Embryonal Carcinoma Cells Transfected with ZP3 Genes Differentially Glycosylate Similar Polypeptides and Secrete Active Mouse Sperm Receptor

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Abstract. Mouse and hamster sperm receptors, called mZP3 (~83,000 M_r) and hZP3 (~56,000 M_r), respectively, are glycoproteins located in the ovulated egg zona pellucida. Certain of the glycoprotein O-linked oligosaccharides are essential for sperm receptor activity. Here, we transfected mouse embryonal carcinoma (EC) cells with mZP3 and hZP3 genes placed under control of a constitutive promoter. Transfected cells synthesized and secreted large amounts of the glycoproteins, called EC-mZP3 and EC-hZP3. Although the primary structures of mZP3 and hZP3 polypeptides (44,000 M_r) are very similar to one another, EC-mZP3 (~83,000 M_r) and EC-hZP3 (~49,000 M_r) were glycosylated to very different extents, such that they resembled their egg counterparts. Like egg mZP3, EC-mZP3 inhibited binding of sperm to ovulated eggs and induced sperm to acrosome-react in vitro. In addition, large numbers of sperm bound to aggregates of mZP3-transfected EC cells in vitro. On the other hand, unlike egg hZP3, EC-hZP3 did not exhibit either sperm receptor or acrosome reaction-inducing activity, and sperm failed to bind to aggregates of hZP3-transfected EC cells. Thus, transfected EC cells not only express sperm receptor genes, but also discriminate between very similar polypeptides with respect to glycosylation and, in the case of mZP3, add specific oligosaccharides essential for biological activity. In addition, the results demonstrate that EC cells can serve as a source for large amounts of functional mouse sperm receptor.

DURING fertilization in mammals, sperm bind in a relatively species-specific manner to the ovulated egg extracellular coat, or zona pellucida (ZP)[1] (Gwatkin, 1977; Yanagimachi, 1988). Binding is supported by "sperm receptors" in the egg ZP and "egg-binding proteins" on the sperm surface (Wassarman, 1987*a*,*b*, 1990). Considerable evidence suggests that mZP3, one of three ZP glycoproteins, serves as sperm receptor in mice (Wassarman, 1987*a*,*b*, 1990). Sperm bind to certain mZP3 serine/threonine- (O-) linked oligosaccharides that are essential for sperm receptor activity (Wassarman, 1987*a*,*b*, 1988, 1989, 1990, 1991).

mZP3 (~83,000 M_r) consists of a 44,000 M_r polypeptide, 3 or 4 complex-type, asparagine- (N-) linked oligosaccharides, and an undetermined number of O-linked oligosaccharides (Wassarman, 1987*a*,*b*, 1988, 1990). mZP3 O-linked oligosaccharides essential for sperm receptor function are ~3,900 M_r, have an essential galactose residue in α-linkage at the nonreducing terminus, and represent a small fraction of total mZP3 O-linked oligosaccharide (Wassarman, 1988, 1989, 1990, 1991). The mZP3 gene has been cloned and characterized, and the mZP3 polypeptide primary structure determined (Kinloch and Wassarman, 1989*a*). There is considerable evidence that expression of the mZP3 gene is restricted to growing oocytes (Kinloch and Wassarman, 1989*a*). *Cis*-acting sequences in the 5'-flanking region of the gene are responsible for oocyte-specific expression of mZP3 (Lira et al., 1990).

Recently, we reported that hZP3 serves as sperm receptor during fertilization in hamsters (Moller et al., 1990) and that the primary structure of hZP3 polypeptide is very similar (81%) to that of mZP3 (Kinloch et al., 1990). The latter is of interest since secreted forms of hZP3 and mZP3 are ~56,000 M_r and ~83,000 M_r, respectively, indicating that the two polypeptides (44,000 M_r) are glycosylated to very different extents. Since purified hZP3 and mZP3 bind to both mouse and hamster sperm, apparently the two sperm receptors have certain O-linked oligosaccharides in common. This is consistent with the fact that mouse sperm bind to the hamster egg ZP and hamster sperm bind to the mouse egg ZP (Schmell and Gulyas, 1980; Cherr et al., 1986; Moller et al., 1990).

In several attempts, we were unable to establish transfected cell lines that secreted functional mammalian sperm receptors. Although transfected cells synthesized sperm

1. *Abbreviations used in this paper*: ZP, zona pellucida; mZP3, mouse ZP3; hZP3, hamster ZP3; EC, embryonal carcinoma; pgk-1, phosphoglycerate kinase-1.

receptor polypeptides, they apparently failed to glycosylate the polypeptides with those O-linked oligosaccharides required for biological activity. Here, we report that embryonal carcinoma (EC) cells transfected with the mZP3 gene secrete large amounts of biologically active mouse sperm receptor (EC-mZP3). Thus, EC cells glycosylate mZP3 polypeptide with oligosaccharides essential for biological activity. On the other hand, EC cells transfected with the hZP3 gene secrete inactive hamster sperm receptor (EC-hZP3). Surprisingly, the extents of glycosylation of EC-mZP3 and EC-hZP3 resemble those of egg mZP3 and hZP3, respectively, indicating that the EC cell glycosylation apparatus can distinguish between two very similar polypeptides.

Materials and Methods

Plasmid Construction

Plasmids used to generate transfected EC cell lines are depicted schematically in Fig. 1. Briefly, pKJ-1 contains the bacterial neomycin resistance gene placed between the mouse phosphoglycerate kinase-1 (pgk-1) 5'- and 3'-regions (Adra et al., 1987; Boer et al., 1990). pPGK/mZP3 contains mouse genomic sequences containing the entire mouse ZP3 (mZP3) coding region (nts −59 to +8,900; Kinloch and Wassarman, 1989*b*), including the polyadenylation and termination signals, placed under control of the pgk-1 promoter (Adra et al., 1987). pPGK/hZP3 contains the entire hamster ZP3 (hZP3) coding region (nts +28 to +8,200; Kinloch et al., 1990), including the polyadenylation and termination signals, placed under control of the pgk-1 promoter. Prior to electroporation, plasmids pKJ-1, pPGK/mZP3, and pPGK/hZP3 were digested with EcoRI and HindIII, ClaI and SstII, or HindIII and ApaI, respectively (Fig. 1).

Cell Culture

EC cells (F9) were cultured in DME containing 10% FBS (Rudnicki and McBurney, 1987). Transfected EC cell lines were cultured in the same medium supplemented with 0.2 mg/ml G418 (Geneticin; Gibco Laboratories B.R.L., Grand Island, NY). In general, routine EC cell culture procedures (Stewart, 1980) were followed. In some cases, EC cells were induced to form aggregates by plating at 10^6 cells/ml onto bacteriological culture dishes (Martin and Evans, 1975; Stewart, 1980). In some cases, EC cells were cultured in the presence of tunicamycin. Cells at 70% confluency were cultured overnight in either medium containing tunicamycin (5 μg/ml; prepared from a 10 mg/ml stock solution made up in DMSO; Sigma Chemical Co., St. Louis, MO) or control medium containing the appropriate concentration of DMSO.

Production of EC-mZP3 and EC-hZP3 Cell Lines

Appropriately digested plasmid combinations (pPGK/mZP3 and pKJ-1; pPGK/hZP3 and pKJ-1; pPGK/mZP3, pPGK/hZP3, and pKJ-1) at either a 5:1 or 5:5:1 molar ratio (25 μg pPGK/mZP3 or pPGK/hZP3:5 μg pKJ-1), were introduced into 5 × 10^6 F9 cells by electroporation (Gene Pulser; 625 V/cm, 500 μFD; Bio-Rad Laboratories, Richmond, CA). Cells were maintained under selection (0.4 mg/ml Geneticin; Gibco Laboratories B.R.L.) until G418-resistant colonies were of a suitable size to be analyzed by polymerase chain reaction (PCR).

Polymerase Chain Reaction

Individual G418-resistant colonies were picked and one-half of each colony was used to establish the cell line and the other half was used to make DNA by resuspending in 25 μl of lysis buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, and 60 μg/ml Proteinase K; Higuchi, 1989) and incubated for 1 h at 55°C, followed by 15 min at 95°C. Three oligonucleotides were used for PCR analysis of transfected EC cells: (*a*) 5'-CACTAGTCTCGTGCAGATGGA-3', specific for the pgk-1 promoter (nts −272 to −251; Adra et al., 1987); (*b*) 5'-ACCTTCACAGGTGATGAGGAC-3', specific for the mZP3 gene (nts 160 to 139; Kinloch and Wassarman, 1989*b*); (*c*) 5'-CTGACCACATCCGTAGCCACGGA-3', specific for the hZP3 gene, (nts 297 to 276; Kinloch et al., 1990). These primers amplify fragments of 526 nts (pgk-1 and mZP3 primers) or 577 nts (pgk-1 and hZP3 primers) from the appropriate plasmid (Fig. 1). A 20 μl PCR reaction mix (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 20 μg/ml gelatin, 0.2 mM nucleotide triphosphates, 0.6 units Taq Polymerase (Perkin-Elmer/Cetus, Norwalk, CT), 0.2 μg of each oligonucleotide primer, and 2 μl of DNA sample) was overlaid with 25 μl of mineral oil and run in a Perkin Elmer/Cetus DNA Thermal Cycler for 30 cycles of 94°C, 90 s; 65°C, 60 s; and 72°C, 60 s. Positive colonies were expanded under G418 selection.

Northern Gel Analyses

Total RNA was isolated from cell lines using RNAzol B, as described by the supplier (Cinna/Biotecx Labs, Friendswood, TX). RNA samples were fractionated on 1.5% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose, and probed with a ^{32}P-labeled cDNA (nts 560 to 1127; Kinloch et al., 1990), according to methods previously described (Maniatis et al., 1982). 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.

Western Immunoblot Analyses

Transfected and untransfected EC cells (1 × 10^7 cells) were extracted in 1 ml of lysis buffer (1% Triton X-100, 1 mM iodoacetamide, 0.2 U/ml aprotinin, 1 mM PMSF, 10 mM Tris-Cl, pH 8, 140 mM NaCl, 0.025% sodium azide), centrifuged at 3,000 *g* to remove nuclei, and the supernatant adjusted to 1% SDS and cleared by centrifugation at 30,000 *g* for 30 min at 4°C. The supernatant was recovered and adjusted to 2% SDS/10% glycerol/50 mM DTT/0.05% Bromphenol blue (protein sample buffer). Alternatively, cells were cultured overnight in serum-free DME, and the medium was recovered, concentrated 10-fold using a Centricon-10 unit (Amicon, Danvers, MA), cleared by centrifugation, and adjusted to protein sample buffer. Aliquots of treated medium and proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membrane. The membrane was processed for Western immunoblotting by first incubating it in a "blocking buffer," then in the presence of a rabbit polyclonal antiserum (Pocono Rabbit Farms, Canadensis, PA) directed against either mZP3 or hZP3 (1:750 dilution), and finally in the presence of a goat anti-rabbit IgG-alkaline phosphatase conjugate (1:3,000 dilution; Bio-Rad Laboratories), essentially according to published procedures (Ausubel, 1987). Blocking buffer contained 3% BSA, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5; washing buffer containing 0.2% Tween-20, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5; and antibody solutions contained 1% BSA, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5, 0.2% Tween-20.

Glycoprotein Purification

Purification of egg mZP3 and hZP3 from ovaries has been described (Bleil et al., 1988; Moller et al., 1990). Purification of EC-mZP3 and EC-hZP3 was carried out in the following manner. Transfected EC cells were cultured in serum-free medium (Heath, 1987) in 150-cm^2 tissue culture flasks. Medium containing either secreted EC-mZP3 or EC-hZP3 was recovered and concentrated 100-fold, in a Centricell-60 unit (Polysciences, Warrington, PA) by centrifugation at 2,500 *g* for 1 h. Concentrated medium was then substituted with buffer containing 0.075 M Tris.HCl, pH 6.8, 0.1% SDS, by centrifugation at 7,000 *g* for 20 min using a Centricon-10 microconcentration unit (Amicon). The sample was dialyzed extensively against 8 M urea, then against distilled water, adjusted to 0.2 M phosphate, pH 6.6, 0.1% SDS, and fractionated by HPLC on a Bio-Rad Laboratories SEC-250 size-exclusion column (mobile phase was 0.2 M phosphate, pH 6.6, 0.1% SDS, and flow rate was 0.1 ml/min). Aliquots of 500 μl fractions were analyzed first by SDS-PAGE, followed by Western immunoblotting and silver staining. Fractions containing either EC-mZP3 or EC-hZP3, together with proteins that co-purified with EC-mZP3 and EC-hZP3, were pooled, dialyzed extensively, lyophilyzed, and stored at −20°C. In some cases, similar procedures were applied to culture medium from EC cells transfected with the hZP3 gene in order to purify those proteins that normally co-purified with EC-mZP3.

Sperm Receptor Assays

Assays for sperm receptor activity ("competition assays") were carried out in vitro essentially as previously described (Bleil and Wassarman, 1980;

Florman and Wassarman, 1985; Moller et al., 1990), using gametes and embryos obtained from randomly bred, Swiss albino mice (CD-1; Charles River Breeding Labs, Wilmington, MA). Assays were carried out in Earles modified medium 199 (Gibco B.R.L) containing 25 mM Hepes, pH 7.3, 30 μg/ml pyruvate, and 4 mg/ml BSA (M-199M), at 37°C in a humidified atmosphere of 5% CO_2 in air. Capacitated (M-199M, 4 mM EGTA, 37°C, 1 h) mouse sperm in 50 μl of medium (5×10^5 sperm/ml, final concentration) were incubated in the presence of either purified EC-mZP3 or EC-hZP3, purified egg mZP3 or hZP3, or in M-199M alone. Then 12–15 ovulated mouse eggs and 3–4 two-cell mouse embryos, obtained as previously described (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; Moller et al., 1990), were added to the cultures and the incubation continued for an additional 30 min. Ovulated eggs and two-cell embryos were washed by mouth pipetting with fresh M-199M, until no sperm remained associated with two-cell embryos, and the cells were fixed in 1% gluteraldehyde and the number of bound sperm per egg determined by light microscopy. In some cases, either transfected or untransfected EC cell aggregates, together with 4–5 ovulated mouse eggs and 3–4 two-cell mouse embryos, were incubated with capacitated mouse sperm for 30 min. At the end of the incubation aggregates, eggs, and embryos were washed as described above. After fixation of cells, the number of bound sperm per aggregate and per egg was determined by light microscopy. In all competition assays, at least 70% of sperm remained highly motile up to the time of fixation.

Acrosome Reaction Assays

Capacitated mouse sperm in 25 μl of M199-M (1×10^6 sperm/ml, final concentration) were mixed with 25-μl samples containing M199-M alone, purified egg mZP3 or hZP3, purified EC-mZP3 or EC-hZP3, or ionophore A23187 (10 μM, final concentration; Sigma Chemical Co.) incubated for

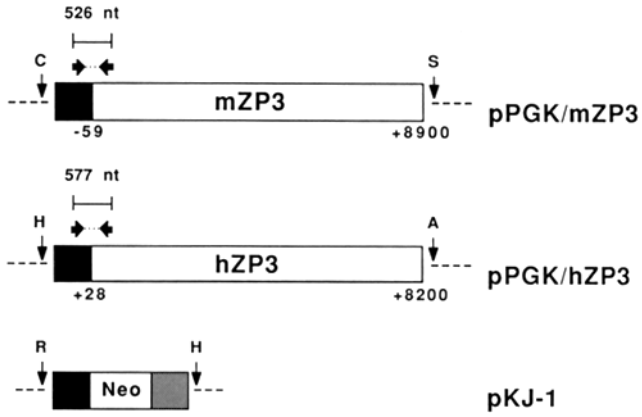


Figure 1. Schematic of appropriately digested plasmids (*pPGK/mZP3, pPGK/hZP3, and pKJ-1*) introduced into EC cells by electroporation. Restriction endonucleases used: A, ApaI; C, ClaI; H, HindIII; R, EcoRI; S, SstII. Coding regions of the mZP3, hZP3, and Neo genes are represented by open boxes and the pgk-1 5′- and 3′-regions are denoted by closed and stippled boxes, respectively. The numbering represents the nt position of the mZP3 and hZP3 coding regions with respect to their transcriptional start sites (+1). Arrows indicate the positions of PCR primers used and lengths of the amplified fragments are given.

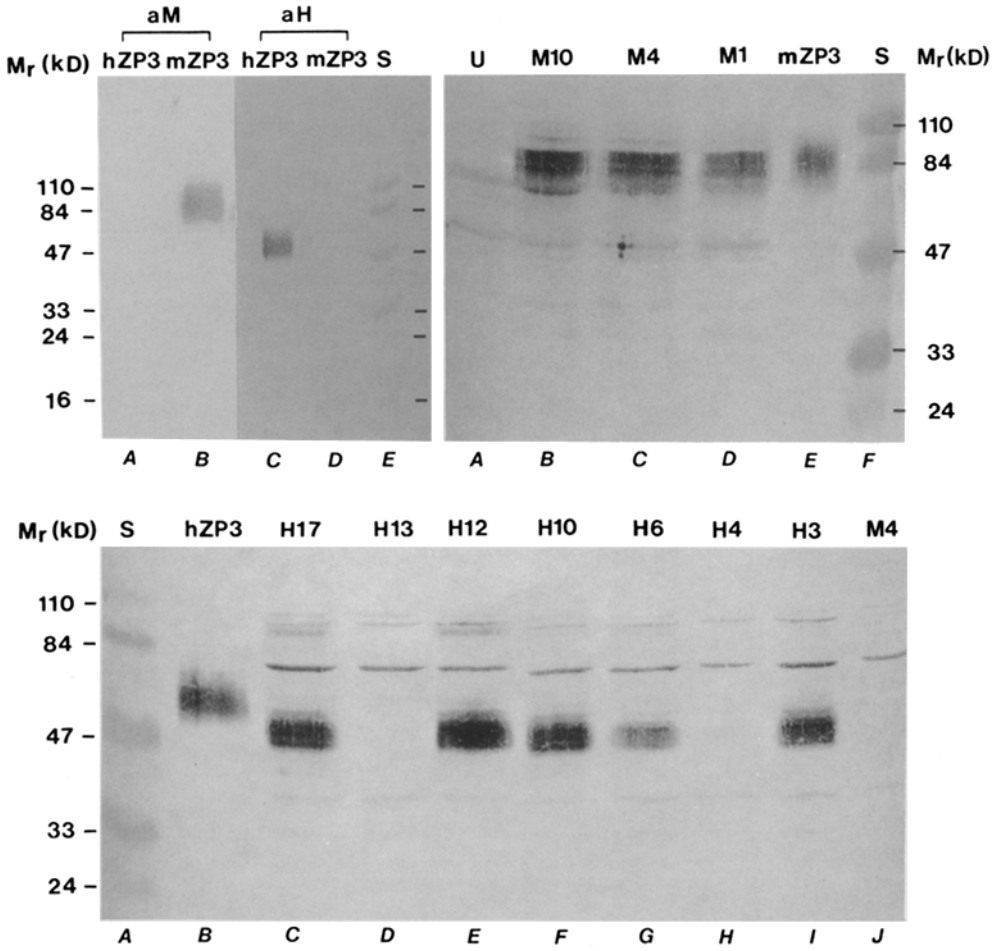


Figure 2. Western immunoblot analysis of transfected EC cells. Purified mZP3 and hZP3, and lysates from transfected and untransfected cells were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and processed for Western immunoblotting using polyclonal antisera directed against either mZP3 or hZP3, as described in Materials and Methods. (*Top left*) Purified hZP3 (100 ng) and mZP3 (100 ng) probed with either anti-mZP3 (*aM*; lanes *A* and *B*) or anti-hZP3 (*aH*; lanes *C* and *D*). Size standards (*S*) are shown in lane *E*. (*Top right*) Lysates from untransfected cells (*U*; lane *A*), cell lines M10, 4, and 1 (lanes *B–D*, respectively), and purified mZP3 (lane *E*; 100 ng), probed with anti-mZP3. Size standards (*S*) are shown in lane *F*. (*Bottom*) Lysates from cell lines H17, 13, 12, 10, 6, 4, and 3 (lanes *C–I*), M4 (lane *J*), and purified hZP3 (lane *B*; 100 ng), probed with anti-hZP3. Size standards (*S*) are shown in lane *A*.

1 h at 37°C, and then fixed and processed, essentially as previously described (Moller et al., 1990). Briefly, treated sperm were fixed with 2% formaldehyde for 30 min, washed by centrifugation at 10,000 rpm for 2 min in 0.15 M ammonium acetate, pH 9, and dried onto gelatin-coated (0.25% gelatin, 0.5% chromium sulphate) glass slides. Sperm were stained with 0.04% Coomassie G-250 (in 3.5% perchloric acid) for 2.5 min, washed with distilled water, and scored for the presence or absence of an acrosome by bright-field microscopy.

Immunohistological Analyses

Transfected and untransfected EC cells were cultured under conditions that led to formation of large aggregates. Cryosections of EC cell aggregates were prepared and processed for immunohistological examination using rabbit antisera directed specifically against either mZP3 or hZP3 and goat anti-rabbit IgG coupled to 5-nm colloidal gold (Jansen Biotec, Piscataway, NJ). Sections were prepared and immunostained essentially as previously described (Griffiths et al., 1983; Polak and Varndell, 1984), except that all solutions contained polyvinylpyrrolidone (5 mg/ml; 40,000 M_r), all incubations were carried out in a 40°C water bath, and sections were reembedded in epon before examination by transmission EM (Mortillo and Wassarman, 1991). Sections mounted on copper grids were examined with a Philips 201 electron microscope at 60 KV (201; Philips Electronic Instruments, Inc., Piscataway, NJ).

Results

Production of Transfected EC Cell Lines

A schematic diagram of appropriately digested plasmids introduced into EC cells by electroporation is presented in Fig. 1. In the case of PGK/mZP3 (KJ-1), genomic DNA was purified from 11 G418-resistant colonies and PCR analysis revealed three positives that were used to produce cell lines M1, 4, and 10. Southern gel analysis suggested that the PGK/mZP3 construct was present as tandem copies (10–15) at a single insertion site that was unique to each transfected EC cell line. In the case of PGK/hZP3 (KJ-1), genomic DNA was purified from 17 G418-resistant colonies and PCR analysis revealed 16 positives, 7 of which were chosen to produce cell lines H3, 4, 6, 10, 12, 13, and 17. Northern gel analysis revealed that all three cell lines transfected with PGK/mZP3 and five of seven cell lines transfected with PGK-hZP3 contained relatively high levels of ZP3 mRNA (~1.5 kb), as compared to mouse ovarian RNA preparations (data not shown). Untransfected EC cells contained undetectable levels of ZP3 mRNA.

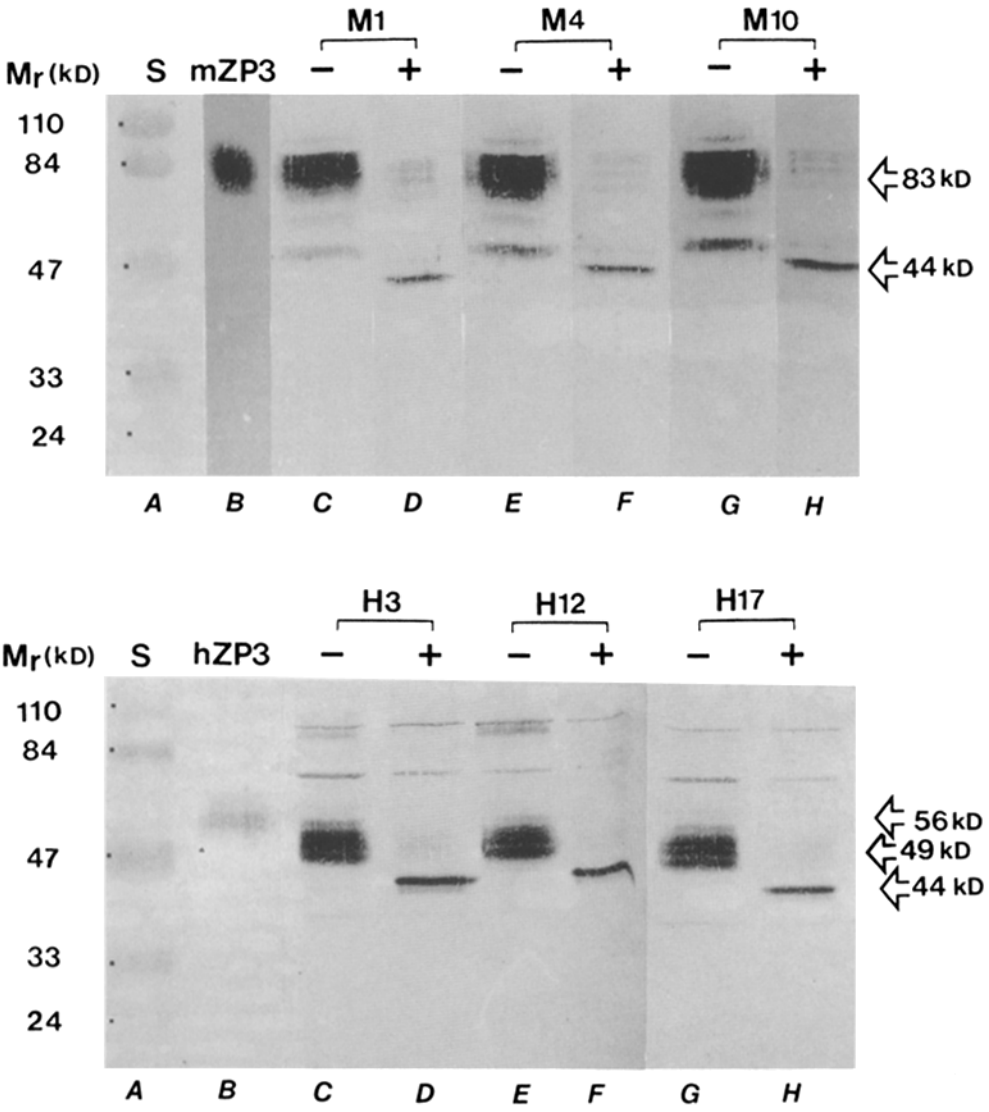


Figure 3. Western immunoblot analysis of transfected EC cells treated with tunicamycin. Transfected EC cell lines were cultured in the presence (+) or absence (−) of tunicamycin, and cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and processed for Western immunoblotting using polyclonal antisera directed against either mZP3 or hZP3, as described in Materials and Methods. (*Top*) Purified mZP3 (lane *B*; 100 ng) and lysates from cell lines M1 (lanes *C* and *D*), M4 (lanes *E* and *F*), and M10 (lanes *G* and *H*), probed with anti-mZP3. Arrows indicate the expected positions of mature mZP3 and EC-mZP3 (~83 kD) and mZP3 and EC-mZP3 polypeptide (44 kD). Size standards (*S*) are shown in lane *A*. (*Bottom*) Purified hZP3 (lane *B*; 50 ng) and lysates from cell lines H3 (lanes *C* and *D*), H12 (lanes *E* and *F*), and H17 (lanes *G* and *H*), probed with anti-hZP3. Arrows indicate the expected positions of mature hZP3 (~56 kD), mature EC-hZP3 (~49 kD), and hZP3 and EC-hZP3 polypeptide (44 kD). Size standards (*S*) are shown in lane *A*.

Synthesis of EC-mZP3 and EC-hZP3

To determine whether or not transfected EC cells synthesized mZP3 and hZP3, cell lysates were prepared and analyzed on immunoblots by using rabbit antisera raised against two different synthetic peptides (16-mers). These peptides were constructed on the basis of mZP3 and hZP3 primary structures (amino acid residues 328–343 and 326–341 for mZP3 and hZP3, respectively). As seen in Fig. 2, on Western immunoblots the antiserum directed against mZP3 peptide recognized purified egg mZP3, but not hZP3, and that directed against hZP3 peptide recognized purified egg hZP3, but not mZP3.

Immunoblots of lysates of the three EC cell lines (M1, 4, 10) transfected with the mZP3 gene and five lines (H3, 6, 10, 12, 17) transfected with the hZP3 gene synthesized protein that was recognized by antisera directed specifically against either mZP3 or hZP3, respectively (Fig. 2). In the case of mZP3 transfected cells, a broad band at ~83,000 M_r (EC-mZP3), approximately the same M_r as purified egg mZP3, was observed on immunoblots of cell lysates. In the case of hZP3 transfected cells, a broad band at ~49,000 M_r (EC-hZP3), a significantly lower M_r than purified egg hZP3 (56,000 M_r), was observed on immunoblots of cell lysates. Lysates of untransfected EC cells did not exhibit either EC-mZP3 or EC-hZP3 on immunoblots.

The sizes of the mZP3 and hZP3 polypeptides are nearly identical (44,000 M_r) (Kinloch et al., 1988, 1990; Ringuette et al., 1988). Therefore, the immunoblot results described above strongly suggest that EC-mZP3 (~83,000 M_r) and EC-hZP3 (~49,000 M_r) are glycosylated to very different extents during intracellular processing. To determine whether or not a 44,000 M_r polypeptide was synthesized in both mZP3- and hZP3-transfected EC cells, experiments were carried out in the presence of tunicamycin, an inhibitor of N-linked glycosylation (Struck and Lennarz, 1980). It had been demonstrated previously that incubation of growing oocytes in the presence of tunicamycin resulted in the appearance of 44,000 M_r mZP3 polypeptide (Salzmann et al., 1983; Roller and Wassarman, 1983). As seen in Fig. 3, when mZP3- and hZP3-transfected EC cell lines were incubated in the presence of tunicamycin and cell lysates subjected to immunoblotting, indeed, a 44,000 M_r species was detected. Cell lines incubated in the absence of tunicamycin exhibited a broad band at the M_rs expected for EC-mZP3 and EC-hZP3.

In view of the results just described, two EC cell lines were produced (called M/H4 and M/H5) by cotransfecting EC cells with both pPGK/mZP3 and pPGK/hZP3, as well as pKJ-1. These lines were produced to determine whether or not mZP3 and hZP3 polypeptides would be glycosylated to different extents when synthesized in the same cell. M/H4 and M/H5 cell lysates were probed on immunoblots with antiserum directed against either mZP3 or hZP3. Lysates of both lines contained both EC-mZP3 (~83,000 M_r) and EC-hZP3 (~49,000 M_r), providing further evidence for differential glycosylation of nascent mZP3 and hZP3 by transfected EC cells (data not shown).

Secretion of EC-mZP3 and EC-hZP3

mZP3 and hZP3 are secreted glycoproteins and each has a 22 amino acid signal sequence (Kinloch et al., 1990). To determine whether or not EC-mZP3 and EC-hZP3 were secreted from transfected EC cells, aliquots of culture medium used to grow mouse lines M1, 4, and 10, hamster lines H3, 12, and 17, and untransfected cells were probed on immunoblots with antiserum directed against either mZP3 or hZP3. Immunoblots revealed that mouse and hamster lines secreted relatively large amounts of EC-mZP3 and EC-hZP3, respectively, into culture medium (Fig. 4). For example, ~5 μg of EC-mZP3 was secreted/10^7 cells per 16 h, as estimated from immunoblots containing known amounts of egg mZP3. As in the case of cellular lysates, secreted EC-mZP3 was ~83,000 M_r and secreted EC-hZP3 ~49,000 M_r. Culture medium used to grow untransfected EC cells was negative on immunoblots probed with either mZP3 or hZP3 antisera.

Immunohistological analysis of transfected EC cells provided further evidence that they secreted EC-mZP3 and EC-hZP3. Transfected and untransfected EC cells were induced to form aggregates (Martin and Evans, 1975; Stewart, 1980) and semi-thin cryosections were prepared and processed for immunohistology using colloidal gold. As seen in Fig. 5, EC

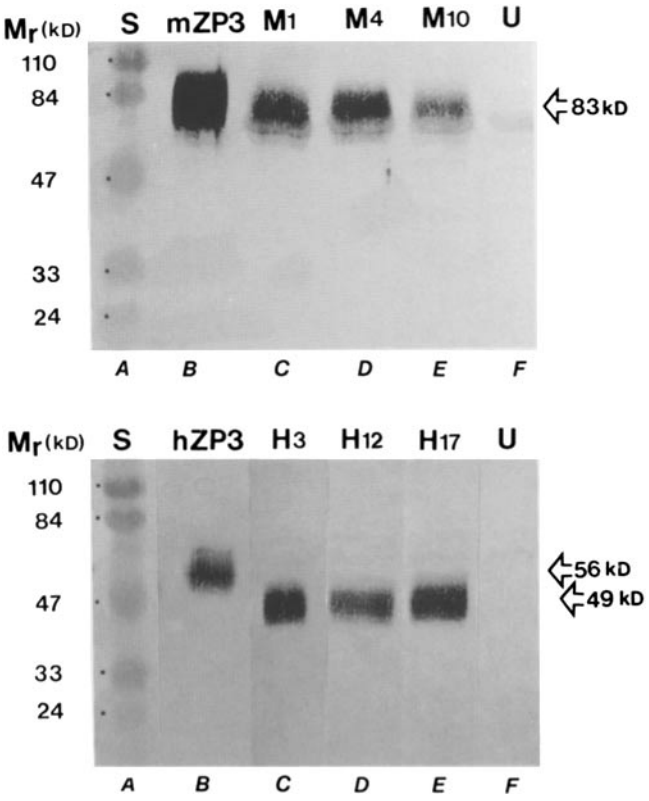


Figure 4. Western immunoblot analysis of secretion by transfected EC cell lines. Transfected and untransfected cell lines were cultured overnight in serum-free medium, and concentrated culture supernatants were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and processed for Western immunoblotting using polyclonal antisera directed against either mZP3 (*top*) or hZP3 (*bottom*), as described in Materials and Methods. (*Top*) Purified mZP3 (lane *B*; 200 ng), cell lines M1, 4, and 10 (lanes *C–E*), and untransfected cells (*U*; lane *F*), probed with anti-mZP3. Size standards (*S*) are shown in lane *A*. (*Bottom*) Purified hZP3 (lane *B*; 100 ng), cell lines H3, 12, and 17 (lanes *C–E*), and untransfected cells (*U*; lane *F*), probed with anti-hZP3. Arrows indicate the expected positions of mZP3 and EC-mZP3 (83 kD), hZP3 (56 kD), and EC-hZP3 (49 kD). Size standards (*S*) are shown in lane *A*.

cells transfected with the mZP3 gene contained large numbers of secretory vesicles and these contained material that was recognized by antiserum directed against mZP3. On average, each secretory vesicle in transfected EC cells contained 40 gold particles (total of 30 measurements; range, 10–101 gold particles), as compared to two gold particles per equivalent area in untransfected EC cells ("background"; total of 30 measurements; range, 0–9 gold particles). Immunoreactive material was also abundant on the surface of transfected EC cells, presumably because of adventitious sticking of secreted EC-mZP3. Similar results were obtained using EC cells transfected with the hZP3 gene (data not shown). These observations confirm that transfected EC cell lines secreted EC-mZP3 and EC-hZP3.

Biological Activities of EC-mZP3 and EC-hZP3 In Vitro

Three experimental approaches were used to determine whether or not EC-mZP3 and EC-hZP3 exhibited biological activity. In the first approach, an in vitro "competition assay" was used. Mouse sperm were exposed to either purified egg

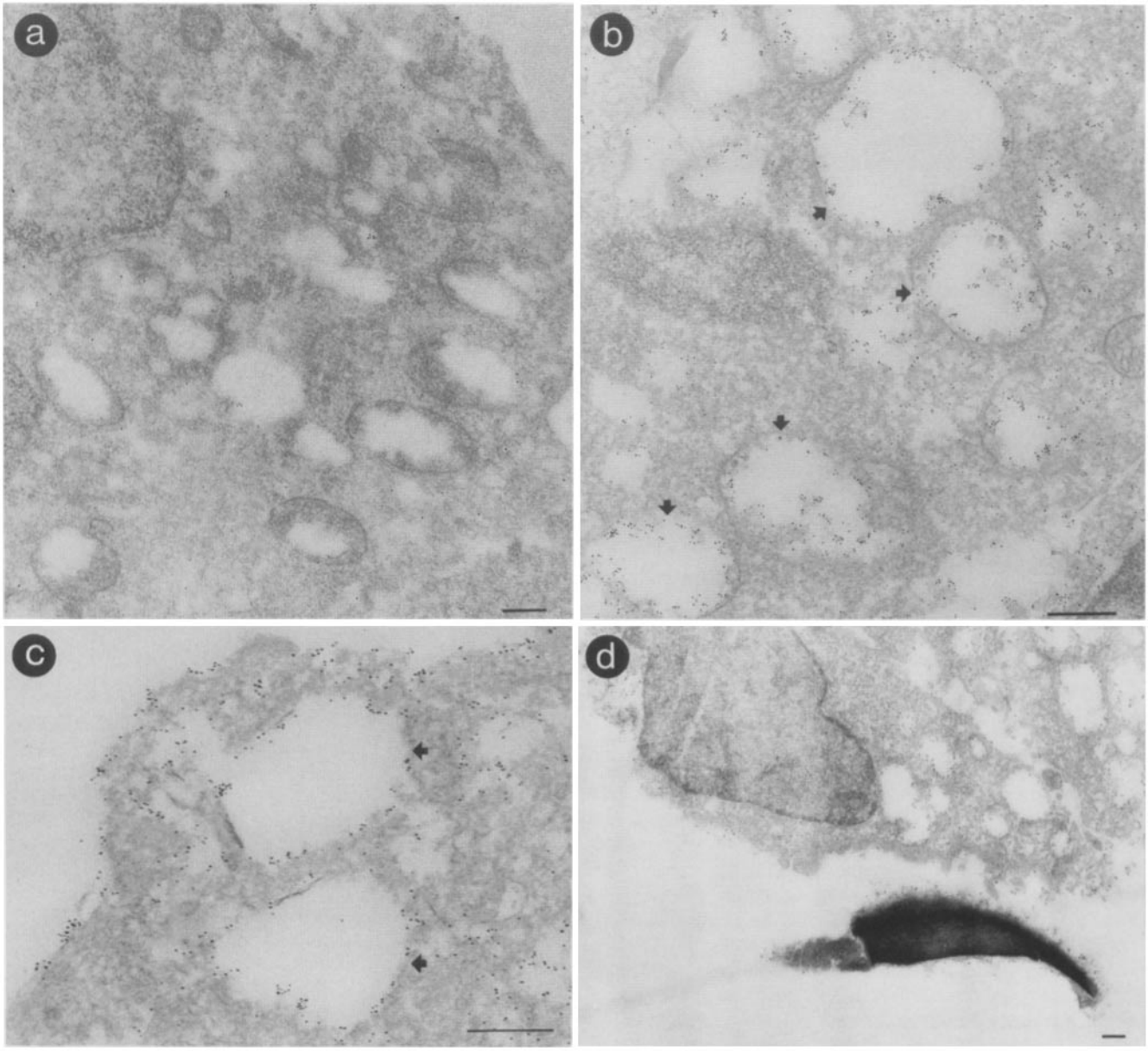


Figure 5. Immunohistochemical analysis of aggregates of EC cells transfected with the mZP3 gene. Cell line M4 and untransfected EC cells were cultured under conditions that led to formation of aggregates, and semi-thin cryosections were prepared and processed for immunohistochemistry using a rabbit polyclonal antiserum directed against mZP3 and goat anti-rabbit IgG coupled to colloidal gold, as described in Materials and Methods. Shown are representative transmission electron micrographs of untransfected (*A*) and transfected (*B–D*) cell aggregates. Secretory vesicles are indicated by arrows and gold particles, indicative of the location of EC-mZP3, appear as small black dots in secretory vesicles and on the surface of transfected EC cell aggregates (*B* and *C*). In *D*, a sperm is shown bound to the transfected EC cell aggregate (see legend to Fig. 7). Bar, 1 μm.

mZP3 or hZP3, to either EC-mZP3 or EC-hZP3 prepared from transfected EC cell culture medium, or to proteins that co-purified with EC-mZP3 during HPLC fractionation. Sperm were then incubated with ovulated eggs and two-cell embryos, and the extent of binding of sperm to eggs was compared with binding of sperm that had been exposed to medium alone ("control"). (Capacitated sperm bind only to ovulated eggs, not to embryos, due to inactivation of sperm receptors shortly after fertilization; Gwatkin, 1977; Bleil and Wassarman, 1980.) Exposure of sperm to egg mZP3, egg hZP3, or EC-mZP3 resulted in about 60% inhibition of sperm binding to eggs, as compared to sperm incubated in medium alone (Fig. 6 *A*). On the other hand, exposure of sperm to EC-hZP3 had no significant effect on binding of sperm to eggs. Proteins that co-purified with EC-mZP3 had no significant effect on binding of sperm to eggs. These results suggest that EC-mZP3, but not EC-hZP3, is active as a sperm receptor in vitro.

In a second approach, to determine whether or not EC-mZP3 and EC-hZP3 also exhibited acrosome reaction-inducing activity, mouse sperm were incubated in culture medium alone and in the presence of ionophore A23187, egg mZP3, egg hZP3, EC-mZP3, EC-hZP3, or proteins that co-purified with EC-mZP3, and then scored for the presence or absence of an acrosome. Sperm incubated in the presence of medium alone and in the presence of ionophore provided "background" and "maximal" values, respectively, for the percentage of sperm that acrosome-reacted during a 1-h period (Fig. 6 *B*). Comparison of these values with those obtained using egg mZP3, egg hZP3, EC-mZP3, and EC-hZP3, revealed that only EC-hZP3 failed to induce sperm to undergo the acrosome reaction in vitro. Proteins that copurified with EC-mZP3 did not induce the acrosome reaction in vitro. Thus, EC-mZP3 exhibited both sperm receptor and acrosome reaction-inducing activities, whereas EC-hZP3 did not exhibit either activity.

In a third approach, transfected and untransfected EC cells were induced to form aggregates. Capacitated sperm were then incubated with aggregates in the presence of ovulated eggs and two-cell embryos, under conditions that supported binding of sperm to eggs. At the end of the incubation, samples were fixed and photographed, and the number of sperm associated with aggregates, eggs, and embryos determined. As seen in Fig. 7, many sperm were bound to ovulated eggs (37 ± 4 sperm/egg) and aggregates of transfected EC cells that secreted EC-mZP3 (28 ± 8 sperm/aggregate), but not to two-cell embryos (0–2 sperm/embryo) and aggregates of untransfected EC cells (3 ± 1 sperm/aggregate) (in each case, the number of sperm bound is an average of either three or four separate experiments). Only background levels of sperm binding (3–5 sperm/aggregate) were observed when aggregates of transfected EC cells that secreted EC-hZP3 were used in similar experiments (data not shown). It was noted that the majority of sperm bound to EC cell aggregates that secreted EC-mZP3 were in the process of undergoing the acrosome reaction. These results support the conclusion that EC-mZP3 is active and EC-hZP3 is inactive as a sperm receptor in vitro.

Discussion

We chose to use EC cells in these experiments since they are derived from mice (Bernstine et al., 1973), have the capacity to synthesize a wide variety of oligosaccharides (Richa and Solter, 1986), can be induced to form cellular aggregates (Stewart, 1980), and can use the pgk-1 promoter to control expression of exogenous genes (C. L. Stewart, unpublished results). Results presented here demonstrate that EC cells, transfected with either the mZP3 or hZP3 gene fused to the pgk-1 promoter (Boer et al., 1990), synthesize, and secrete relatively large amounts (~5 μg EC-mZP3 secreted/10^7 cells per 16 h) of recombinant EC-mZP3 or EC-hZP3. The former is approximately the same size as egg mZP3 (~83,000 M_r), whereas the latter is significantly smaller (~49,000 M_r) than egg hZP3 (~56,000 M_r). However, based on results of experiments carried out in the presence of tunicamycin, both EC-mZP3 and EC-hZP3 have virtually the same size polypeptide (44,000 M_r) as their egg ZP3 counterparts. Further-

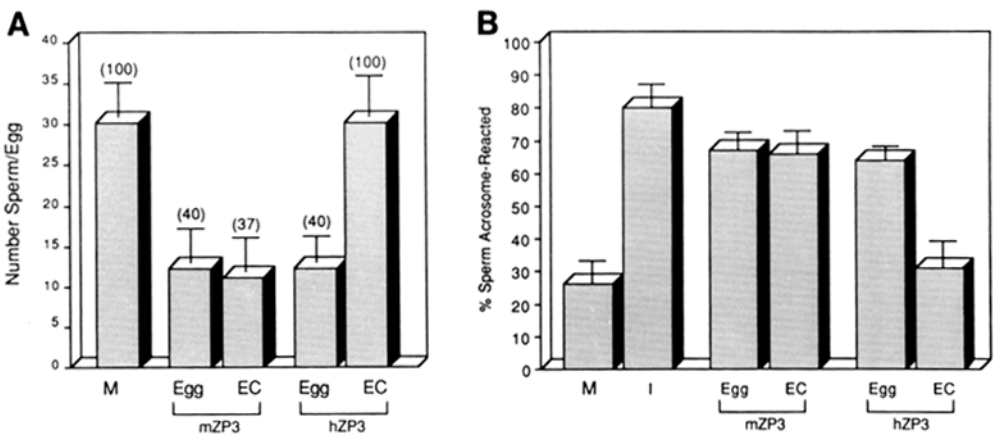
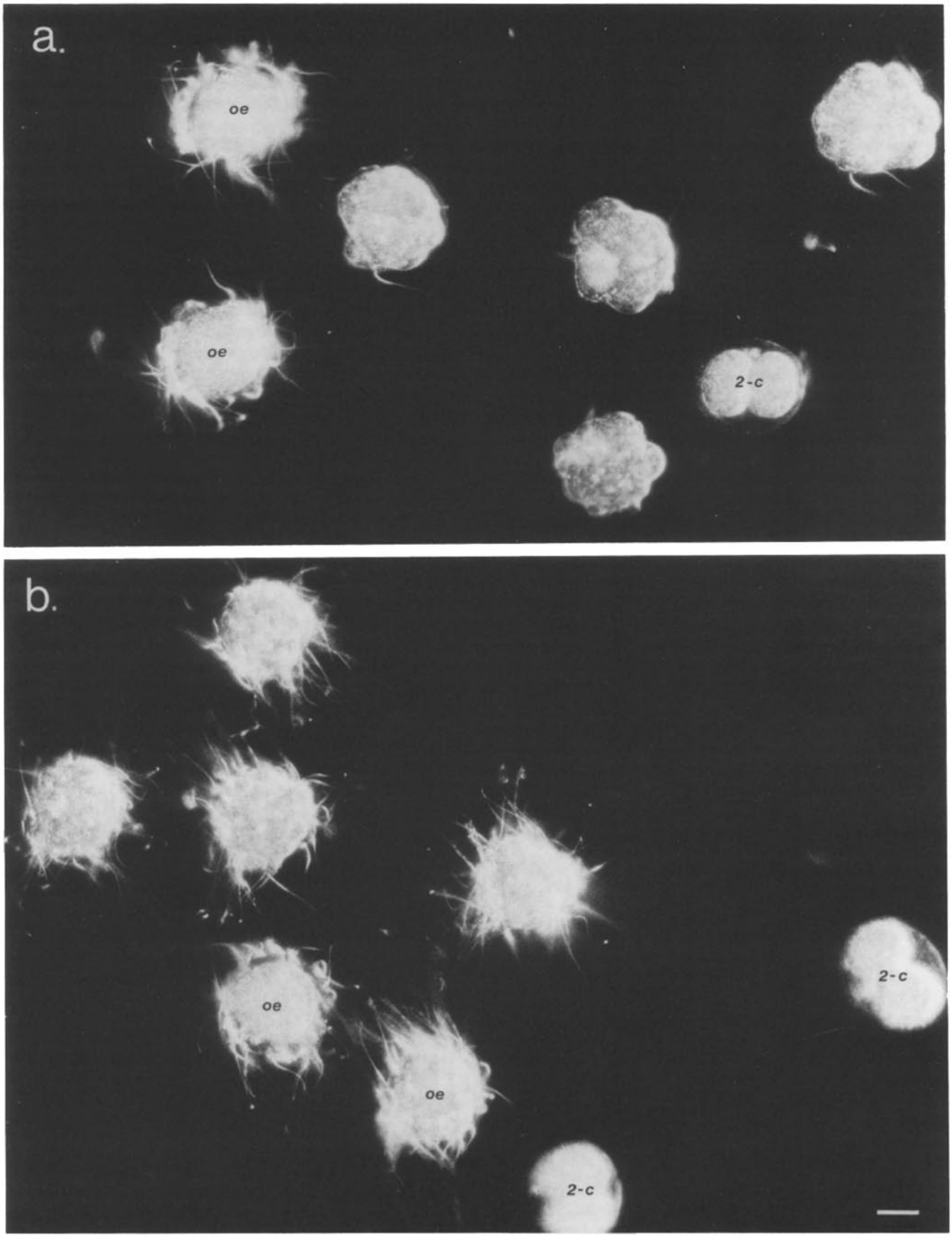


Figure 6. Analysis of sperm receptor activity and acrosome reaction-inducing activity in vitro. (*A*) Capacitated mouse sperm were exposed to culture medium (*M*), egg (~4 ng/μl) and EC-mZP3 (~4 ng/μl), or egg (~4 ng/μl), and EC-hZP3 (~4–10 ng/μl), incubated with at least 12 ovulated eggs and three two-cell embryos, and the number of sperm bound per egg determined, as described in Materials and Methods. The values in parentheses represent the percentage of sperm bound to eggs as compared to the control (*M*; 100%). Each bar of the histogram represents the average of at least three individual experiments. (*B*) Capacitated mouse sperm were exposed to culture medium (*M*), ionophore A23187 (*I*; 10 μM), egg (~4 ng/μl) and EC-mZP3 (~1–4 ng/μl), or egg (~4 ng/ μl) and EC-hZP3 (~2–10 ng/μl), and scored for the presence or absence of an intact acrosome, as described in Materials and Methods. Each bar of the histogram represents the average of at least three individual experiments and a total of at least 590 sperm (range 590–1,540).


a.
oe
oe
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more, based on results of in vitro assays, EC-mZP3 possesses both sperm receptor and acrosome reaction-inducing activities. Consequently, these transfected EC cells provide further evidence that egg mZP3 serves as sperm receptor during gamete adhesion in mice and, more importantly, provide a rich source of biologically active mouse sperm receptor for biochemical and structural studies. In the latter context, each mouse egg ZP contains only 1–2 ng of mZP3, whereas the culture medium from a single 60-mm plate of transfected EC cells contains several micrograms of biologically active EC-mZP3 (equivalent to ~3,000 eggs).

At least three points can be highlighted regarding glycosylation of mouse and hamster sperm receptor polypeptides in transfected EC cells. First, EC cells glycosylate nascent mZP3 polypeptide in a manner that apparently mimics the process in growing mouse oocytes. EC-mZP3 has the same apparent M_r and possesses the same biological activities in vitro as egg mZP3. These biological activities have been shown to be absolutely dependent on correct O-linked glycosylation (Wassarman, 1989, 1991). Second, EC cells glycosylate nascent mZP3 and hZP3 polypeptides to very different extents (even in cotransfected cells), such that secreted products resemble those synthesized by mouse (~83,000 M_r) and hamster (~56,000 M_r) oocytes, respectively (Moller et al., 1990); although EC-hZP3 (~49,000 M_r) apparently is glycosylated to a lesser extent than egg hZP3. This suggests that the EC cell glycosylation apparatus can distinguish between mZP3 and hZP3 polypeptides, despite the fact that their primary structures are very similar (81%) to one another (Kinloch et al., 1990). Third, unlike EC-mZP3, EC-hZP3 apparently does not possess the specific O-linked oligosaccharides that are essential for sperm receptor and acrosome reaction-inducing activities. This is somewhat surprising, since egg mZP3 and egg hZP3 probably have certain receptor O-linked oligosaccharides in common (Moller et al., 1990) and EC-mZP3 is active as a sperm receptor. However, it is important to realize that even relatively small changes in egg mZP3 O-linked oligosaccharides (e.g., either removal of a terminal galactose residue or conversion of its C-6 hydroxyl to an aldehyde) can result in complete loss of sperm receptor activity (Bleil and Wassarman, 1988; Wassarman, 1989). In view of the sensitivity of sperm receptor activity to very small changes in O-linked oligosaccharide structure, the absence of EC-hZP3 sperm receptor activity is consistent with the significant relative molecular weight difference between egg hZP3 and EC-hZP3.

In the case of N- and O-linked oligosaccharides, species specific and tissue-specific glycosylation have been demonstrated and the basis of these specificities has been investigated, especially with respect to the influence of protein structure and distribution of processing enzymes on glycosylation (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987; Rademacher et al., 1988; Hubbard, 1988; Smith and Baenziger, 1988; Carraway and Hull, 1989). Less information is available about rules that govern O-linked, as compared with N-linked glycosylation of glycoproteins (e.g., no consensus sequence for O-linked glycosylation of polypeptides has been identified; Wilson et al., 1991), although studies of the low-density lipoprotein receptor in mutant CHO cell lines have begun to provide information about the processing and functions of O-linked oligosaccharides (Krieger et al., 1985; Yoshimura et al., 1987; Kozarsky et al., 1988; Shite et al., 1988; Kuwano and Ono, 1989; Kuwano et al., 1991).

Based on observations cited above, there are several factors that could account for the differences between EC-mZP3 and EC-hZP3. For example, F9 cells were derived from mice and this could lead to faulty oligosaccharide processing of glycoproteins from mammals other than mice. As in the case of glycosylation of closely related viral glycoproteins, a combination of polypeptide conformation and host-dependent factors, such as differences in cellular processing enzymes and differences in the intracellular transit times, could account for abnormal oligosaccharide processing of hZP3 when present in a heterologous cellular system (Kornfeld and Kornfeld, 1985). We are currently examining these and other aspects of O-linked glycosylation of mammalian sperm receptors.

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Figure 7. Analysis of binding of mouse sperm to transfected and untransfected EC–cell aggregates in vitro. Capacitated mouse sperm were incubated with EC–cell aggregates, ovulated eggs, and two-cell embryos, and the number of sperm bound was determined, as described in Materials and Methods. (*A*) Photomicrograph of a sample containing four untransfected EC–cell aggregates, two ovulated eggs (*OE*), and one two-cell embryo (*2-C*). Note that sperm are bound to ovulated eggs, but not to EC–cell aggregates or the embryo. (*B*) Photomicrograph of a sample containing four transfected EC–cell aggregates, two ovulated eggs (*OE*), and two two-cell embryos (*2-C*). Note that sperm are bound to ovulated eggs and EC-cell aggregates, but not to two-cell embryos. Bar, 30 μm.

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