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Recombinant Human Zona Pellucida Protein ZP3 Produced by Chinese Hamster Ovary Cells Induces the Human Sperm Acrosome Reaction and Promotes Sperm-Egg Fusion

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ABSTRACT

Studies on the role of specific molecules in the human fertilization process and additional assessments of potential applications for these proteins are hampered by the limited amount of available biological material. However, this drawback might be circumvented by the recent cloning of several gamete-specific genes, which opens possibilities for the production of recombinant proteins.

By use of cDNA and genomic DNA fragments of the human ZP3 gene, which encodes a major constituent of the zona pellucida surrounding the oocyte, a 2.7-kb minigene was constructed containing the natural third and fourth introns of the gene and a truncated intron between exons 2 and 3. This ZP3 DNA was transfected to Chinese hamster ovary cells, and a single-cell clone producing the recombinant ZP3 protein (recZP3) was generated. Western blot analysis of culture medium from these cells showed that recZP3 has a molecular mass ± 5 kDa smaller than that of natural ZP3. Under reducing conditions, it migrates at an apparent molecular mass of 55–60 kDa. RecZP3 induced the sperm acrosome reaction and promoted fusion of human spermatozoa with zona-free hamster oocytes, indicating that the recombinant protein is biologically active.

RecZP3 provides an attractive tool for studying the initial stage of the human fertilization process. Furthermore, it might have clinical applications in the development of diagnostic tests for male infertility and serve as target antigen in the design of contraceptive vaccines.

INTRODUCTION

Human fertilization consists of at least three fundamental stages, comprising sperm-egg recognition, zona pellucida penetration, and finally sperm-oocyte fusion (for review see [1, 2]). This sequence of events is initiated by a species-specific receptor-ligand interaction between male and female gametes, which results in the activation of the spermatozoon and the induction of an exocytotic event known as the acrosome reaction. Proteolytic enzymes released during the course of the acrosome reaction then facilitate the penetration of the fertilizing spermatozoon through the zona pellucida and into the perivitelline space. Finally, subsequent fusion of the sperm and oocyte plasma membranes results in the completion of fertilization and the initiation of embryonic development.

At the molecular level, the mechanisms underlying this series of events are still elusive, although recent years have seen some progress in the understanding of the molecules involved in sperm-zona interaction. With regard to the spermatozoon's receptor for the zona pellucida, a number of different candidate molecules have been put forward [3, 4], including a murine tyrosine kinase [5–7] and galactosyl transferase [8]. As far as the zona pellucida is concerned, the glycoprotein ZP3 is generally believed to serve as a ligand for receptors on the sperm surface that, after ZP3 binding, mediate a G protein-coupled signal transduction event leading to the acrosome reaction [9]. In the case of murine and porcine ZP3, the O-linked carbohydrate side chains have been implicated as key elements in gamete recognition [10, 11]. A second zona glycoprotein, ZP2, has been proposed as the mediator of secondary sperm binding, whereby the inner acrosomal membrane of the acrosome-reacted spermatozoon contacts the zona pellucida during the process of gamete interaction [1]. A third zona glycoprotein, designated ZP1, appears to be primarily involved in maintaining the three-dimensional structure of the zona pellucida [1].

Much of our current understanding of the functional roles played by individual zona glycoproteins has been obtained by use of the mouse as an animal model. The extent to which these functional roles can be assigned to the ZP glycoproteins identified in other animal models has been hampered by the heterogeneous molecular weights exhibited by these molecules and the corresponding differences in nomenclature. However, this situation is rapidly being resolved as progress is made with the molecular cloning of ZP genes. For the ZP3 glycoprotein, mouse, hamster, human, and marmoset cDNA sequences have been elucidated with interspecies homologies ranging from 67–91% [12–15]. Comparable similarities have been found for the cloned sequences of human and mouse ZP2 and the homologous rabbit rc75 protein [16–18]. Finally, sequences have been reported for a third group of ZP proteins, i.e., rabbit rc55

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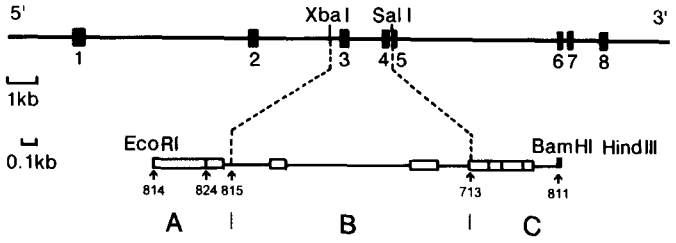

FIG. 1. Schematic presentation of construction of ZP3 minigene. Top part of the figure shows the genomic organization of the human ZP3 gene with exons (numbered 1 to 8) indicated as black boxes [25]. Below, the structure of the ZP3 minigene is given with exons 1 to 8 shown as open boxes. The assembling of segments A, B, and C, and subsequent insertion of the DNA in a mammalian expression vector is described in *Materials and Methods*. Arrows with numbers indicate the positions of oligonucleotides used. Only restriction sites relevant for the cloning strategy are given.

and the homologous porcine ZP3α. For the rabbit protein, a cross-linking function has been hypothesized akin to the putative role of ZP1 in the ZP matrix [19]. In addition, for the pig protein, a direct role in sperm-binding has also been reported [20].

ZP3 is a particularly important constituent of the zona pellucida because it is this molecule that serves as the recognition site for spermatozoa and provides the stimulus that induces the acrosome reaction. In a human context ZP3 is important as both a potential target for contraceptive vaccine development and as a possible tool in the diagnosis and treatment of male infertility. In order to explore this clinical potential of ZP3 and to study the cellular mechanisms by which the human spermatozoon becomes activated on making contact with ZP3, it is necessary to generate a biologically active recombinant form of this molecule. In the studies reported in this article, we describe strategies for the expression and purification of human ZP3 that have resulted in a biologically active product with the capacity to stimulate human spermatozoa to acrosome react and initiate sperm-oocyte fusion.

MATERIALS AND METHODS

General Methods

For cloning procedures, DNA, RNA, and protein analysis standard protocols were followed [21].

Biological Material

The study population comprised 24 unselected normospermic donors [22] who had been clinically examined and shown to be free of any detectable pathology, including hepatitis and sexually transmitted diseases such as Human Immunodeficiency Virus (HIV), that might have influenced the quality or cellular composition of their semen. The semen samples were produced by masturbation and collected into sterile containers. After allowing at least 30 min for liquefaction to occur, the spermatozoa were fractionated on a discontinuous 2-step Percoll gradient comprising a 3-ml volume of 100% isotonic Percoll [23] overlaid with a further 3-ml volume of 50% Percoll in a 10-ml conical-based sterile centrifuge tube. The 50% Percoll was produced by diluting isotonic Percoll with Biggers, Whitten, and Whittingham (BWW) medium [24]. Semen (1–3 ml) was layered on the top of each gradient and centrifuged at 500 × *g* for 20 min. Thereafter the seminal plasma was discarded, and the cells were collected from the base of the 100% Percoll fraction. These spermatozoa were then resuspended in 7 ml of BWW medium + 0.3% human serum albumin (HSA), centrifuged at 500 × *g* for 5 min, resuspended in the same solution at a sperm concentration of 20×10^6 cells/ml, and used for the experiments shown in Figures 6 and 7. With respect to the experiments shown in Figure 5, semen was purified over 70% Percoll and after one wash step was resuspended in Hepes-buffered (15 mM, pH 7.3) Ham's F-10 medium at a concentration of 5×10^6 cells/ml and left overnight at 20°C until further use.

Human zonae pellucidae were obtained from in vitro fertilization (IVF) programs. Briefly, the oocytes that had failed to be fertilized after incubation with spermatozoa were stored at 4°C in 20 mM Hepes (pH 7.2–7.4), 1.5 M $MgCl_2$, 0.1% dextran (D-4876, Sigma, St. Louis, MO), 10% glycerol, and 0.1% polyvinylpyrrolidone (PVP-40, Sigma).

Chinese hamster ovary (CHO) cells were cultured in Dulbecco's Minimum Eagle's medium (DMEM)/Ham's F-12 medium supplemented with 5% calf serum and antibiotics.

Oligonucleotides

Sequence primers and oligonucleotides for polymerase chain reaction (PCR) experiments were synthesized on an Applied Biosystems 381A DNA synthesizer (Applied Biosystems Inc., Foster City, CA). The following oligonucleotides were synthesized (see also Fig. 1):

org713 CTGTCTTGTCGACGGTCTCACTGATGCCTC

org811 CGACAAGCTTGGATCCTTTATTCGGAAGCAGACACAGG

org814 GTCGAAGCTTGAATTCGCAGGTACCATGGAGCTGAGCTAT

org815 CGCAAAGCTTCTAGACCCAACTTGGCCTGCAGCCACAC

org824 CACCAGGGCATCGTCAGTTACCTGCATGCTGTTGCCACACT

Construction of a Human ZP3 Minigene

For making a ZP3 expression construct, both genomic and 3′ cDNA fragments [25] were used. With respect to the latter, cDNAs of exons 5–8 corresponding to both ZP3 alleles (as recently reported [25]) have been used, i.e., harboring a truncated open reading frame (ZP3–372) and encoding a normal ZP3 protein of 424 amino acids. The following three DNA fragments (schematically shown in Fig. 1) were assembled to a so-called ZP3 minigene: a PCR-gen-

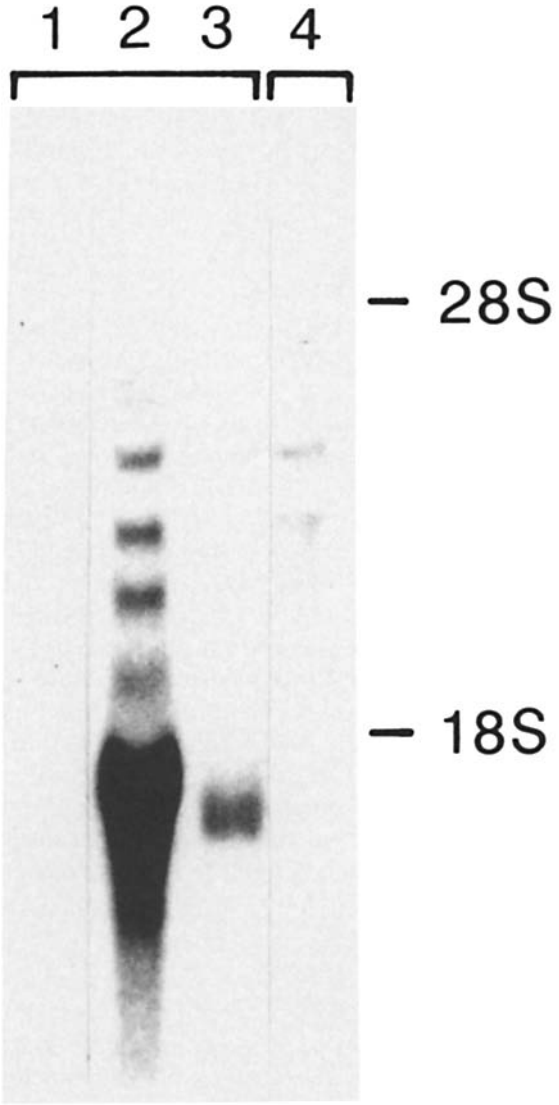

FIG. 2. Autoradiograph of Northern blot with 20 μg total RNA from control CHO cells (lane 1), geneticin-resistant CHO transformant carrying a ZP3 minigene (construct pNKSI.ZP3-minigene-372; lanes 2 and 4), and 5 μg poly(A)$^+$ RNA from human ovary tissue (lane 3). The following ^{32}P-labeled probes were used: ZP3 *Sal* I-*Hin*dIII cDNA fragment (lanes 1–3); 260-bp *Xba* I-*Apa* LI fragment from truncated intron between exon 2 and 3 (lane 4).

erated *Eco*RI-*Xba* I fragment with exon 1 and 2; a genomic *Xba* I-*Sal* I fragment containing exons 3, 4, and a part of exon 5 (from lambda clone I1 described in van Duin et al. [25]; and a *Sal* I-*Hin*dIII cDNA part of ZP3–372 harboring the rest of exons 5–8. These were cloned in pGEM-3Z (Promega, Madison, WI) opened with *Eco*RI and *Hin*dIII. The 5′ and 3′ fragments of this construct were obtained as follows: with several genomic subclones carrying exons 1 and 2 (derived from previously described lambda EMBL clones [25], a reported PCR procedure was used [26] to join exon 1 and 2 with use of primers org814 (creating a 5′ *Eco*RI site), org824 (overlapping exon 1 and 2), and org815 (derived from 2nd intron, creating *Xba* I site); standard PCR conditions [27] were used to provide ZP3–372 cDNA of exons 5 to 8 with 3′ *Bam*HI and *Hin*dIII sites using primers org713 (5′ part exon 5) and org811 (creating *Bam*HI and *Hin*dIII sites). The resulting 2.7-kb minigene was isolated from the pGEM-3Z vector after digestion with *Eco*RI and *Bam*HI and placed behind the SV40 early promoter in a pKCR [28], yielding construct pNKSI.ZP3-minigene-372. This vector also carries the geneticin-resistance gene aminoglycosyl phosphotransferase [29]. A *Sal* I-*Bam*HI fragment of this construct (harboring the ZP3–372 specific mutation) was replaced by the corresponding 3′ part of the wild type ZP3 cDNA [25] (yielding a open reading frame of 424 amino acids) to yield plasmid pNKSI.hZP3-minigene-424. The construct corresponding to the polymorphic ZP3–372 allele [25] was used only to show that the artificial intron between exons 2 and 3 was correctly spliced (see Fig. 2). The integrity of all PCR-generated DNA fragments was verified by DNA sequence analysis to exclude mutations introduced by the *Taq*-polymerase (Perkin-Elmer, Norwalk, CT).

Expression of Human ZP3 in Chinese Hamster Ovary (CHO) Cells

Three dishes with 5.10^5 CHO cells were co-transfected with ZP3 minigene DNA (10 μg/dish) and plasmid pMT2 (carrying the human metallothionein IIa gene, 1 μg/dish) according to the standard calcium phosphate precipitation method. Transformants were first selected in medium containing 800 μg/ml geneticin (Life Technologies Inc., Gaithersburg, MD), and a pooled mass population (consisting of 300–500 clones) was stored in liquid nitrogen and in part further selected with medium containing increasing concentrations of cadmium chloride [30] to select transfected cells with the highest ZP3 production levels. Subsequently, approximately 90 single-cell clones were generated from a pool of geneticin and 2.5 μM $CdCl_2$-resistant ZP3 transformants. Ten days after the cells were plated on microtiter plates by limiting dilution, wells containing single-cell clones were incubated for two days with 200 μl serum-free medium, after which 100 μl was subjected to dot blot analysis using a rabbit anti-human ZP antiserum. On the basis of visual inspection of the intensity of immunostaining, clone ZP3–424.14 was selected and used for production of recombinant ZP3 (recZP3).

Production of Anti-ZP/ZP3 Antibodies

A rabbit anti-ZP antiserum was generated by five immunizations, with three-week intervals, of male rabbits with 20–25 heat-solubilized (incubation at 70°C for 30 min) human salt-stored ZPs with complete and incomplete Freund's adjuvant for the primary and booster immunizations, respectively. Likewise, mice were immunized with similar ZP material to allow selection of hybridomas producing human ZP3-specific monoclonal antibodies (mAbs). Conditioned culture media of nontransfected CHO cells and ZP3 expressing CHO cells were used for hybridoma selection.

The production and characterization of this and other ZP3-specific mAbs will be reported separately (van Duin et al., in preparation).

Detection of Recombinant ZP3

For Western blot analysis, proteins were separated by SDS PAGE and blotted to PVDF membranes (Biorad, Hercules, CA). When appropriate, culture medium samples were first concentrated on Centricon 30 columns (Amicon, Beverly, MA). Immunodetection was carried out with a 1:500–1:1000 dilution of a rabbit antiserum against human ZP and/or with a ZP3-specific mAb at a concentration of 1 μg/ml followed by incubation with an appropriate second antibody conjugated to alkaline phosphatase. Similar conditions were used for immunostaining of dot blots of serum-free culture medium of ZP3-expressing CHO cells.

For Western blot analysis, salt-stored human ZPs were washed 4–5 times in 1.5 ml PBS, collected in the smallest possible volume (± 20 μl), and heat-solubilized in sample buffer [31].

Affinity Purification

A single-cell clone of CHO cells expressing recZP3 (clone ZP3–424.14) was grown to 60–80% confluency in 850-cm^2 roller bottles (Corning Glass Works, Corning, NY) or to a concentration of 1–2 × 10^6/ml in a 250-ml spinner culture on CultiSpher-G microcarriers (Hyclone Laboratories, Logan, UT) in medium containing 2.5 μM $CdCl_2$ and 50 μM $ZnSO_4$. The cells were washed twice with serum-free medium for 15–30 min each and subsequently incubated with 100 ml serum-free medium in the case of roller bottles, or 225 ml medium supplemented with 5 mg/ml bovine transferrin (Miles, Kankakee, IL) and 0.5 mg/ml porcine insulin (Diosynth, Oss, The Netherlands) in the case of the spinner culture, for 1–2 wk; medium was refreshed every 2–3 days. After the medium was harvested, it was centrifuged and filtered through a 0.2-μm membrane. Finally, the medium was stored at −20°C until further use.

Anti-human ZP3 mAb was purified from serum-free hybridoma culture medium by use of protein G coupled to Sepharose beads (Pharmacia, Uppsala, Sweden). Subsequent covalent linkage of the antibodies to protein G was achieved by incubation in 50 mM DMP (dimethyl pimelimidate; Pierce, Rockford, IL) in 0.2 Hepes buffer (pH 8.5) for 1 h at room temperature. Four to five milligrams of antibodies per milliliter of protein G beads were coupled without significant antibody leakage during the elution steps.

Serum-free ZP3-containing medium was incubated with the affinity matrix at 4°C for 16–24 h and subsequently transferred to a C10/10 column (Pharmacia) and washed with at least 10 bed volumes of PBS (flow rate 1.0 ml/min). More than 95% of recZP3 did bind to the column material as measured by immunoassay (see below). ZP3-containing fractions were obtained by elution with 0.1 M glycine (pH 2.7) and immediately neutralized with 2.0 M Tris. ZP3-containing fractions were dialyzed against Milli Q water and stored at −20°C until further use. The affinity column could be used at least four times without significant reduction of ZP3 recovery (usually 90–95%). From 1 L of serum-free culture medium, 1–2 mg (roller bottle culture) to ± 5 mg (spinner culture) recZP3 could be purified.

Quantitative ZP3 Determination

The protein concentration of purified ZP3 samples was measured by the BCA protein quantification test (Pierce, Rockfort, IL). In addition, all ZP3-containing samples were analyzed by an immunoassay using two protein G-purified ZP3-specific mAbs. In this immunoassay, a first mAb was coated to maxisorb plates (NUNC, Roskilde, IL) in 50 mM sodium carbonate buffer (pH 9.5), and a second mAb was biotinylated with sulfo-NHS-LC-Biotin as described by the manufacturer (Pierce). Streptavidin conjugated to Europium (Eu) was used to measure binding of the conjugated second ZP3 mAb. Eu-fluorescence was determined by means of the DELFIA system (Pharmacia). Heat-solubilized human ZPs were used as a provisional standard in these experiments. By combining the data obtained with purified ZP3 batches (containing known amounts of recZP3 as measured by protein determination assay), it was possible to estimate that a single salt-stored human zona pellucida contains on the average approximately 5 ng ZP3. This information was used in subsequent ZP3 immunoassays in which human ZPs were used as standard.

Human Sperm Acrosome Reaction Assay

The acrosome reaction was assessed by use of fluorescein-conjugated *Arachis hypogaea* lectin (Sigma) in conjunction with an assessment of viability using the hypoosmotic swelling test, as described by Aitken et al. [32]. Cells that were viable and exhibited dispersal of the label from the acrosomal region of the cell were classified as acrosome-reacted [32, 33]. All experiments were done "blind" with coded samples and contained either concentrated culture media of CHO cells or affinity-purified recZP3 samples (see also legends to Figs. 5 and 6, respectively), apart from the positive and negative controls for which the calcium ionophores 4-bromo A23187 (Sigma) or the free acid A23187 form (Calbiochem, San Diego, CA) dissolved in DMSO or DMSO alone were used, respectively. In all cases, at least 100 viable cells were scored.

In the experiments with culture media, spermatozoa were capacitated overnight at 20°C followed by 3 h at 37°C. In these experiments, 1–10 μl of test sample was added to 100 μl of 3 × 10^5 sperm cells in BWW + 0.3% HSA and incubated for 1 h at 37°C.

In the time-course experiment, the Percoll-purified spermatozoa were incubated at 37°C for the indicated time periods. In this case, recZP3, A23187, or buffer alone was added

to the sperm suspensions at T = 0, and the acrosome reaction was assessed at the following time points: 0, 0.25, 0.5, 1, 3, 6, and 24 h. The stimulants were added in a volume of 10–190 μl of Percoll purified spermatozoa at a concentration of 2×10^7/ml. The final concentrations of A23187 and recZP3 were 2.5 μM and ± 15–20 ng/μl, respectively. The experiment was repeated six times with independent donors.

Hamster Egg Penetration Assay

In order to determine whether spermatozoa that had been induced to undergo the acrosome reaction in response to recZP3 had also acquired the competence for sperm-oocyte fusion, the zona-free hamster oocyte test was performed as recently reported [32]. For this experiment, the spermatozoa were capacitated for 5 h before the addition of recZP3 at final concentrations ranging from 2 to 32 ng/ml to a suspension containing 10^7 spermatozoa/ml. After 30 min had elapsed, 18–20 zona-free hamster oocytes were introduced into the sperm suspensions, prepared as 50-μl droplets under liquid paraffin and incubated for a period of 2 h. At the end of this period, the oocytes were removed and examined for the presence of decondensing sperm heads with an attached or closely associated tail.

RESULTS

Expression of Human ZP3 in CHO Cells

The construction of a ZP3 minigene is schematically depicted in Figure 1. Both genomic and cDNA fragments of the cloned human ZP3 gene have been assembled to a 2.7-kb *Eco*RI-*Bam*HI DNA-fragment carrying a truncated intron between exon 2 and 3 and the natural third and fourth ZP3 introns (for details see *Materials and Methods*). The decision to make a minigene construct rather than a vector carrying the complete ZP3 cDNA was based on experiments showing that intervening sequences of, for example, β-globin, adenovirus, and thymidylate synthase, stimulate gene expression [34–36]. It was suggested that the presence of introns leads to enhanced efficiency of RNA 3′ processing resulting, in an accumulation of cytoplasmic RNA [35].

The ZP3 minigene was placed behind an SV40 early promoter in a vector that also harbored the geneticin resistance gene. CHO cells were transfected with the minigene DNA and a geneticin- and cadmium-resistant mass population was examined for ZP3 expression. To investigate whether transcripts derived from the minigene construct were correctly spliced, a pool of CHO ZP3 transfectants was analyzed by Northern blot analysis by using probes specific for the ZP3 coding region and for the intron between exon 2 and 3. Figure 2 presents an example of an autoradiograph showing a relatively high level of ZP3 transcription in transfected cells (equivalent to actin mRNA levels, not shown), whereas no hybridization was detected in RNA from non-transfected cells (compare lanes 1 and 2). A probe from the truncated intron between exon 2 and 3 detected only precursor transcripts (lane 4) that are also recognized by the coding ZP3 probe. These signals probably represent partially processed mRNA molecules. The size of the recombinant ZP3 mRNA is slightly larger than the natural ZP3 transcript in human ovarian tissue (compare lanes 2 and 3), which might be explained by 5′ and 3′ flanking DNA in the expression vector and/or a difference in length of poly-A tail. From these results it can be inferred that the ZP3 minigene-derived precursor RNA is correctly spliced. This was further supported by PCR amplification of cDNA fragments containing the correct sequence of exons 1 to 5 from total RNA of CHO ZP3 transformants, and amplification of fragments of similar size from human ovarian poly(A)$^+$ RNA (not shown).

By limiting dilution of a pool of geneticin-resistant CHO cells carrying the ZP3 construct with an open reading frame of 424 amino acids, a single-cell clone, designated ZP3–424.14, was generated that secreted relatively high amounts of recZP3 into the culture medium. Western blot analysis of concentrated serum-free culture medium of this clone with a rabbit anti-human ZP antiserum is presented in Figure 3A. Under nonreducing conditions, recZP3 migrated at 45–53 kDa (lanes 2 and 3), which is slightly smaller than natural ZP3 (lane 1). The band migrating at 90–100 kDa (lane 1) probably represents ZP1 and/or ZP2, the other constituents of the human ZP [37]. Both natural ZP3 and recZP3 were hardly detectable under reducing conditions (not shown), indicating that the anti-ZP antiserum primarily recognizes conformational ZP-epitopes. Western blot analysis of recZP3 in the presence of β-mercaptoethanol with a ZP3-specific mAb revealed an immunostained protein migrating at 55–60 kDa.

Affinity Purification of recZP3

A ZP3-specific mAb was used for affinity purification of recZP3 directly from serum-free culture medium of ZP3–424.14 cells. In Figure 4, protein and immunostaining are shown after SDS-PAGE of the protein fractions eluting from an antibody column at pH 2.7 (lanes 3 and 6) and of the serum-free culture medium before and after incubation with the immobilized anti-ZP3 antibodies (lanes 1 and 2, and lanes 4 and 5, respectively). On the basis of these experiments, it can be inferred that affinity chromatography with a ZP3-specific mAb has yielded a considerable purification of the recZP3 protein.

The amount of protein in the purified ZP3 fractions was measured with BSA used as the standard. In addition, all ZP3-containing fractions were also analyzed by an immunoassay with anti-ZP3-specific antibodies, with a batch of 10 heat-solubilized human zonae used as a reference. This revealed that, depending on the culture conditions, i.e., in roller bottles or on microcarriers, from 1 L of serum-free culture medium 1–5 mg of recZP3 could be purified.

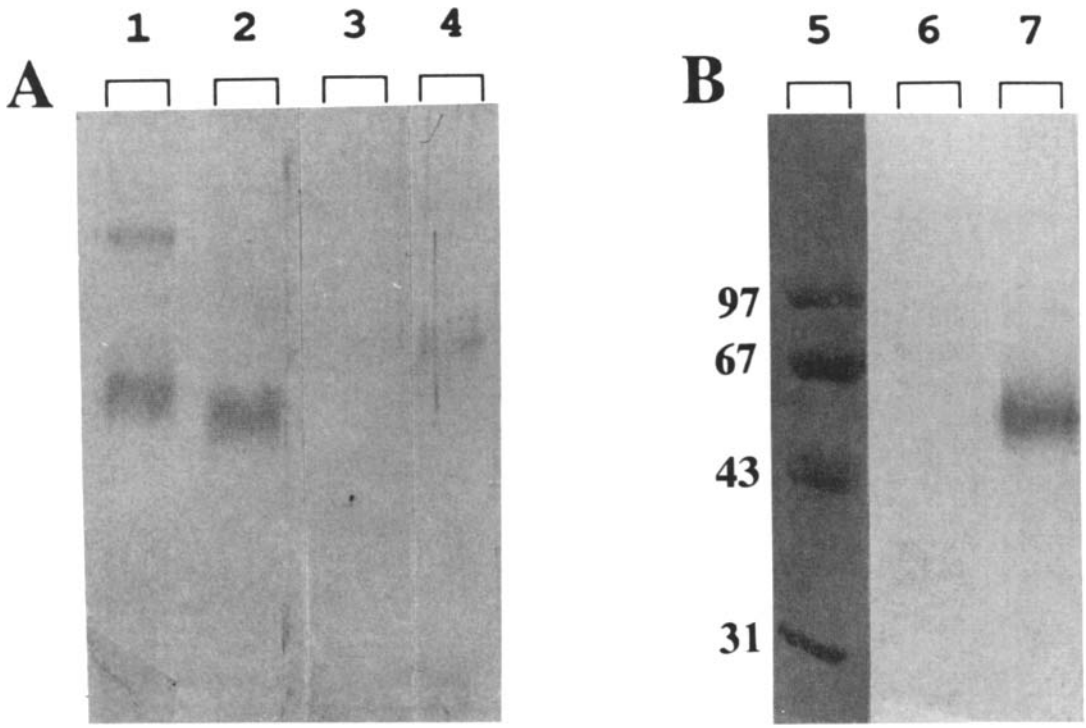

FIG. 3. Western blot analysis of recombinant and natural ZP3. Panel A: lanes 1 and 4, five human ZPs without and with β-mercaptoethanol (β-meOH), respectively; lanes 2 and 3 (both without β-meOH), 1 and 2 μl of 100× concentrated medium of ZP3 CHO-transformants and CHO control cells, respectively. The blot was incubated with rabbit anti-human ZP antiserum, 1:1000 dilution. Panel B: lane 5, molecular weight markers; lanes 6 and 7, 2 and 5 μl of 100× concentrated medium (+ β-meOH) of control CHO cells and ZP3 transformants, respectively. The blot was incubated with an anti-ZP3 mAb. Blots in both A and B were stained with the appropriate anti-rabbit and anti-mouse antibodies conjugated with alkaline phosphatase.

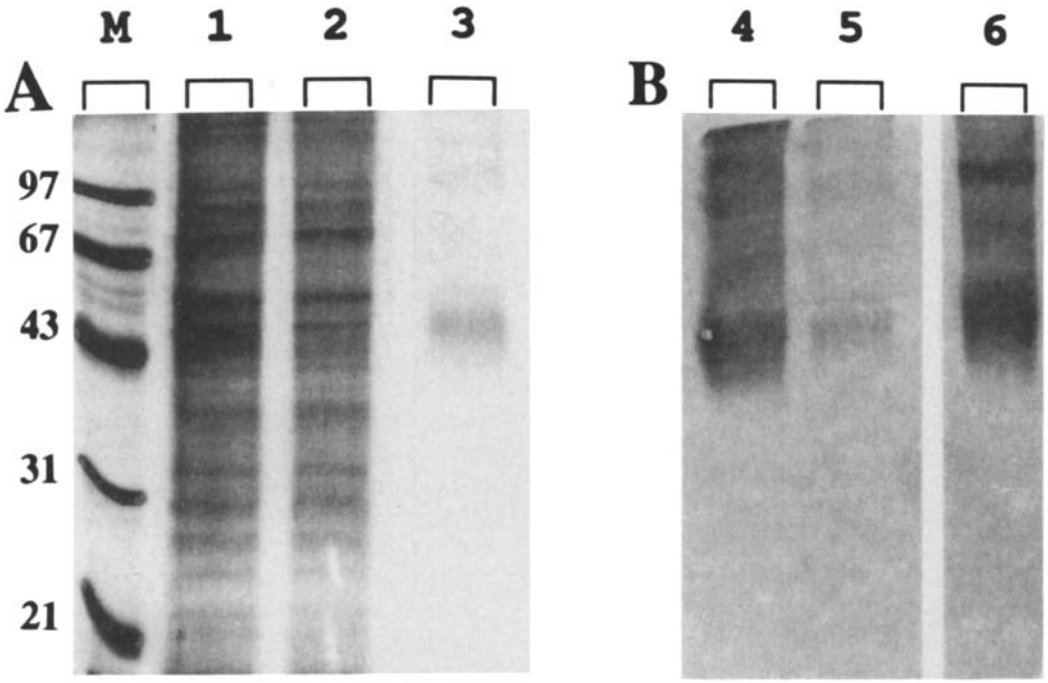

FIG. 4. Affinity purification of recZP3. Coomassie brilliant blue staining (panel A) and Western blot analysis (panel B) stained with rabbit anti-human ZP antiserum and an alkaline phosphatase conjugate) of an immunoaffinity purification of recZP3 (see also *Materials and Methods*). Results are shown of a purification from 200 ml serum-free medium, of which 150 μl before and after treatment with protein G-mAb affinity beads were concentrated (centricon 30; Amicon, Beverly, MA) to allow analysis by SDS PAGE (lanes 1 and 2, and 4 and 5, respectively). Volume of the recZP3-containing fraction was 10 ml, of which 10 μl was loaded on gel (lanes 3 and 6).

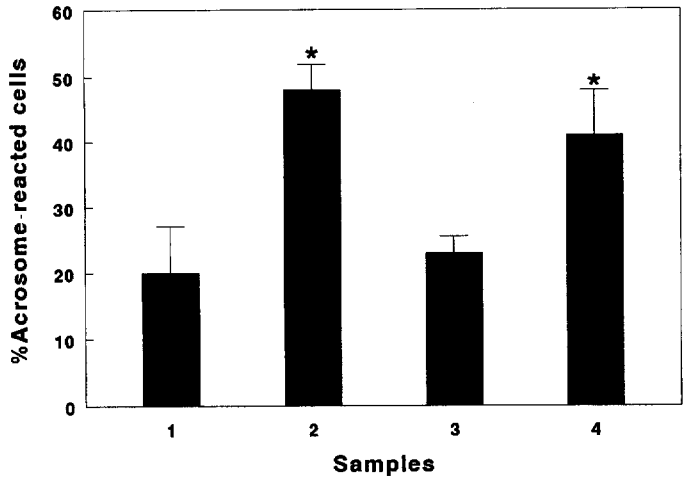

FIG. 5. Analysis of recZP3 secreted in culture medium by transfected CHO cells for potential to induce the human sperm acrosome reaction (see also *Materials and Methods*). Serum-free culture media (samples 3 and 4) were concentrated by centricon 30 spin columns and, together with negative and positive controls (samples 1 and 2, respectively), were analyzed in independent experiments using sperm samples from 5 different donors. Average results ± standard errors of means are given. Stars refer to significant differences at 5% level as determined by an analysis of variance followed by a *t*-test on adjusted means. Samples tested: 1, DMSO control; 2, DMSO + 4-bromo A23187 (10 μM); 3 and 4, 10× concentrated serum-free medium of CHO control and CHO ZP3-expressing cells, respectively (ZP3 concentration: 10–20 ng/μl). Before start of experiment spermatozoa were capacitated overnight at 20°C and 3 h at 37°C.

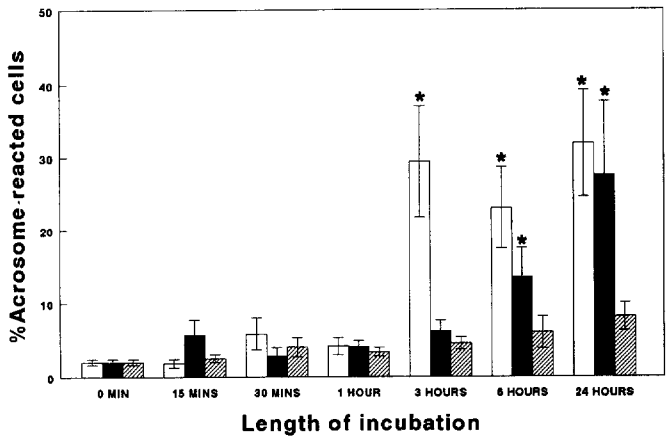

FIG. 6. Effect of increasing durations of incubations of human sperm cells with affinity-purified recZP3 (see also *Materials and Methods*) on percentage of acrosome-reacted cells. Black bar, purified recZP3 ± 15–20 ng/μl; open bar, 2.5 μM free acid A23187; shaded bar, BWW control. Error bars represent standard error of means of six experiments. Stars refer to significant differences at 5% level according to an analysis of variance and X^2 test.

By comparison of the data obtained from both the protein determination assay and the ZP3 immunoassay (data not shown), it could be deduced that a single human ZP contains approximately 5 ng of ZP3 protein. Given the comparable sizes of porcine and human zonae pellucidae, this amount of human ZP3 corresponds reasonably well with the estimated ZP3 content of a pig ZP [38]. However, further determination of the absolute amount of protein in affinity-purified recZP3 will be necessary to establish the ZP3 content of a single human oocyte more accurately.

Biological Activity of recZP3

The biological activity of recZP3, i.e., the potential of this molecule to induce the sperm acrosome reaction and initiate sperm-oocyte fusion, was assessed with both the medium of ZP3-secreting CHO cells and the affinity-purified recombinant protein as stimulant. The results of these experiments are shown in Figures 5 and 6, respectively.

The time-course analysis of the acrosome reaction rates observed in response to recZP3 and A23187 revealed that both of these reagents were capable of inducing significant increases in acrosome reaction rates compared with the control incubations. However, the kinetics of the response varied with the agonist employed. Thus, A23187 induced a significant increase in acrosome reaction rates after 3 h to a mean value of around 30% and remained at that level over the ensuing 21 h (Fig. 6). The response to recZP3 was much slower: a significant increase in acrosome reaction rates was not observed until the spermatozoa had been exposed to this reagent for 6 h, although by 24 h the biological response was not significantly different from that achieved with ionophore (Fig. 6). This latency period before the spermatozoa responded to recZP3 appeared to be a reflection of the capacitation status of the cells. If the sper-

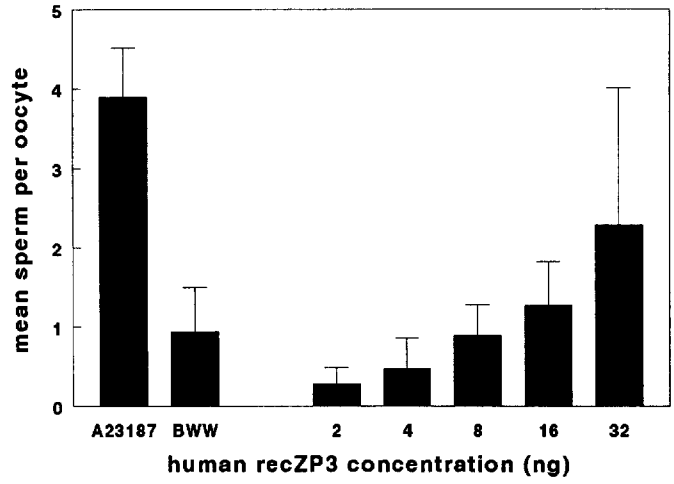

FIG. 7. Effect of several amounts of recZP3 on sperm-hamster zona-free oocyte fusion. Experiment was carried out as recently described [32] (see also *Materials and Methods*). Spermatozoa were capacitated for 5 h before addition of recZP3 at final concentrations ranging from 2–32 ng/μl. After 30 min had elapsed, 18–20 zona-free hamster oocytes were introduced into sperm suspensions and incubated for 2 h. Cells treated with free acid A23187 (2.5 μM) and BWW medium represent negative and positive controls, respectively. From three independent experiments mean number of penetrated spermatozoa per egg are given, plus or minus standard error of means, indicated by error bars. Results obtained with highest and lowest amount of recZP3 are significantly different at 1% level as calculated by X^2-analysis; furthermore, a nonparametric test against trent using ranking numbers of individual data indicated a relationship between increasing dose and response ($\alpha = 0.05$).

matozoa were first capacitated for 16–20 h at room temperature followed by 3 h at 37°C prior to the addition of recZP3, then a much more rapid response to recZP3 was observed, with a significant increase in acrosome reaction rates being recorded within 3 h of treating the spermatozoa with the concentrated medium of CHO clone ZP3–424.14 (see Fig. 5). This effect was comparable to the acrosome reaction rates triggered by the calcium ionophore A23187 and was not obtained with medium of nontransfected cells. In order to determine whether the acrosome reactions induced by the recZP3 were biologically meaningful in the sense that the cells had also acquired the competence to engage in sperm-oocyte fusion, a further series of experiments was conducted in which this aspect of sperm function was assessed. The results of those hamster egg penetration tests are presented in Figure 7. After a capacitation period of 5 h at 37°C it was found that recZP3 could induce a dose-dependent increase in the rates of sperm-oocyte fusion that at the highest dose examined was not significantly different from the results obtained with A23187 (Fig. 7).

DISCUSSION

In this report we have demonstrated that purified human recombinant ZP3 produced in CHO cells is biologically active, as evidenced by its capacity to induce the acrosome reaction and to generate a fusogenic equatorial segment capable of initiating sperm-oocyte fusion. In the latter experiment, a dose-response relationship was found; and in additional experiments with independent batches of recZP3, a significant biological activity was found (data not shown). It has been shown that the protein can be affinity-purified, and it appears that, in terms of some of the biochemical and biological characteristics, there are no apparent differences between recZP3 produced in roller bottles or in spinner cultures (data not shown). It is furthermore concluded that the ZP3-producing CHO cell line will allow the production and purification of milligram quantities of protein, which is roughly equivalent to the amount of ZP3 protein in at least 2×10^5 human ova.

The interaction between mammalian gametes is a highly specific process in which the carbohydrate moieties of the molecules involved are thought to play a major role in the recognition events associated with fertilization. In principle, this specificity could limit the possibilities for expressing biologically active recombinant proteins involved in sperm-egg recognition, since such recognition requires a specific glycosylation mechanism similar to that present in the developing sperm cell and oocyte. However, from the data presented here, it appears that for the biosynthesis of bioactive recombinant human ZP3, the amino acid sequence of the protein rather than the genetic background of the expression system is important. A similar finding has also been reported recently for mouse ZP3. Expression of this gene in at least four different mammalian cell lines yielded, in all cases, recombinant protein that could induce the murine acrosome reaction [39, 40]. In addition, it was found that hamster ZP3 could be functionally expressed in transgenic mice [41].

The molecular size of human recZP3 expressed in CHO cells was slightly smaller than that of the native protein present in salt-stored human ova. This was probably due to differences in the glycosylation pattern, although such disparities did not influence the capacity of this molecule to recognize complementary molecules at the sperm surface. Similarly, biologically active mouse recZP3 is smaller than the natural protein [40], suggesting that the post-translational processing of these proteins during oogenesis differs to some extent from that observed in the expression systems used. The four mammalian ZP3 genes that have been cloned thus far, i.e., mouse, human, hamster, and marmoset ZP3, all encode homologous proteins of 422–424 amino acids. Post-translational processing of the protein leads in the case of mouse ZP3 to a protein of ± 83 kDa, whereas in the other species, ZP3 has a size of approximately 55 kDa. The explanation for this difference is presently unknown, although it is conceivable that variation in the extent and/or patterns of O-linked glycosylation accounts for the disparate molecular weights. For the murine protein, it was found that O-linked carbohydrate moieties are especially responsible for sperm recognition [10]. The mouse protein contains three clusters of three or more serine residues, which have been suggested to be potential sites for O-linked oligosaccharides [42]. Only in the case of hamster ZP3 is one of these regions conserved. Studies on porcine ZP proteins have also suggested O-linked sugars as important structures in sperm-egg interaction [11]. An O-glycosylated domain of 25 amino acids has recently been mapped in the porcine ZP3 homologue [43], although nothing is known about the specific role of these structures in pig gamete interaction. In contrast, preliminary data from experiments with tunicamycin and N-glycanase, which inhibit N-linked glycosylation and cleaves N-linked sugars, respectively, have yielded a human recZP3 protein with a size almost identical to that calculated for the naked protein backbone (data not shown), suggesting a small contribution of O-linked glycosylation to the processing of human ZP3. Further experiments involving mutagenesis of ZP3, expression of recZP3 in glycosylation mutant cell lines, and/or studies in which the purified recZP3 proteins are digested with deglycosylating enzymes, such as N- and O-glycanases, will have to be conducted to reveal the role of the carbohydrate moieties in the bioreactivity of human recZP3.

The induction of the acrosome reaction and the assessment of sperm-oocyte fusion are frequently used as diagnostic tests in cases of male infertility [22, 32]. In such circumstances, reagents such as the ionophore A23187 [32, 33] are used to trigger a biological response by generating the second messengers, calcium, and pH needed to activate these cells. Although this reagent is effective, it suffers from the

inherent disadvantage that it bypasses the signal transduction pathway normally activated by ZP3. In order to address this problem, several studies have used natural human ZP proteins for the bioassay of human sperm function, at concentrations that are on the same order of magnitude as those used in this report [9, 44, 45]. However, it appears from those experiments that the level of acrosome reaction induced with native zona proteins is, in most cases, lower than with the ionophore A23187. Such differences probably reflect the capacitation status of the spermatozoa. Whereas A23187 largely bypasses the need for capacitation, this process is thought to be a prerequisite for the biological action of ZP3. Significantly, human spermatozoa appear to be particularly difficult to capacitate, as evidenced by the low frequencies of hyperactivated motility [46] and spontaneous acrosome reactions [47] observed with these cells, even when they have been incubated for prolonged periods of time in vitro. A poor state of capacitation would explain the refractoriness of human spermatozoa to stimulation with recZP3, as reflected in the moderate magnitude and slow kinetics of the biological responses observed in vitro. Experiments involving the receptor-mediated activation of human spermatozoa with solubilized human ZP proteins [9, 44, 45] or progesterone [48–50] have revealed a similar dependence on sperm capacitation, in association with slow reaction kinetics. The importance of sperm capacitation may be to influence the level of receptor expression of the surface of the spermatozoa, as indicated by independent studies using mannose [51] and progesterone receptor [52] as models. Murine spermatozoa, in contrast, are extremely easy to capacitate [53] and readily exhibit biological responses to recZP3 [40]. Thus, the possible mechanistic explanation for the observed biological activity of recZP3 could be the slow exposure and/or activation of ZP3-binding receptors at the sperm surface during the capacitation process. In addition, however, several other explanations might (in part) account for the somewhat unexpected limited bioactivity of recZP3 as compared to the calcium ionophore. First, the apparently different glycosylation of recZP3 compared to that of the native protein might have impaired the bioactivity of the recombinant protein. Expression in other mammalian cell lines might overcome this problem. Second, it is possible that soluble recZP3 per se can also be regarded as a nonphysiological or incomplete trigger of the acrosome reaction. In the in vivo situation, the interaction between spermatozoa and all ZP proteins in the zona-matrix, rather than the isolated ZP3 molecule, might provide the stimulus for sperm cells to undergo the acrosome reaction (see for example [44]). In this respect, it is intriguing that for the nonhomologous porcine ZP3α protein, sperm receptor activity has been claimed [20], suggesting that there could be more than one ZP protein with the capacity to contribute to the induction of the acrosome reaction. Moreover, Töpfer-Petersen et al. [54] have recently speculated in this direction by hypothesizing that the sperm receptor activity is associated with different ZP proteins in different species. Third, additional natural inducers and/or promoters of the acrosome reaction like follicular fluid, the cumulus oophorus, and progesterone are absent from the in vitro systems and yet might be important contributors to the efficiency of sperm activation in vivo.

The obvious disadvantage of the native ZP proteins from a diagnostic point of view is their limited availability and lack of standardization, since the native ZP that might be used for this purpose will come from ova at different stages of maturity and, frequently, will have been exposed to spermatozoa during a failed IVF attempt. Clearly the advent of the production of biologically active recombinant human ZP3 and perhaps mixtures of recombinant ZP proteins will have a considerable impact on the design and biological relevance of sperm function bioassays. Moreover, these reagents may have an important therapeutic role in the stimulation of human spermatozoa during micromanipulation procedures, such as sub-zonal injection, that are currently being used to facilitate IVF in cases of male factor infertility.

In terms of the fundamental cell biology of human spermatozoa, the availability of biologically active ZP3 may provide an important contribution to our understanding of the cellular mechanisms by which the ZP activates these cells during the early stages of fertilization [44]. In particular, this material should help us to identify the components on the sperm surface that are involved in binding to ZP3 and transducing this recognition signal. In this respect, a confusing plethora of candidate proteins have been put forward, including a group of low molecular weight zona binding proteins identified by O'Rand et al. [3], a sperm surface β-1,4-galactosyltransferase which was found to bind murine ZP3 [8], the human sperm antigen FA-1, which has been claimed to bind porcine ZP3 [55], a 56-kDa sperm protein identified in photo-affinity labeling experiments involving murine ZP3 [56], and, finally, a 95-kDa tyrosine kinase that is activated on interaction with murine ZP3 [6]. It seems unlikely that all these proteins are involved in ZP3 recognition, and the present uncertainty emphasizes the importance of purified bioactive recombinant ZP3 for the definitive identification of the sperm surface proteins involved in human fertilization.

Finally, both ZP3 and the complementary zona-binding sperm antigens are, in principle, attractive target antigens for the development of contraceptive vaccines that would prevent conception by disrupting the gamete recognition events that initiate fertilization. With respect to the ZP approach, most research in this direction has been carried out with either porcine or murine zona proteins as antigens [57]. The cloning and expression of the human ZP3 gene therefore represents an important intermediate step in generating the tools that are necessary to further the investigation of ZP3-based immunocontraception for the regulation of human fertility.

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REFERENCES

1. Wassarman PM. Zona pellucida glycoproteins. Annu Rev Biochem 1988; 57:415–442.
2. Wassarman PM. Profile of a mammalian sperm receptor. Development 1990; 108:1–17.
3. O'Rand MG, Widgren EE, Nikolajczyk BS, Richardson RT, Shabanowitz RB. Receptors for zona pellucida on human spermatozoa. In: Alexander N (ed.), Gamete interaction: Prospects for immunocontraception. New York: Wiley-Liss; 1990: 213–226.
4. Richardson RT, Nikolajczyk G, Beavers J, Widgren E, O'Rand M. Zona pellucida binding proteins of spermatozoa: mouse anti-RSA cross-reactive proteins. Comp Spermatol 20 Years After 1991; 75:587–591.
5. Leyton L, Saling P. Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. J Cell Biol 1989; 108:2163–2168.
6. Leyton L, Saling P. 95 kd sperm proteins bind ZP3 and serve as tyrosine kinase substrates in response to zona binding. Cell 1989; 57:1123–1130.
7. Leyton L, LeGuen P, Bunch D, Saling P. Regulation of mouse gamete interaction by a sperm tyrosine kinase. Proc Natl Acad Sci USA 1992; 89:11692–11695.
8. Miller DJ, Macek MB, Shur BD. Complementarity between sperm surface β-1,4-galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. Nature 1992; 357:589–593.
9. Lee MA, Check JH, Kopf GS. A guanine nucleotide-binding regulatory protein in human sperm mediates acrosomal exocytosis induced by the human zona pellucida. Mol Reprod Dev 1992; 31:78–86.
10. Florman HM, Wassarman PM. O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. Cell 1985; 41:313–324.
11. Yurewicz EC, Pack BA, Sacco AG. Isolation, composition, and biological activity of sugar chains of porcine oocyte zona pellucida 55k glycoproteins. Mol Reprod Dev 1991; 30:126–134.
12. Ringuette MJ, Chamberlin ME, Baur AW, Obieski DA, Dean J. Molecular analysis of cDNA coding for ZP3, a sperm binding protein of the mouse zona pellucida. Dev Biol 1988; 127:287–295.
13. Kinloch RA, Mortillo S, Wassarman PM. Transgenic mouse eggs with functional hamster sperm receptors in their zona pellucida. Development 1992; 115:937–946.
14. Chamberlin ME, Dean J. Human homolog of the mouse sperm receptor. Proc Natl Acad Sci USA 1990; 87:6014–6018.
15. Thillai-Koothan P, van Duin M, Aitken RJ. Cloning, sequencing and oocyte specific expression of the marmoset sperm receptor protein, ZP3. Zygote 1993; 1:93–101.
16. Liang L-F, Chamow SM, Dean J. Oocyte-specific expression of mouse Zp-2: developmental regulation of the zona pellucida genes. Mol Cell Biol 1990; 10:1507–1515.
17. Liang L-F, Dean J. Conservation of mammalian secondary sperm receptor genes enables the promoter of the human gene to function in mouse oocytes. Dev Biol 1993; 156:399–408.
18. Lee VH, Schwoebel E, Prasad S, Cheung P, Timmons TM, Cook R, Dunbar B. Identification and structural characterization of the 75-kDa rabbit zona pellucida protein. J Biol Chem 1993; 268:12412–12417.
19. Schwoebel E, Prasad S, Timmons TM, Cook R, Kimura H, Niu E-M, Cheung P, Skinner S, Avery SE, Wilkins B, Dunbar B. Isolation and characterization of a full-length cDNA encoding the 55-kDa rabbit zona pellucida protein. J Biol Chem 1991; 266:7214–7219.
20. Yurewicz EC, Hibler D, Fontenot GK, Sacco AG, Harris J. Nucleotide sequence of cDNA encoding ZP3α, a sperm-binding glycoprotein from zona pellucida of pig oocyte. Biochim Biophys Acta 1993; 1174:211–214.
21. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor; 1989.
22. World Health Organisation. WHO Semen Manual for the Examination of Human Semen and Cervical Mucus Interaction. Cambridge, UK: Cambridge University Press; 1992.
23. Lessley BA, Garner DL. Isolation of motile spermatozoa by density gradient centrifugation in Percoll. Gamete Res 1983; 7:49–61.
24. Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In: Daniel JC (ed.), Methods in Mammalian Embryology. San Francisco: Freeman; 1971: 86–94.
25. Van Duin M, Polman JEM, Verkoelen CCEH, Bunschoten H, Olijve W, Aitken RJ. Cloning and characterization of the human sperm receptor ligand ZP3: evidence for a second polymorphic allele with a different frequency in the Caucasian and Japanese populations. Genomics 1992; 14:1064–1070.
26. Yon J, Fried M. Precise gene fusion by PCR. Nucleic Acids Res 1989; 17:4895.
27. Erlich HA, Gibbs R, Kazazian HH. Current Communications in Molecular Biology. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
28. O'Hare K, Benoist C, Breathnach R. Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a prokaryotic dihydrofolate reductase. Proc Natl Acad Sci USA 1981; 78:1527–1531.
29. Colbère-Garapin F, Horodniceaunu F, Kourilsky P, Garapin A-C. A new dominant hybrid selective marker for higher eukaryotic cells. J Mol Biol 1981; 150:1–14.
30. Kushner PJ, Hort E, Shine J, Baxter JD, Greene GL. Construction of cell lines that express high levels of the human estrogen receptor and are killed by estrogens. Mol Endocrinol 1990; 4:1465–1473.
31. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 277:680–685.
32. Aitken RJ, Buckingham DW, Fang HG. Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. J Androl 1993; 14:132–141.
33. Aitken RJ, Brindle JP. Analysis of the ability of three probes targeting the outer acrosomal membrane or acrosomal contents to detect the acrosome reaction in human spermatozoa. Hum Reprod 1993; 8:1663–1669.
34. Buchman AR, Berg P. Comparison of intron-dependent and intron independent gene expression. Mol Cell Biol 1988; 8:4395–4405.
35. Huang MTF, Gorman CM. Intervening sequences increase efficiency of RNA 3′ processing and accumulation of cytoplasmic RNA. Nucleic Acids Res 1990; 18:937–947.
36. Ash J, Ke Y, Korb M, Johnson LF. Introns are essential for growth-regulated expression of the mouse thymidylate synthase gene. Mol Cell Biol 1993; 13:1565–1571.
37. Shabanowitz RB. Mouse antibodies to human zona pellucida: evidence that human Zp3 is strongly immunogenic and contains two distinct isomer chains. Biol Reprod 1991; 43:260–270.
38. Timmons TM, Dunbar BS. Antigens of mammalian zona pellucida. In: Mathur S, Fredericks CM (eds.), Perspectives in Immunoreproduction. New York: Hemisphere Publishing Corporation; 1988: 242–260.
39. Kinloch RA, Mortillo S, Stewart CL, Wassarman PM. Embryonal carcinoma cells transfected with ZP3 genes differentially glycosylate similar polypeptides and secrete active mouse sperm receptor. J Cell Biol 1991; 115:655–664.
40. Beebe SJ, Leyton L, Burks D, Ishikawa M, Fuerst T, Dean J, Saling P. Recombinant mouse Zp3 inhibits sperm binding and induces the acrosome reaction. Dev Biol 1992; 151:48–54.
41. Kinloch RA, Mortillo S, Wassarman P. Transgenic mouse eggs with functional hamster sperm receptors in their zona pellucida. Development 1992; 115:937–946.
42. Do S-I, Cummings RD. Presence of O-linked oligosaccharide on a threonine residue in the human transferrin receptor. Glycobiology 1992; 2:345–353.
43. Yurewicz EC, Pack BA, Sacco AG. Porcine oocyte zona pellucida M_r 55 000 glycoproteins: identification of o-glycosylated domains. Mol Reprod Dev 1992; 33:182–188.
44. Hoshi K, Sugano T, Endo CE, Yoshimatsu N, Yanagida K, Sato A. Induction of the acrosome reaction in human spermatozoa by human zona pellucida and effect of cervical mucus on zona-induced acrosome reaction. Fertil Steril 1993; 60:149–153.
45. Tesarik J, Carreras A, Mendoza C. Differential sensitivity of progesterone- and zona pellucida-induced acrosome reactions to pertussis toxin. Mol Reprod Dev 1993; 34:183–189.
46. Burkman LJ. Discrimination between nonhyperactivated and classical hyperactivated motility patterns in human spermatozoa using computerized analysis. Fertil Steril 1991; 55:363–371.
47. Stock CE, Fraser LR. The acrosome reaction in human sperm from men of proven fertility. Hum Reprod 1987; 2:109–119.
48. Uhler ML, Leung A, Chan SYW, Wang C. Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction. Fertil Steril 1992; 58:1191–1198.
49. Mendoza C, Moos J, Tesarik J. Progesterone action on the human sperm surface is potentiated by an egg-associated acrosin activator. FEBS Lett 1993; 326:149–152.

50. Sueldo CE, Oehninger S, Subias E, Mahony M, Alexander NJ, Burkman LJ, Acosta AA. Effect of progesterone on human zona pellucida sperm binding and oocyte penetrating capacity. Fertil Steril 1993; 60:137–140.
51. Benoff S, Cooper GW, Hurley I, Napolitano B, Rosenfeld DL, Scholl GM, Hershlag A. Human sperm fertilizing potential in vitro is correlated with differential expression of a head-specific mannose-ligand receptor. Fertil Steril 1993; 59:854–862.
52. Tesarik J, Mendoza C, Moios J, Carreras A. Selective expression of a progesterone receptor on the human sperm surface. Fertil Steril 1992; 58:784–792.
53. Saling PM. Mammalian sperm interaction with extracellular matrices of the egg. Oxf Rev Reprod Biol 1989; 11:339–388.
54. Töpfer-Petersen E, Mann K, Calvete JJ. Identification of porcine oocyte 55 kDa α and β proteins within the zona pellucida glycoprotein families indicates that oocyte sperm receptor activity is associated with different zona pellucida proteins in different mammalian species. Biol Chem Hoppe-Seyler 1993; 374:411–417.
55. Naz RK, Sacco AG, Yurewicz EC. Human spermatozoal FA-1 binds with ZP3 of porcine zona pellucida. J Reprod Immunol 1991; 20:43–58.
56. Bleil JD, Wassarman PM. Identification of a ZP3-binding protein on acrosome intact mouse sperm by photoaffinity crosslinking. Proc Natl Acad Sci USA 1990; 87:5563–5567.
57. Paterson M, Aitken RJ. Development of vaccines targeting the zona pellucida. Curr Opin Immunol 1990; 2:7443–7447.