid pHSG/ZP3[h]. This plasmid was partially digested with *Sal* I and completely digested with *Xho* I, and then it was religated, creating plasmid pHSG/ZP3[h]*. (These steps were carried out to destroy the *Sal* I site present in hamster intron 7.) pPGK/mZP3 was digested with *Sal* I, and the ≈7.7-kb fragment, containing the mouse *pgk-1* promoter and mZP3 exons 1–5, was ligated to *Sal* I-cleaved pHSG/ZP3[h]*, generating plasmid pPGK/ZP3[m/h].

Plasmid Construction for Site-Directed Mutagenesis. pGEM-7Zf(+) was digested with *Apa* I and *Eco*RI, the recessed ends were filled-in by using T4 DNA polymerase, and the plasmid was religated (needed to destroy the *Apa* I site). This plasmid was digested with *Bam*HI and ligated to a 3.8-kb *Bam*HI fragment of mZP3 (nt 6140–10,048), generating plasmid pGEM-7*/ZP3BHI. An 800-nt PCR fragment was generated by using 1 ng of pGEM-7*/ZP3BHI as the template and the following oligonucleotides: mZP3-5′-deletion, 5′-CCCCTGCAGCGACGTCTGGTCCAAGCT-AGTTTCTCG-3′; mZP3-3′-deletion, 5′-ATCAGGGGC-CCTACAGTGACA-3′. PCR was carried out in a 25-μl reaction mixture (50 mM KCl/10 mM Tris·HCl, pH 8.3/2 mM $MgCl_2$/gelatin, 20 μg/ml/0.2 mM NTPs/0.6 unit *Taq*/ DNA polymerase/0.2 μg of each oligonucleotide primer) overlaid with 25 μl of mineral oil in a Perkin–Elmer/Cetus DNA Thermal Cycler for 30 cycles where each cycle consists of 94°C for 30 sec, 68°C for 30 sec, and 72°C for 30 sec. The PCR-generated fragment was digested with *Pst* I and *Apa* I and then ligated to *Pst* I and *Apa* I double-digested pGEM-7*/ZP3BHI, creating plasmid pZP3BHId. Plasmid pZP3BHId has mZP3 nt, 7456–7513 deleted, and the deletion is flanked by a *Pst* I site

Abbreviations: EC, embryonal carcinoma; mZP3, mouse ZP3; hZP3, hamster ZP3.

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on the 5′-side and an *Aat* II site on the 3′-side. Plasmids containing mutated ZP3 sequences were made by annealing complementary oligonucleotides (designed to possess *Pst* I and *Aat* II sites at their 5′ and 3′ ends, respectively), digesting them with *Pst* I and *Aat* II, and then ligating them to *Pst* I and *Aat* II double-digested pZP3BHId. For example, the following oligonucleotides (sense strand only), 5′-CTGCAGCCATGGCAACTGTGCTACTGTAGGAGCTGCTCAGGTACAGATCCATGGACCCGACGTC-3′ and 5′-CTGCAGCCATGGCCAGTGTGCTCAGGTAGAGCAGAAGCTAATATCTGAGGAGGATCTAGACGTC-3′, were processed as described above to generate plasmids pZP3BHI[ser] and pZP3BHI[myc], respectively. Sequencing of these two plasmids confirmed that the predicted changes had occurred. pZP3BHI[ser] and pZP3BHI[myc] were digested with *Bam*HI and the 3.8-kb *Bam*HI fragments were ligated to a 9-kb *Bam*HI fragment [isolated from a *Bam*HI digest of pGEM-G9/R-A (10)], producing plasmids pZP3[ser] and pZP3[myc], respectively. pZP3[ser] and pZP3[myc] were digested with *Eco*RI and the 10-kb *Eco*RI fragments were ligated to *Eco*RI-digested pGEM-7Zf(+), creating plasmids pGEM/mZP3[ser] and pGEM/mZP3[myc], respectively. pGEM/mZP3[ser] and pGEM/mZP3[myc] were digested with *Xba* I and the 9-kb *Xba* I fragments were ligated to a 4.3-kb *Xba* I fragment of pPGK/mZP3, producing plasmids pPGK/mZP3[ser] and pPGK/mZP3[myc], respectively.

Cell Culture. EC cell culture was carried out essentially as previously described (14).

Transfected Cell Lines. Appropriately digested plasmid combinations at a 5:1 molar ratio {25 μg of pPGK/mZP3[ser], pPGK/mZP3[myc], pPGK/ZP3[h/m], or pPGK/ZP3[m/h]: 5 μg of pKJ-1 (this plasmid contains the bacterial neomycin resistance gene placed between the 5′ and 3′ regions of *pgk-1*)} were introduced into F9 cells (5×10^6) by electroporation (Gene Pulser; 625 V/cm, 500 μF; Bio-Rad). Cells were maintained under G418 selection (0.4 mg/ml) until G418-resistant colonies were of a suitable size for PCR analysis.

PCR. PCR analyses were carried out essentially as previously described (14). Individual G418-resistant colonies were picked and cultured until semiconfluent. Cells were removed from the culture plates by trypsinization. Half of the cells were used to establish the cell line, and half of the cells were used to make DNA by resuspending them in 25 μl of lysis buffer (50 mM KCl/10 mM Tris·HCl, pH 8.3/2 mM $MgCl_2$/0.45% Nonidet P-40/0.45% Tween 20/proteinase K, 60 μg/ml) and incubating the mixture for 1 h at 55°C, followed by 45 min at 95°C. Three oligonucleotides were used for PCR analysis of transfected EC cells: (*i*) 5′-CACTAGTCTCGTGCAGATGGA-3′, specific for the *pgk-1* promoter (nt −272 to −251) (16, 17); (*ii*) 5′-ACCTTCACAGGTGATGAGGAC-3′, specific for the mZP3 gene (nt 160–139); (*iii*) 5′-CTGACCACATCCGTAGCCACGGA-3′, specific for the hZP3 gene (nt 297–275). These primers amplify fragments of 526 nt (*pgk-1* and mZP3 primers) or 577 nt (*pgk-1* and hZP3 primers) from the appropriate plasmid. A 25-μl PCR reaction mixture [50% DNA sample in lysis buffer, 50% PCR buffer (16.6 mM ammonium sulfate/67 mM Tris·HCl, pH 8.8/bovine serum albumin, 0.1 mg/ml), plus 0.2 mM NTPs, 1 unit *Taq* DNA polymerase, and 0.2 μg of each oligonucleotide primer] was overlaid with 25 μl of mineral oil and the reaction carried out in a Perkin–Elmer/Cetus DNA thermal cycler for 40 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec. Colonies containing amplified fragments of the appropriate size were cultured under G418 selection.

Northern Analyses. Northern blots were prepared essentially as previously described (13). RNA was fractionated on 1.5% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose, and probed with a ^{32}P-labeled cDNA (nt 560–1127). Hybridizations were carried out at 42°C for 16 h in 50% formamide, with two final washes at 60°C for 30 min each in 0.2× SSC/0.1% SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate).

Western Analyses. Western immunoblots were prepared essentially as previously described (13). Cell lysates (lysis buffer = 1% Triton X-100/1 mM iodoacetamide/0.1 mM aprotinin/1 mM phenylmethylsulfonyl fluoride/10 mM Tris·HCl, pH 8.0/140 mM NaCl/0.025% sodium azide) were cleared by centrifugation and adjusted to 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 50 mM dithiothreitol, and 0.05% bromphenol blue (protein sample buffer). Alternatively, cells were cultured overnight in serum-free DMEM, culture supernatants were concentrated and fractionated by HPLC, and aliquots of pooled HPLC fractions were adjusted with protein sample buffer. Samples were separated on SDS/10% polyacrylamide gels and transferred to nitrocellulose membrane, and the membrane was processed by incubating it in a "blocking buffer," then in the presence of a rabbit polyclonal antiserum (1:1000 dilution; Pocono Rabbit Farms, Canadensis, PA) directed against mZP3, and finally in the presence of a goat anti-rabbit IgG-alkaline phosphatase conjugate (1:3000 dilution; Bio-Rad), essentially according to published procedures (18). Blocking buffer contained 3% (wt/vol) bovine serum albumin, 0.5 M NaCl, and 20 mM Tris·HCl, pH 7.5; washing buffer contained 0.2% Tween 20, 0.5 M NaCl, and 20 mM Tris·HCl, pH 7.5; and antibody solutions contained 1% bovine serum albumin, 0.5 M NaCl, 20 mM Tris·HCl, pH 7.5, and 0.2% Tween 20. Control immunoblots were prepared with nonimmune rabbit serum.

Glycoprotein Purification. Purification of the various EC-ZP3 glycoproteins was carried out essentially as previously described (13). Transfected EC cells at 80% confluency were cultured in serum-free medium (19) in 150-cm^2 tissue culture flasks for 24–48 h. Culture medium containing secreted EC-ZP3 was recovered and concentrated ≈100-fold, by centrifugation in a Centricell-60 unit (Polyscience) at $2500 \times g$ for 1 h. Concentrated medium was diluted with 60 ml of 0.2 M sodium phosphate, pH 6.7/0.1% SDS in the Centricell unit and reconcentrated by centrifugation. The sample was fractionated over an HPLC SEC-250 size-exclusion column (Bio-Rad; mobile phase = 0.2 M sodium phosphate, pH 6.7/0.1% SDS; flow rate = 0.2 ml/min). Aliquots of the 200-μl fractions were analyzed by SDS/PAGE and Western immunoblotting. Fractions containing EC-ZP3 were pooled, dialyzed extensively first against 8 M urea and then against distilled water, lyophilized, and stored at −20°C. Similar procedures were applied to culture medium from untransfected EC cells to purify those proteins/glycoproteins that copurified with EC-ZP3. Total protein concentrations were estimated spectrophotometrically and EC-ZP3 concentrations were estimated by comparison with known amounts of pure mZP3 on Western immunoblots (i.e., construction of a standard curve).

Sperm-Binding Assay. Assays for sperm-binding activity were carried out essentially as previously described (13, 14, 20–23) with gametes and embryos from randomly bred, Swiss albino mice (CD-1; Charles River Breeding Laboratories). Assays were carried out in Earle's modified medium 199 (M199; GIBCO/BRL) containing 25 mM Hepes at pH 7.3, 30 μg of pyruvate per ml, and 4 mg of bovine serum albumin per ml (M199-M) at 37°C in a humidified atmosphere of 5% CO_2 in air. Sperm (capacitated in M199-M containing 4 mM EGTA at 37°C for 1 h) in 50 μl of M199-M (5×10^5 sperm per ml, final concentration) were incubated in the presence of partially purified EC-ZP3 (≈4 ng/μl; EC-mZP3[ser] was tested in a range from ≈4 to 10 ng/μl) or in M199-M containing proteins/glycoproteins that copurified with EC-ZP3. Then 10–15 ovulated mouse eggs and 3 two-cell mouse embryos were added to the cultures and the incubation was continued for 30 min. Ovulated eggs and two-cell embryos were washed with fresh M199-M by using a mouth-operated micropipette until no sperm remained associated with embryos. The cells were

fixed in 1% glutaraldehyde, and the number of bound sperm per egg was determined by light microscopy. At least 70% of sperm remained highly motile up to the time of fixation.

Acrosome Reaction Assay. Capacitated mouse sperm in 25 μl of M199-M (1×10^6 sperm per ml, final concentration) were mixed with 25-μl samples containing HPLC-purified EC-ZP3 ($\approx$4 ng/μl; EC-mZP3[ser] was tested in a range of $\approx$4 to 10 ng/μl), M199-M, or Ca^{2+} ionophore A23187 (10 μM, final concentration; Sigma), incubated for 1 h at 37°C, and then fixed and processed essentially as previously described (13, 14, 23).

RESULTS

Experimental Rationale. Experiments described here are based on several observations: (*i*) the sperm-binding and acrosome reaction-inducing activities of mZP3 are dependent on specific O-linked oligosaccharides (20, 24, 25); (*ii*) mouse EC cells stably transfected with mZP3 and hZP3 genes secrete active EC-mZP3 and inactive EC-hZP3 (13); (*iii*) limited digestion of purified mZP3 by certain proteases yields a $M_r \approx$ 55,000 glycopeptide that originates from the C-terminal third of the polypeptide and is active (15); and (*iv*) more extensive digestion of the $M_r \approx$ 55,000 glycopeptide results in removal of amino acid residues 328–343 and, concomitantly, in loss of activity (15).

To determine whether proper glycosylation of the C-terminal third of the mZP3 polypeptide is, indeed, essential for sperm-binding and acrosome reaction-inducing activities, exon swapping and site-directed mutagenesis experiments were carried out. Exons 6–8 of the mZP3 gene were replaced with exons 6–8 of the hZP3 gene, and exons 6–8 of the hZP3 gene were replaced with exons 6–8 of the mZP3 gene, and the hybrid glycoproteins secreted by transfected EC cells (EC-ZP3[m/h] and EC-ZP3[h/m], respectively) were analyzed. It was anticipated on the basis of previous results (15) that EC-ZP3[h/m] would be active and EC-ZP3[m/h] would be inactive. In addition, potential O-linked glycosylation sites in the region of mZP3 polypeptide encompassing amino acids 328–343 were converted to glycine, alanine, or valine, or the region was replaced by a c-myc peptide by site-directed mutagenesis, and mutated glycoproteins that were secreted by transfected EC cells (EC-ZP3[ser] and EC-ZP3[myc], respectively) were analyzed. It was anticipated that, if this region of mZP3 polypeptide contains O-linked oligosaccharides essential for activity, the mutated glycoproteins would be inactive.

Exon-Swapping Experiments. Construction of plasmids pPGK/ZP3[h/m] and pPGK/ZP3[m/h], used to generate stably transfected EC cell lines, is described in *Materials and Methods*. Primary structures of EC-ZP3[h/m] and EC-ZP3[m/h] polypeptides are shown in Fig. 1. EC-ZP3[h/m] consists of the first 276 amino acids hZP3 and amino acids 279–424 of mZP3. EC-ZP3[h/m] consists of the first 278 amino acids of mZP3 and amino acids 277–424 of hZP3. PCR was used to identify stably transfected EC cells harboring the hybrid ZP3 genes and Northern blotting was used to identify cell lines that expressed the hybrid ZP3 genes (data not shown; EC-ZP3[h/m], three positive cell lines; EC-ZP3[m/h], seven positive cell lines).

To determine whether cell lines that expressed hybrid ZP3 genes also synthesized and secreted the recombinant glycoproteins, cell lysates and HPLC-fractionated culture medium were analyzed by Western immunoblotting. As seen in Fig. 2*A*, cell lysates contained EC-ZP3[h/m] (lane e) and EC-ZP3[m/h] (lane f) that migrated as broad bands at $M_r \approx$ 69,000 and $\approx$ 58,000, respectively (as compared with EC-hZP3, $M_r \approx$ 49,000, and EC-mZP3, $M_r \approx$ 80,000). Very similar results were obtained by immunoblotting HPLC-fractionated culture medium, in the absence of EC cells (Fig. 2*B*, lanes e and f). The M_r differences between hybrid and wild-type EC-ZP3 probably

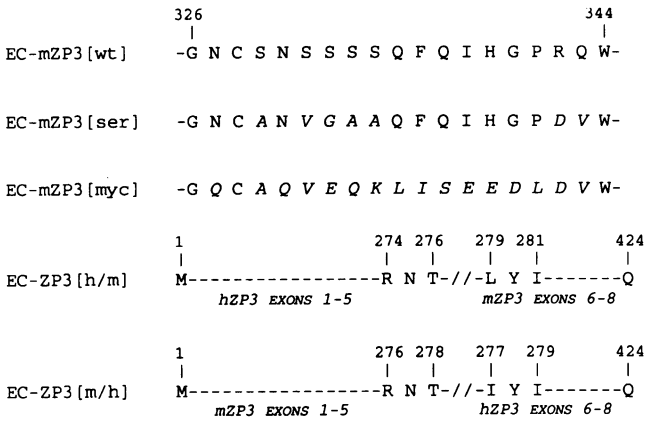

FIG. 1. Exon swapping and site-directed mutagenesis. The single-letter amino acid code is used to show the region of wild-type (wt) EC-mZP3 polypeptide (amino acids 326–344) that was modified by site-directed mutagenesis to produce EC-mZP3[ser] and EC-mZP3[myc]. Mutated amino acids are italicized. Also shown are the hybrid polypeptides produced by fusing hZP3 exons 1–5 to mZP3 exons 6–8 (EC-ZP3[h/m]) and mZP3 exons 1–5 to hZP3 exons 6–8 (EC-ZP3[m/h]). EC-ZP3[h/m] has T-276 of hZP3 joined to L-279 of mZP3 and EC-ZP3[m/h] has T-278 of mZP3 joined to I-277 of hZP3; in each case a polypeptide of 424 amino acids is produced.

reflect the fact that, as compared with hZP3, the C-terminal third of mZP3 possesses more asparagine-linked (N-linked) oligosaccharides (ref. 15 and M. Weetall, E. Litscher, and P.M.W., unpublished results).

Two different *in vitro* assays were employed to assess the activities of secreted EC-ZP3[h/m] and EC-ZP3[m/h] after partial purification by HPLC. These assays examined sperm-binding and acrosome reaction-inducing activities (see legends to Figs. 3 and 4). Purified mZP3 and hZP3, at nanomolar concentrations, possess both activities (20–22). As reported previously (13), we found that EC-mZP3 inhibited binding of sperm to eggs and induced sperm to undergo the acrosome reaction, whereas EC-hZP3 was inactive in both assays (Figs. 3 and 4). On the other hand, EC-ZP3[h/m] was about as effective as EC-mZP3, and EC-ZP3[m/h] was as ineffective as EC-hZP3 in these assays (Figs. 3 and 4). These results are consistent with the proposal that O-linked oligosaccharides essential for activity are located within the region of polypeptide encoded by exons 6–8 of the mZP3 and hZP3 genes. While the overall three-dimensional structures of mZP3 and hZP3 probably are very similar, differences in the C-terminal third of the polypeptides apparently determine whether or not transfected EC cells can add essential O-linked oligosaccharides to nascent ZP3.

Site-Directed Mutagenesis Experiments. To characterize more fully the mZP3 combining site for sperm, site-directed mutagenesis was carried out on a small region of polypeptide that is encoded by mZP3 gene exon 7 and contains five serine residues that are potential O-linked glycosylation sites (Fig. 1) (10, 11, 26). This portion of the polypeptide was chosen for mutagenesis since previous experiments revealed that proteolysis of this region results in inactivation of mZP3 *in vitro* (15) and that antibodies directed specifically against this region prevent binding of sperm to eggs both *in vivo* (27) and *in vitro* (ref. 15 and S. Mortillo and P.M.W., unpublished results). Accordingly, Ser-329, -331, -332, -333, and -334 were converted to glycine, alanine, or valine (also Arg-342 and Gln-343 were converted to asparagine and valine, respectively) (EC-mZP3[ser]; Fig. 1). In addition, site-directed mutagenesis was carried out such that a portion of c-myc sequence, 10 amino acids in length (Glu-332 to Leu-341) (28), was introduced into the region including amino acids 326–344 (EC-mZP3[myc]; Fig. 1). Two potential N-linked glycosylation sites (Asn-327

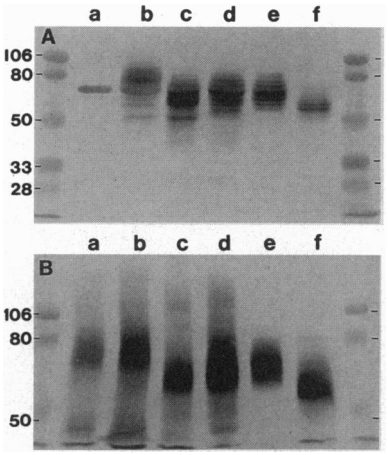

FIG. 2. Western immunoblots of transfected EC cells. EC cell lysates (*A*) or HPLC-fractionated culture medium from transfected EC cells (*B*) was subjected to SDS/PAGE, transferred to nitrocellulose membrane, and processed for Western immunoblotting using a polyclonal antiserum directed against mZP3. (*A*) Lane a, EC-mZP3[myc], line 1; lane b, EC-mZP3[wt], line 4; lane c, EC-mZP3[ser], line 7; lane d, EC-mZP3[myc], line 7; lane e, EC-ZP3[h/m], line 9; and lane f, EC-ZP3[m/h], line 19. (*B*) Lane a, EC-mZP3[wt], line 4, 100 ng; lane b, EC-mZP3[wt], line 4, 200 ng; lane c, EC-mZP3[ser], line 7; lane d, EC-mZP3[myc], line 7; lane e, EC-ZP3[h/m], line 9; and lane f, EC-ZP3[m/h], line 19. Note that EC-mZP3[myc], line 1 (lane a), possesses undetectable levels of recombinant glycoprotein. M_r standards ($\times 10^{-3}$) are present in the first and last lanes of each gel.

and -330) were destroyed by site-directed mutagenesis of mZP3; however, the glycoprotein's activities are not dependent on N-linked oligosaccharides (8, 15, 20).

Construction of plasmids pPGK/mZP3[ser] and pPGK/mZP3[myc], used to produce stably transfected EC cell lines, is described in *Materials and Methods.* PCR analysis was used to identify transfected EC cells harboring mutated mZP3

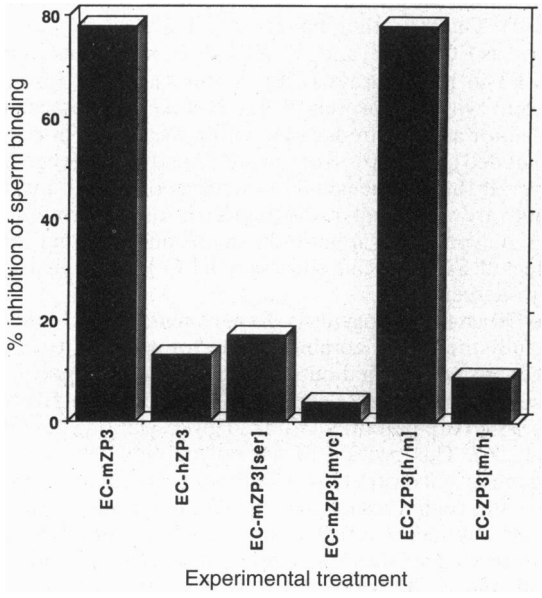

FIG. 3. Sperm-binding activity. Assays for sperm-binding activity were carried out as described in *Materials and Methods.* Shown is the average percent inhibition of sperm binding for various ZP3 preparations (≈4 ng/μl). These values were calculated by setting the average "control" value of 23 ± 4 sperm bound per egg (i.e., in the presence of only M199-M) as 100% and represent the average of four separate experiments with each test sample. The standard deviation of the mean for all test samples ranged from ±2% to ±6%.

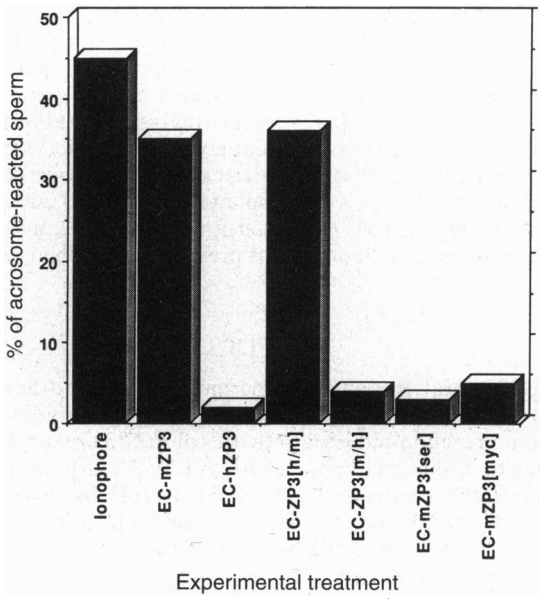

FIG. 4. Acrosome reaction-inducing activity. Assays for acrosome reaction-inducing activity were carried out as described in *Materials and Methods.* Shown is the average percentage of acrosome-reacted sperm above "background" (i.e., sperm exposed to only M199-M) for at least three separate experiments with each test sample (≈4 ng/μl) and with Ca^{2+} ionophore A23187 (10 μM; ionophore provides a "maximal" value). A total of at least 600 sperm were scored for each test sample. In these experiments, the average background value was 27% ± 2% acrosome-reacted sperm and the standard deviation of the mean for all test samples ranged from ±1% to ±5%.

genes and Northern blotting was used to identify cell lines that expressed the mutated genes (data not shown; EC-mZP3[ser], three positive cell lines; EC-mZP3[myc], three positive cell lines). EC cell lysates and HPLC-fractionated culture medium were analyzed by Western immunoblotting to detect mutated glycoproteins. As seen in Fig. 2*A*, cell lysates contained EC-mZP3[ser] (lane c) and EC-mZP3[myc] (lane d) that migrated as broad bands at $M_r \approx 63{,}000$ and $M_r \approx 66{,}000$, respectively. Very similar results were obtained by immunoblotting HPLC-fractionated culture medium, freed of EC cells to detect secreted glycoproteins (Fig. 2*B*, lanes c and d).

EC-mZP3[ser] and EC-mZP3[myc] were partially purified by HPLC and assessed for activity *in vitro.* As compared with wild-type EC-mZP3, EC-mZP3[ser] and EC-mZP3[myc] had little, if any, effect on either binding of sperm to eggs (Fig. 3) or induction of the acrosome reaction (Fig. 4). These findings suggest that elimination of potential O-linked glycosylation sites in this polypeptide region results in the synthesis of an inactive form of mZP3.

DISCUSSION

Previous studies revealed that the sperm-binding activity of mZP3 is dependent on a relatively small proportion of its O-linked oligosaccharides (20, 24, 25) that have either a galactose in α-linkage (24) or an *N*-acetylglucosamine in β-linkage (25) at their nonreducing end. These oligosaccharides can be selectively released from mZP3 by alkaline reduction and, like intact mZP3 and small mZP3 glycopeptides, shown to bind to free-swimming sperm and to prevent them from binding to ovulated eggs (20, 24, 29). Furthermore, there is some evidence to suggest that the O-linked oligosaccharides recognized by sperm are located on the C-terminal third of the mZP3 polypeptide (13, 15, 27). While the precise nature of the sperm component that binds to mZP3 remains a controversial issue, it should be noted that some of the

principal candidates recognize carbohydrate determinants (9, 30).

Here, exon swapping and site-directed mutagenesis were used to map the mZP3 combining site for sperm. Results of these experiments provide further support for the proposal that the mZP3 combining site for sperm (i.e., the region of mZP3 polypeptide carrying the O-linked oligosaccharides to which free-swimming sperm bind) is located on the C-terminal third of the polypeptide. Furthermore, consistent with certain other findings (refs. 15 and 27 and S. Mortillo and P.M.W., unpublished results), they suggest that the O-linked oligosaccharides recognized by sperm are located on a region of mZP3 polypeptide that includes amino acids 328–343. There are five serine residues in this region and no threonine residues. Four of the five serine residues occur consecutively and the fifth is separated from the others by a single amino acid, an arrangement of hydroxyamino acids typical of heavily O-glycosylated regions of many glycoproteins (31, 32). On the other hand, proline residues that are often located at positions −1 and +3 relative to glycosylated serine/threonine residues (32) are not present. It should be noted, however, that the five serine residues are located adjacent to a stretch of nine amino acids that includes four cysteine residues that are conserved in all ZP3 genes characterized thus far (6, 7). The local 3-dimensional structure generated by the cysteine residues may render this region of mZP3 polypeptide accessible to *N*-acetylgalactosaminyltransferase, the first enzyme required in the O-linked glycosylation pathway (31).

It is interesting that the putative mZP3 combining site for sperm is located in a polypeptide region that has undergone considerable sequence divergence during evolution. For example, whereas the amino acid sequences of mZP3 and human ZP3 (huZP3) (33) polypeptides are 67% identical overall, the region including amino acids 329–342 is only 28% identical—i.e., 4 of 14 residues are identical. Two of five serine residues of mZP3 (Ser-332 and -334) are conserved and huZP3 has an additional serine (Ser-342) and threonine (Thr-330) residue. Furthermore, although the amino acid sequences of mZP3 and hZP3 polypeptides are 82% identical overall, the region including amino acids 329–342 has only six residues in common (43% identical). Three of five serine residues of mZP3 (Ser-331, -332, and -334) are conserved and hZP3 has an additional serine residue (Ser-342). In fact, a comparison of the primary structures of mZP3 and hZP3 polypeptides, carried out in 40-residue increments from Pro-35 to Leu-394, reveals that the region including amino acids 315–354 has undergone twice as many changes as any other region. In addition, there is evidence to suggest that the combining site of mZP3 is located in one of two structural domains that are separated by a hinge region located in about the middle of the mZP3 polypeptide (34).

It is tempting to suggest that the relatively extensive, local changes in polypeptide primary structure just described are related to the presence of O-linked oligosaccharides involved in sperm recognition and binding. In this context, we suggested previously that species specificity of sperm–egg adhesion is determined by ZP3 oligosaccharides (5–9). Differences in ZP3 oligosaccharide structure among species could account for the frequent failure of sperm from one mammalian species to bind to eggs of another. While the rules that govern placement and structure of O-linked oligosaccharides on glycoproteins remain unclear (31, 32), it is likely that changes in polypeptide primary structure in and around the ZP3 combining site influence the location and nature of oligosaccharides added to nascent ZP3 and, in this manner, could determine species specificity of gamete adhesion. In this context, protein–carbohydrate interactions now are thought to be employed in a number of instances of cellular adhesion, in systems as diverse as binding of pathogenic bacteria to animal cells (35, 36), neuronal development (37, 38), and lymphocyte homing (39, 40). The potential for enormous variation in oligosaccharide structure, which permits extreme fine-tuning of recognition determinants, is an appealing feature of carbohydrate-mediated cellular adhesion in general.

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