BIOLOGY OF REPRODUCTION **81,** 7–15 (2009)
Published online before print 25 February 2009.
DOI 10.1095/biolreprod.108.074716

Functional Activity of Human ZP3 Primary Sperm Receptor Resides Toward Its C-Terminus[1]

Pankaj Bansal, Kausiki Chakrabarti, and Satish K. Gupta[2]

Reproductive Cell Biology Laboratory, National Institute of Immunology, New Delhi, India

ABSTRACT

Zona pellucida glycoprotein 3 (*ZP3*) has been ascribed as a putative primary sperm receptor during fertilization in humans. Herein, attempts have been made to delineate the functional domain of human ZP3. *ZP3* has been cloned and expressed in a baculovirus expression system as N-terminal fragments (amino acid [aa] residues 1–175 [pAc-ZP3$_{(1–175\ aa)}$] and 23–175 [pBg-ZP3$_{(23–175\ aa)}$]) and as C-terminal fragments (aa residues 214–305 [pBg-ZP3$_{(214–305\ aa)}$] and 214–348 [pBg-ZP3$_{(214–348\ aa)}$]). ZP3 encompassing both N- and C-terminal fragments corresponding to aa residues 1–370 (pAc-ZP3$_{[1–370\ aa]}$) has also been expressed. Lectin-binding analysis with these recombinant proteins revealed the presence of *N*- and *O*-linked glycosylation. Significant induction of acrosomal exocytosis was observed when capacitated sperm were incubated with pBg-ZP3$_{(214–348\ aa)}$, pBg-ZP3$_{(214–305\ aa)}$, and pAc-ZP3$_{(1–370\ aa)}$ ($P < 0.05$), whereas incubation with pAc-ZP3$_{(1–175\ aa)}$ and pBg-ZP3$_{(23–175\ aa)}$ failed to do so under similar experimental conditions. However, N- and C-terminal fragments labeled with fluorescein isothiocyanate revealed binding to the anterior head of capacitated human spermatozoa. *Escherichia coli*-expressed ZP3 C-terminal fragments and chemically deglycosylated pBg-ZP3$_{(214–348\ aa)}$ failed to induce a significant ($P > 0.05$) increase in acrosomal exocytosis, suggesting the relevance of glycosylation in imparting functional activity to ZP3 C-terminal fragments. pBg-ZP3$_{(214–348\ aa)}$-mediated induction of acrosomal exocytosis is regulated by G_i protein, extracellular calcium, GABA(A) [gamma aminobutyric acid (A)] receptor-mediated Cl^- channel, and T-type voltage-operated calcium channels. Taken together, the results of these studies suggest that the functional activity of human ZP3 resides in its C-terminal domain.

acrosome reaction, baculovirus expression system, recombinant human ZP3 fragments, sperm, zona pellucida domain

INTRODUCTION

The mammalian oocyte is surrounded by an acellular glycoproteinaceous matrix termed the zona pellucida (ZP). It has a pivotal role in species-specific sperm-egg recognition and binding, induction of acrosomal exocytosis in the ZP-bound spermatozoa, avoidance of polyspermy, and protection of the embryo before implantation [1]. In contrast to mouse ZP, human ZP matrix is composed of four glycoproteins designated as ZP1, ZP2, ZP3, and ZP4, with ZP4 being a pseudogene in mouse [2–4]. In mice and humans, ZP3 is a putative primary sperm receptor, as it has a critical role in initial binding of the spermatozoa to the oocyte and in induction of acrosomal exocytosis [5–10].

Human ZP3 is a 424-amino acid (aa)-long polypeptide encoded by eight exons. It has a 22-aa-long N-terminal hydrophobic signal peptide sequence that drives the protein into the secretory pathway [11]. A tetrabasic consensus furin cleavage site, RNRR (349–352 aa), is present upstream of a hydrophobic transmembrane-like domain (TMD [387–409 aa]). TMD anchors the glycoproteins in secretory vesicles and plasma membrane [12]. ZP3 is heterogeneously glycosylated with complex-type asparagine-linked (*N*-linked) and serine(Ser)/threonine-linked (*O*-linked) oligosaccharides [13]. Mass spectrometric analysis of recombinant human ZP3 has revealed three (Asn^{125}, Asn^{147}, and Asn^{272}) of four potential *N*-linked glycosylation sites and two clusters (156–173 aa and 260–281 aa) of potential *O*-linked glycosylation sites [14]. Mature human ZP3 has 12 cysteine (Cys) residues. Of these, eight are conserved across species in ZP3 polypeptide, forming four disulfide bonds. The first four Cys residues (Cys^{46}/Cys^{140} and Cys^{78}/Cys^{99}) form a loop-within-loop motif (1 to 4 and 2 to 3), and the second four (Cys^{217}/Cys^{282} and Cys^{239}/Cys^{300}) participate in the formation of a crossover motif (1 to 3 and 2 to 4). The remaining four Cys residues (Cys^{319}, Cys^{321}, Cys^{322}, and Cys^{327}) lying within a tight cluster toward the C-terminus are linked by two unassigned disulfide linkages, which could not be determined because of the absence of any proteolytic cleavage site [14]. In addition, ZP3 has a homologous region, spanning 45–304 aa, known as the ZP domain, which is conserved in other zona proteins across species, along with a variety of other extracellular proteins of diverse functions such as uromodulin (UMOD or Tamm-Horsefall protein), transforming growth factor, beta receptor III, and tectorin alpha and beta [15]. The ZP domain in human ZP3 consists of two conserved subdomains, an N-terminus that is followed by an internal hydrophobic patch (IHP [167–173 aa]) and a C-terminus that is followed by an external hydrophobic patch (EHP [362–368 aa]) separated by a short protease-sensitive hinge. In mice, IHP and EHP are essential for incorporation of ZP3 into the ZP matrix [12]. The N-terminal subdomain of the ZP domain is involved in polymerization of murine ZP3 [16]. The functional importance of different domains of human ZP3, based on disulfide linkages of conserved Cys residues, needs more elaboration.

In this study, human ZP3 polypeptide was cloned and expressed in baculovirus as the following fragments: 1) ZP3 corresponding to aa residues 1–175, including signal peptide and IHP (pAc-ZP3$_{[1–175\ aa]}$); 2) aa residues 23–175, excluding signal peptide but including IHP (pBg-ZP3$_{[23–175\ aa]}$) from the N-terminal region; 3) ZP3 corresponding to aa residues 214–305 spanning the C-terminus of the ZP domain (pBg-ZP3$_{[214–305\ aa]}$); and 4) aa residues 214–348, which include

[1]Supported by the Indian Council of Medical Research and Department of Biotechnology, Government of India.
[2]Correspondence: Satish K. Gupta, Reproductive Cell Biology Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110 067, India. FAX: 91 11 26742125; e-mail: skgupta@nii.res.in

Received: 10 November 2008.
First decision: 2 December 2008.
Accepted: 18 February 2009.

eISSN: 1259-7268 http://www.biolreprod.org
ISSN: 0006-3363

the C-terminus of ZP3 up to the consensus furin cleavage site (pBg-ZP3$_{[214–348\ aa]}$). ZP3 corresponding to aa residues 1–370 (pAc-ZP3$_{[1–370\ aa]}$), encompassing both domains was used as a positive control in the baculovirus expression system. Both ZP3 C-terminal fragments were also expressed in *Escherichia coli* to obtain these in nonglycosylated forms. These recombinant ZP3 fragments were investigated for induction of acrosomal exocytosis in capacitated human sperm, as well as their binding characteristics. Furthermore, using various pharmacological inhibitors, the downstream signaling events associated with acrosomal exocytosis mediated by ZP3 fragments were delineated.

MATERIALS AND METHODS

Cloning and Expression of cDNA Encoding Human ZP3 Fragments in Baculovirus and E. coli

The cDNAs encoding human ZP3 corresponding to aa residues 1–175 and 1–370 were cloned in baculovirus transfer vector pAcHLT-A (PharMingen, San Diego, CA) downstream of the polyhistidine tag and under the control of late polyhedrin promoter by following standard procedures as described previously [8] and were designated as pAc-ZP3$_{(1–175\ aa)}$ and pAc-ZP3$_{(1–370\ aa)}$, respectively. ZP3 N- and C-terminal fragments encompassing 23–175 aa (pBg-ZP3$_{[23–175\ aa]}$), 214–348 aa (pBg-ZP3$_{[214–348\ aa]}$), and 214–305 aa (pBg-ZP3$_{[214–305\ aa]}$) were cloned in baculovirus transfer vector pBacGus-6 (Novagen, Madison, WI) with gp64 signal peptide sequence downstream of a modified gp64 tandem promoter and a polyhistidine tag at the N-terminus according to the manufacturer's instructions. The purified recombinant baculovirus transfer vectors were used to cotransfect *Spodoptera frugiperda* (*Sf21*) insect cells grown in Grace Insect Cell Culture Medium (Invitrogen Corp., Carlsbad, CA) supplemented with 0.33% Lactalbumin Hydrolysate (Invitrogen Corp.), Yeastolate Ultrafiltrate (Invitrogen Corp.), and 10% fetal calf serum using the BD Baculogold transfection kit (PharMingen) or the BacVector-2000 DNA kit (Novagen), facilitating in vivo recombination of the zona insert into the AcNPV genome. To produce a recombinant clone, *Sf21* cells were infected with increasing dilutions of transfection supernatant for 1 h, overlaid with 1.5% AgarPlaque Plus agarose (PharMingen), and incubated at 27°C for 96 h. Plaques were visualized by staining with 0.03% neutral red solution, picked up, and used to infect *Sf21* cells. After 96 h of incubation, the cell lysate and supernatant were checked for the presence of the recombinant protein by Western blot analysis as described previously [8]. For large-scale production of the recombinant proteins, a suspension culture of 50×10^6 *Sf21* insect cells growing in a spinner bottle (Thermolyne; Barnstead International, Dubuque, IA) was incubated with the respective recombinant virus at a multiplicity of infection of three at 42 rotations/min on a biological stirrer (Thermolyne) at 27°C for 96 h, after which the cells were pelleted at $1000 \times g$ for 15 min, and recombinant proteins were purified from cell pellet or from culture supernatants using Ni-NTA resin essentially as described previously [8]. Protein concentration was determined using bicinchoninic acid assay (Pierce, Rockford, IL) using bovine serum albumin (BSA) (Sigma-Aldrich Inc., St. Louis, MO) as the standard.

In addition, cDNAs encoding human ZP3 corresponding to aa residues 214–348 (ZP3$_{[214–348\ aa]}$) and 214–305 (ZP3$_{[214–305\ aa]}$) were cloned downstream of the T7 promoter in pRSET-A vector (Invitrogen Corp.) in frame with a polyhistidine tag at its N-terminus. For expression, these constructs were used to transform the BL21[DE3] pLysS strain of *E. coli* (Stratagene, La Jolla, CA) deficient in *ompT* and *lon* proteases, and one of the positive clones was used for expression of the respective recombinant protein. Recombinant proteins were expressed at the shake flask level in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside after the cell density reached an absorbance of approximately 0.6 at 600 nm (A_{600}) for induction of expression of the protein for 2.5 h. The recombinant proteins were purified using Ni-NTA affinity chromatography under denaturing conditions and were renatured by extensive dialysis in the presence of oxidized/reduced glutathione essentially as described previously [8].

Characterization of Recombinant Human ZP3 Fragments

SDS-PAGE and Western blot. To characterize the *E. coli*- and baculovirus-expressed recombinant ZP3 fragments, the respective cell pellets, culture supernatants, or purified proteins were boiled for 10 min in 2× SDS-PAGE sample buffer (62.5 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue) and were resolved on a 0.1% SDS-10% polyacrylamide gel essentially as described previously [8]. The proteins were stained using Coomassie brilliant blue or were processed for Western blot as described previously using rabbit polyclonal antibodies against recombinant human pAc-ZP3$_{(1–424\ aa)}$ [9].

Lectin-binding analysis. To characterize the nature of glycosylation, microtitration plates (Nunclon, Roskilde, Denmark) were coated with purified baculovirus-expressed pAc-ZP3$_{(1–175\ aa)}$, pAc-ZP3$_{(1–370\ aa)}$, pBg-ZP3$_{(23–175\ aa)}$, pBg-ZP3$_{(214–348\ aa)}$, and pBg-ZP3$_{(214–305\ aa)}$ at a concentration of 500 ng/well in 50 mM PBS (pH 7.4) for 1 h at 37°C, followed by overnight incubation at 4°C. Using similar experimental conditions, microtitration plates were coated with *E. coli*-expressed ZP3$_{(214–305\ aa)}$ and ZP3$_{(214–348\ aa)}$. All subsequent washings were performed three times in 50 mM PBS with 0.1% Tween-20 (PBST). The plates were blocked with PBST (200 µl/well) for 1.5 h at 37°C, followed by incubation with various biotinylated lectins (100 ng/well; Vector Laboratories, Burlingame, CA) at 37°C for 1 h. The bound lectins were revealed by incubation with horseradish peroxidase-conjugated streptavidin (1:3000, 100 µl/well; Pierce) at 37°C for 1 h. Plates were subsequently processed by determination of enzyme activity as described previously [8]. The specificity of reactivity of lectins with baculovirus-expressed ZP3 fragments was further confirmed by lectin blot [8].

Chemical deglycosylation of pBg-ZP3$_{(214–348\ aa)}$. Baculovirus-expressed recombinant pBg-ZP3$_{(214–348\ aa)}$ was chemically deglycosylated using trifluoromethanesulphonic acid (TFMS) (Sigma-Aldrich Inc.) as described previously [17]. In brief, recombinant protein (250 µg) was dialyzed extensively against distilled water to remove Tris, followed by lyophilization in a reaction vial (Borosil Glass Works Ltd., Mumbai, India). The lyophilized protein was further dried overnight under vacuum. To the dried protein, 15 µl of anhydrous TFMS and 5 µl of anisole (Sigma-Aldrich Inc.) were added, and the reaction mixture was incubated at 0°C for 2 h under nitrogen with intermittent mixing. After incubation, the contents of the reaction vial were transferred to a microfuge tube, and 1.5 ml of a 9:1 mixture of precooled (−20°C) ether:pyridine was added dropwise, followed by centrifugation at $10\,000 \times g$ for 5 min. The pellet was resuspended in 150 µl of 0.1 M ammonium bicarbonate (pH 7.4) and dialyzed using the Tube-O-DIALYZER (G-Biosciences, St. Louis, MO) extensively against the same buffer, followed by two changes with 20 mM Tris (pH 7.4).

Induction of the Acrosome Reaction by Recombinant Human ZP3 Fragments in Capacitated Human Spermatozoa

All experiments using human spermatozoa were performed with informed consent and after clearance from the institutional biosafety and human ethical committee. Semen samples were collected from healthy donors after 3 days of sexual abstinence and were subjected to liquefaction at room temperature for 30 min. The motile sperm were isolated by two-step Percoll density gradient as described previously [18]. The sperm (10×10^6 cells/ml) were capacitated in Biggers-Whitten-Whittingham (BWW) medium [19] supplemented with 2.6% BSA for 16 h at 37°C with 5% CO_2 in humidified air in aliquots of 500 µl. Capacitated sperm (1×10^6 in BWW plus 0.3% BSA) were incubated at 37°C with 5% CO_2 in humidified air for varying times and in the presence of various concentrations of different recombinant ZP3 fragments in a total reaction volume of 100 µl. To account for spontaneous induction of the acrosome reaction, sperm were also incubated with BWW plus 0.3% BSA alone. Calcium ionophore (10 µM, A23187; Sigma-Aldrich Inc.) served as a positive control in all experiments. After incubation, the sperm were washed with 50 mM PBS (pH 7.4) and fixed in chilled methanol for 30 sec, and 20-µl aliquots were spotted on poly-L-lysine-coated slides (Sigma-Aldrich Inc.) in duplicates. The spots were air dried and stained with 5 µg/ml of trimethylrhodamine isothiocyanate-conjugated *Pisum sativum* agglutinin (TRITC-PSA; Vector Laboratories) for 30 min at room temperature. Any spermatozoa that demonstrated complete loss of TRITC-PSA staining in the acrosome or that revealed staining at the equatorial region were classified as acrosome reacted. Sperm showing TRITC fluorescence in the acrosomal region of the head were classified as acrosome intact. All slides were read "blind" with coded samples under an Eclipse 80i epifluorescence microscope (Nikon, Chiyoda-ku, Tokyo, Japan) using an oil immersion objective. Two hundred sperm were scored for every spot, and the percentage of the acrosome reaction was calculated by dividing the number of acrosome-reacted sperm by the total number of sperm counted and multiplying by 100. Each experiment was repeated at least five times by collecting semen samples from at least three individual donors.

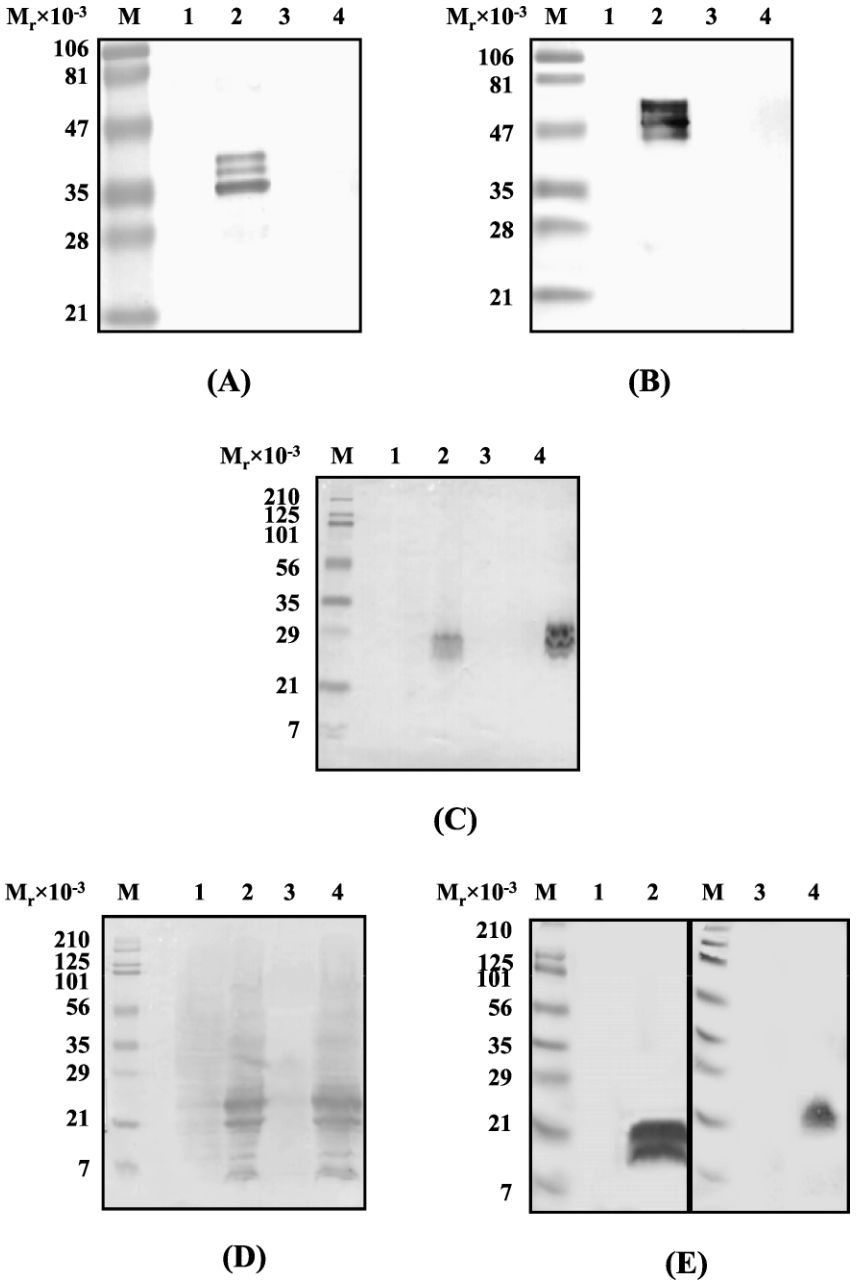

FIG. 1. Analysis of baculovirus-expressed human ZP3 fragments by Western blot. *Sf21* (1×10^6/well) cells were transfected with wild-type AcNPV or recombinant virus and were incubated at 27°C for 96 h. The cell pellet lysate and supernatant were resolved by 0.1% SDS-10% PAGE, followed by Western blot using rabbit polyclonal antibodies raised against recombinant pAc-$ZP3_{(1–424\ aa)}$ as described in *Materials and Methods*. Panels **A**, **B**, **C**, **D**, and **E** depict the expression of pAc-$ZP3_{(1–175\ aa)}$, pAc-$ZP3_{(1–370\ aa)}$, pBg-$ZP3_{(23–175\ aa)}$, pBg-$ZP3_{(214–348\ aa)}$, and pBg-$ZP3_{(214–305\ aa)}$, respectively. Lanes 1 and 2: wild-type AcNPV and recombinant virus-infected cell pellet lysate, respectively; Lanes 3 and 4: wild-type AcNPV and recombinant virus-infected cell supernatant, respectively. M, molecular weight markers.

Delineation of Downstream Signaling Events Associated with ZP3 Fragment-Mediated Induction of Acrosomal Exocytosis

To understand the mechanism of action of functionally active recombinant human ZP3 fragments, the acrosome reaction was induced in presence of different pharmacological inhibitors. Capacitated spermatozoa were pretreated for 10 min at 37°C with 5% CO_2 in humidified air before addition of the recombinant protein: pimozide (10 μM; Sigma-Aldrich Inc.) T-type Ca^{2+} channel blocker (CCB), picrotoxin (100 μM; Sigma-Aldrich Inc.) GABA(A) [gamma aminobutyric acid (A)] receptor antagonist, ethyleneglycoltetracetic acid (EGTA) (8 mM; Sigma-Aldrich Inc.) extracellular calcium chelator, and verapamil (10 μM; Sigma-Aldrich Inc.) and diltiazem (10 μM; Sigma-Aldrich Inc.) L-type CCBs. In addition, pretreatment with pertussis toxin (PTX) (0.1 μg/ml; Sigma-Aldrich Inc.), an inhibitor of G_i protein, was performed for 30 min.

Evaluation of the Binding of Recombinant ZP3 Fragments to Human Spermatozoa

Recombinant ZP3 fragments were conjugated with fluorescein isothiocyanate (FITC), and the fluorescein:recombinant protein molar ratio was determined as described previously [9]. The FITC-labeled recombinant proteins were used for studying their binding characteristics with capacitated and acrosome-reacted human spermatozoa as described previously [9]. The ability of recombinant pBg-$ZP3_{(23–175\ aa)}$ and pBg-$ZP3_{(214–348\ aa)}$ to compete with FITC-labeled pAc-$ZP3_{(1–370\ aa)}$ for binding to capacitated human spermatozoa was also evaluated by competitive inhibition studies. Essentially, capacitated sperm (5×10^6) were incubated with 10 pmol of FITC-labeled pAc-$ZP3_{(1–370\ aa)}$ alone or in the presence of 100 pmol of cold pAc-$ZP3_{(1–370\ aa)}$, pBg-$ZP3_{(23–175\ aa)}$, or pBg-$ZP3_{(214–348\ aa)}$ in a reaction volume of 50 μl at 37°C and 5% CO_2 in humidified air for 15 min, followed by 30 min at 4°C. After incubation, sperm were processed for binding of FITC-labeled pAc-$ZP3_{(1–370\ aa)}$ as described previously [9].

Statistical Analysis

The results pertaining to binding of recombinant human ZP3 fragments to the capacitated/acrosome-reacted spermatozoa and to induction of the acrosome reaction are presented as the mean ± SEM of a minimum three independent experiments using semen samples from at least two different donors. Statistical analysis was performed by comparing the means of the control medium (BWW plus 0.3% BSA) and experimental sets or within two experimental groups by

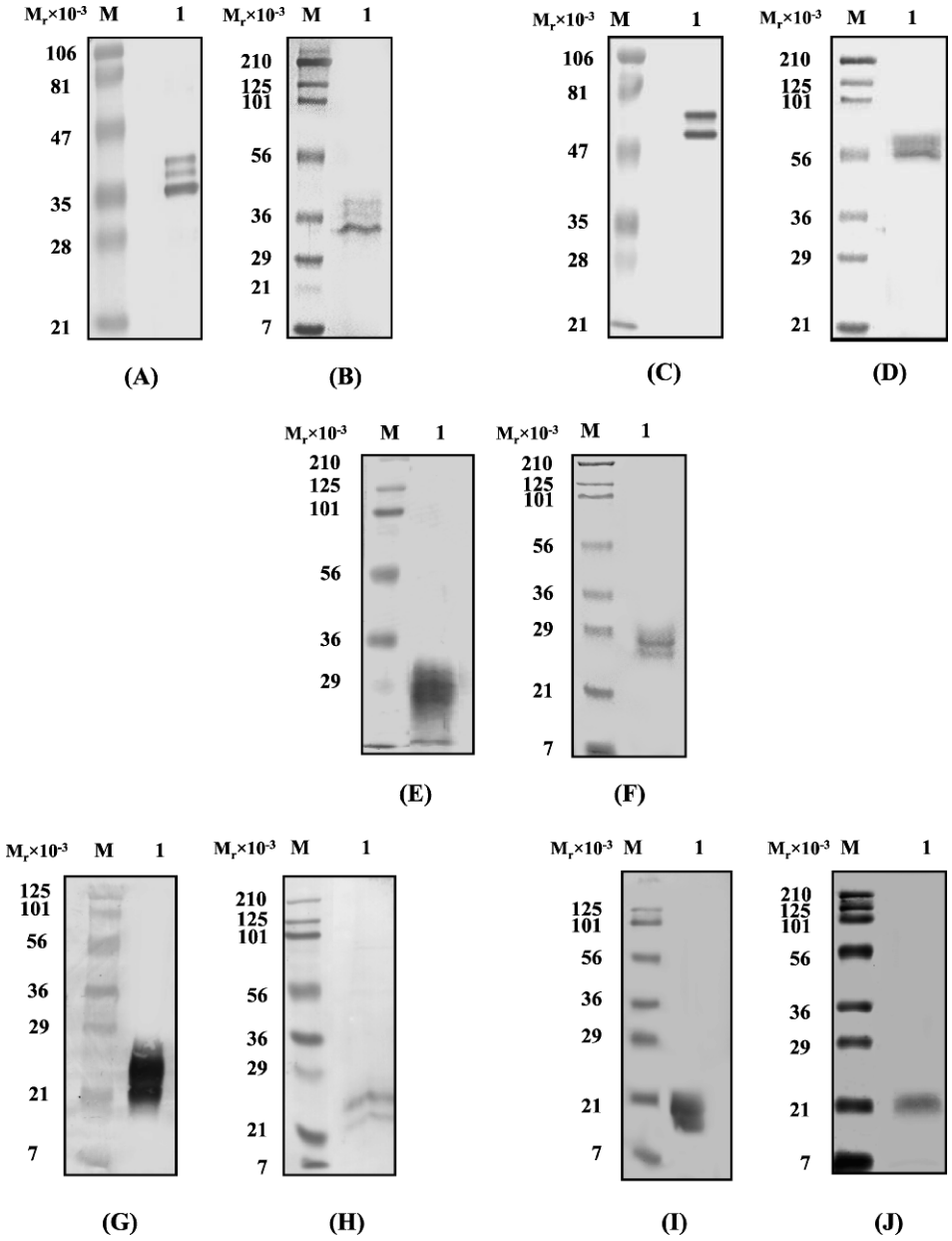

FIG. 2. Analysis of purified recombinant human ZP3 proteins expressed in the baculovirus expression system by SDS-PAGE and Western blot. The Ni-NTA affinity-purified baculovirus-expressed recombinant human ZP3 proteins were resolved by 0.1% SDS-10% PAGE under reducing conditions. Lane 1 in **B**, **D**, **F**, **H**, and **J** shows the SDS-PAGE profile (5 μg of recombinant protein per lane) and in **A**, **C**, **E**, **G**, and **I** shows the Western blot profile (1 μg of recombinant protein per lane) of purified recombinant pAc-ZP3$_{(1–175\ aa)}$, pAc-ZP3$_{(1–370\ aa)}$, pBg-ZP3$_{(23–175\ aa)}$, pBg-ZP3$_{(214–348\ aa)}$, and pBg-ZP3$_{(214–305\ aa)}$, respectively. M, molecular weight markers.

using one-way ANOVA, followed by Newman-Keuls multiple comparisons test. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of Recombinant Human ZP3 Fragments

The recombinant human pAc-ZP3$_{(1–175\ aa)}$ and pAc-ZP3$_{(1–370\ aa)}$ (cloned in pAcHLT-A transfer vector) and pBg-ZP3$_{(23–175\ aa)}$, pBg-ZP3$_{(214–348\ aa)}$, and pBg-ZP3$_{(214–305\ aa)}$ (cloned in pBac-Gus-6 transfer vector) were expressed as polyhistidine-tagged fusion proteins using the baculovirus expression system. Western blot analysis using rabbit polyclonal antibodies generated against baculovirus-expressed recombinant pAc-ZP3$_{(1–424\ aa)}$ [9] revealed that pAc-ZP3$_{(1–175\ aa)}$ and pAc-ZP3$_{(1–370\ aa)}$ were expressed in cell pellet only (Fig. 1, A and B; lane 2) and were absent in culture supernatant (Fig. 1, A and B; lane 4). However, pBg-ZP3$_{(23–175\ aa)}$, pBg-ZP3$_{(214–348\ aa)}$, and pBg-ZP3$_{(214–305\ aa)}$ were expressed in cell lysate and were secreted in the culture supernatant by insect cells infected with respective recombinant virus (Fig. 1, C–E; lanes 2 and 4). Polyclonal antibodies against pAc-ZP3$_{(1–424\ aa)}$ failed to show any reactivity with cell lysate or culture supernatant of *Sf21* cells infected with wild-type AcNPV (Fig. 1, A–E; lanes 1 and 3). Expressed recombinant pAc-ZP3$_{(1–175\ aa)}$ and pAc-ZP3$_{(1–370\ aa)}$ were purified from cell pellet and pBg-ZP3$_{(23–175\ aa)}$, pBg-ZP3$_{(214–348\ aa)}$, and pBg-ZP3$_{(214–305\ aa)}$ from culture supernatant using Ni-NTA affinity chromatography. SDS-PAGE and Western blot analysis of the purified pAc-ZP3$_{(1–175\ aa)}$ revealed a dominant band corresponding to ~35 kDa, with two minor bands of higher molecular weight (Fig. 2, A and B). The purified pAc-ZP3$_{(1–370\ aa)}$ resolved as a doublet corresponding to ~56–58 kDa (Fig. 2, C and D). Analysis of the purified pBg-ZP3$_{(23–175\ aa)}$ (Fig. 2, E and F) revealed two bands in the range of ~25–29 kDa, and analysis of the purified pBg-ZP3$_{(214–348\ aa)}$ (Fig. 2, G and H) revealed two bands in the range of 21–25 kDa. The purified pBg-ZP3$_{(214–305\ aa)}$ (Fig. 2, I and J) resolved as a broad band corresponding to ~21 kDa.

TABLE 1. Lectin binding characteristics with various recombinant ZP3 fragments in ELISA.

Recombinant protein	Absorbance at 490 nm with lectins[a,b]		
	ConA	Jacalin	PSA
pAc-ZP3$_{(1–175\ aa)}$	2.73	1.40	0.33
pAc-ZP3$_{(1–370\ aa)}$	2.91	2.48	0.59
pBg-ZP3$_{(23–175\ aa)}$	2.81	2.67	1.99
pBg-ZP3$_{(214–348\ aa)}$	2.82	2.97	1.09
pBg-ZP3$_{(214–305\ aa)}$	2.72	2.68	1.32
E. coli ZP3$_{(214–305\ aa)}$	0.18	0.07	0.14
E. coli ZP3$_{(214–348\ aa)}$	0.20	0.04	0.11

[a] Values are expressed as absorbance obtained with various lectins binding to the respective recombinant protein, after deducting the nonspecific binding of the lectins to the uncoated wells.
[b] Each value represents a mean of duplicate experiments.

ZP3 C-terminal fragments ZP3$_{(214–348\ aa)}$ and ZP3$_{(214–305\ aa)}$ were expressed as polyhistidine-tagged fusion proteins in *E. coli*. The SDS-PAGE and Western blot analysis of the purified *E. coli*-expressed ZP3$_{(214–305\ aa)}$ and ZP3$_{(214–348\ aa)}$ revealed dominant bands of ~21 kDa and ~24 kDa, respectively (Supplemental Fig. S1 available at www.biolreprod.org).

Baculovirus-Expressed Recombinant Human ZP3 Fragments Have Both N- *and* O*-Linked Glycosylation*

To determine the nature of glycosylation, purified recombinant proteins were analyzed for binding characteristics with various lectins in an ELISA (Table 1). Baculovirus-expressed recombinant ZP3 fragments pAc-ZP3$_{(1–175\ aa)}$, pAc-ZP3$_{(1–370\ aa)}$, pBg-ZP3$_{(23–175\ aa)}$, pBg-ZP3$_{(214–348\ aa)}$, and pBg-ZP3$_{(214–305\ aa)}$ showed reactivity with concanavalin A (Con A [mannose α 1–3/ mannose α 1–6]), jacalin (α-O-glycosides of Gal or GalNAc moieties), and PSA (α-linked mannose). Under similar conditions, *E. coli*-expressed ZP3$_{(214–305\ aa)}$ and ZP3$_{(214–348\ aa)}$ did not reveal any reactivity with these lectins, suggesting that these are not glycosylated. The presence of both *O*- and *N*-linked glycosylation on baculovirus-expressed recombinant ZP3 fragments was con-

TABLE 2. Induction of acrosomal exocytosis in capacitated human sperm by recombinant human ZP3 fragments.

Treatment[a]	Percentage induction of acrosomal exocytosis (mean ± SEM)[b]	Statistical significance
Group I		
Control (BWW + 0.3% BSA)	15.1 ± 0.9	
Fetuin	16.9 ± 0.3	$P = 0.4900$[c]
Human pAc-ZP2$_{(1–745\ aa)}$	18.2 ± 0.6	$P = 0.1842$[c]
Calcium Ionophore (10 μM)	52.2 ± 2.9	$P = 0.0024$[c]*
Group II		
pAc-ZP3$_{(1–175\ aa)}$	18.4 ± 2.1	$P = 0.1711$[c]
pBg-ZP3$_{(23–175\ aa)}$	18.0 ± 0.9	$P = 0.2217$[c]
pBg-ZP3$_{(214–348\ aa)}$	48.9 ± 2.7	$P = 0.0013$[c]*
pBg-ZP3$_{(214–305\ aa)}$	32.4 ± 2.3	$P = 0.0069$[c]*
pAc-ZP3$_{(1–370\ aa)}$	45.3 ± 2.2	$P = 0.0013$[c]*
Group III		
E. coli-expressed ZP3$_{(214–348\ aa)}$	18.5 ± 0.5	$P = 0.2737$[c]
E. coli-expressed ZP3$_{(214–305\ aa)}$	16.7 ± 1.4	$P = 0.6045$[c]
Group IV		
Control (BWW + 0.3% BSA)	12.8 ± 1.2	
Untreated pBg-ZP3$_{(214–348\ aa)}$	48.0 ± 2.2	
TFMS-deglycosylated pBg-ZP3$_{(214–348\ aa)}$	19.4 ± 0.5	$P = 0.0048$[d]*

[a] Capacitated sperm were incubated with the recombinant proteins at a concentration of 10 pmol/100 μl for 60 min.
[b] Percentage induction of acrosomal exocytosis was calculated by dividing the number of acrosome reacted sperm by total number of sperm counted and multiplying by 100; values represent mean ± SEM of at least three independent experiments.
[c] Statistical significance with respect to the medium control was calculated by one-way ANOVA followed by Newman-Keuls multiple comparison test.
[d] Statistical significance with respect to untreated pBg-ZP3$_{(214–348\ aa)}$ was calculated by paired *t*-test.
* Values are statistically significant.

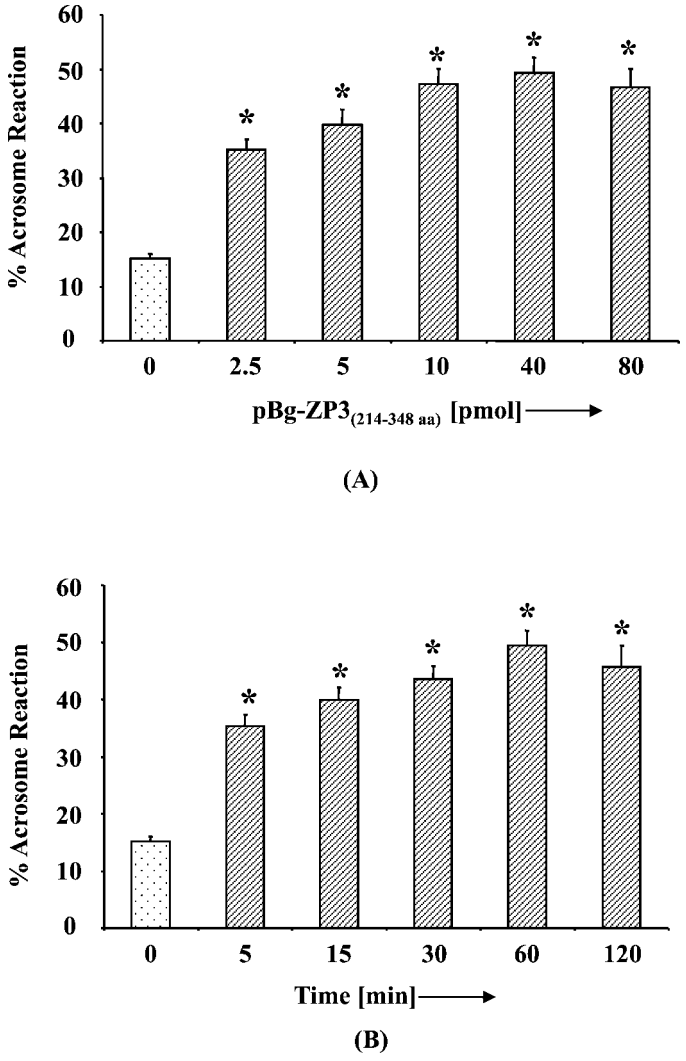

FIG. 3. Dose-response and time kinetics studies of pBg-ZP3$_{(214–348\ aa)}$-mediated induction of acrosomal exocytosis in capacitated human spermatozoa. **A**) Capacitated human sperm (1×10^6/100 μl) were incubated with recombinant pBg$_{(214–348\ aa)}$ at varying concentrations for 60 min and were analyzed for acrosomal status by TRITC-PSA staining as described in *Materials and Methods*. Bar with dotted line represents medium control. **B**) To study the optimum time required for induction of acrosomal exocytosis, capacitated sperm (1×10^6/100 μl) were incubated with 10 pmol of pBg$_{(214–348\ aa)}$ per reaction for varying times. Bar with dotted line represents analysis of acrosome reaction immediately after adding the recombinant protein. The y-axis in **A** and **B** represents percentage induction of acrosomal exocytosis calculated by dividing the number of acrosome-reacted sperm by the total number of sperm counted and multiplying by 100. Values are the mean ± SEM of five different experiments using semen samples from at least three different donors. *Values are statistically significant when compared with respective controls.

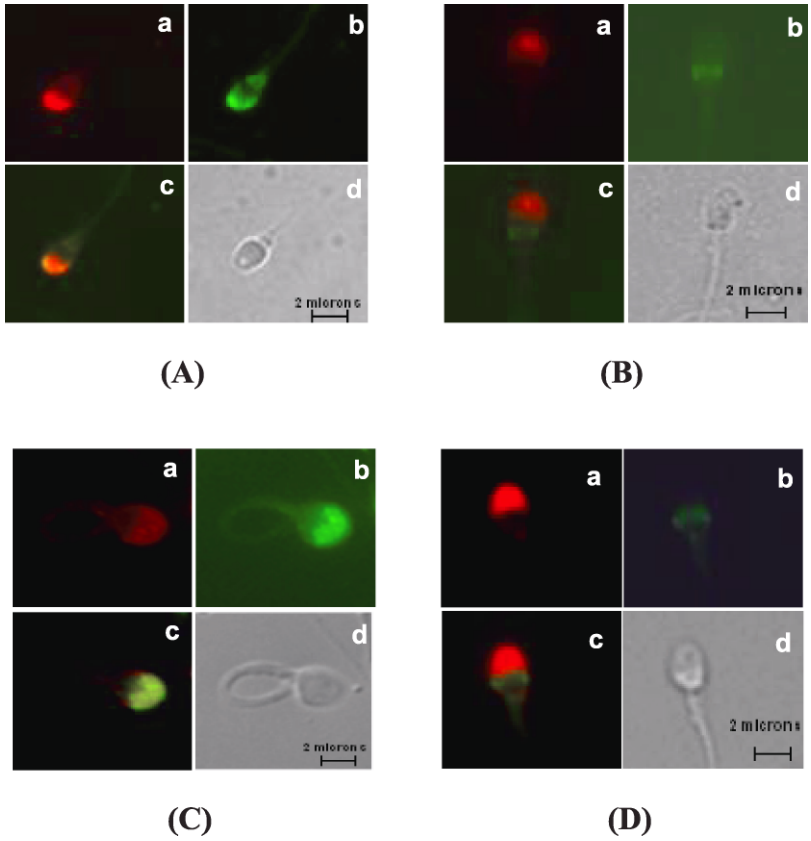

FIG. 4. Representative binding profile of baculovirus-expressed human ZP3 fragments with human spermatozoa. Capacitated sperm (5×10^6/50 μl) were incubated with FITC-conjugated baculovirus-expressed recombinant pBg-ZP3$_{(23–175\ aa)}$ and pBg-ZP3$_{(214–348\ aa)}$ and were processed as described in *Materials and Methods*. The acrosomal status was determined by labeling the sperm with TRITC-PSA. **A** and **B**) Binding profile of pBg-ZP3$_{(23–175\ aa)}$ with capacitated sperm. **C** and **D**) Binding profile of pBg-ZP3$_{(214–348\ aa)}$ with capacitated spermatozoa. The images were captured using a Nikon Eclipse 80i epifluorescence microscope. The following is shown in each panel: PSA-TRITC fluorescence (a), FITC-ZP3 protein fluorescence (b), overlap of the fluorescent frames (c), and phase contrast (d).

firmed by lectin blots using Con A (*N*-linked glycosylation) and jacalin (*O*-linked glycosylation) (Supplemental Fig. S2).

Baculovirus-Expressed C-Terminal ZP3 Fragment Induces Acrosomal Exocytosis in Capacitated Human Sperm

The *E. coli*- and baculovirus-expressed ZP3 fragments were evaluated for their ability to induce acrosomal exocytosis in the capacitated human spermatozoa. Treatment of capacitated sperm with various recombinant human ZP3 fragments did not result in any significant decrease in the viability of the sperm as determined by one-step eosin-nigrosin staining method [20]. Incubation of the capacitated sperm with baculovirus-expressed pAc-ZP3$_{(1–175\ aa)}$, pBg-ZP3$_{(23–175\ aa)}$, and *E. coli*-expressed ZP3$_{(214–305\ aa)}$ and ZP3$_{(214–348\ aa)}$ did not induce a significant increase ($P > 0.05$) in acrosomal exocytosis compared with the control medium (15.5% ± 0.6%) (Table 2). However, when capacitated sperm were incubated with pBg-ZP3$_{(214–348\ aa)}$ and pBg-ZP3$_{(214–305\ aa)}$, a significant increase (50.5% ± 1.3% and 32.4% ± 2.3%, respectively) in induction of acrosomal exocytosis was observed ($P = 0.0013$ and $P = 0.0069$, respectively). The extent of induction of acrosomal exocytosis mediated by recombinant pBg-ZP3$_{(214–348\ aa)}$ was similar to that of recombinant pAc-ZP3$_{(1–370\ aa)}$, which incorporates both N- and C-terminal domains. Chemical deglycosylation of pBg-ZP3$_{(214–348\ aa)}$ resulted in a significant decrease ($P = 0.0048$) in its ability to induce the acrosome reaction compared with untreated recombinant protein. Calcium ionophore (A23187), a chemical agonist of acrosomal exocytosis used as a positive control, also showed a significant increase in the percentage of sperm undergoing the acrosome reaction (51.6% ± 0.7%). Fetuin and pAc-ZP2$_{(1–745\ aa)}$ generated as described earlier [8] and used as internal controls failed to induce any significant increase in acrosomal exocytosis compared with control medium. Dose-response studies with pBg-ZP3$_{(214–348\ aa)}$ revealed that a dose as low as 2.5 pmol/reaction induced a significant increase in acrosomal exocytosis in capacitated sperm (Fig. 3A). No further increase in acrosomal exocytosis was observed if the concentration of ZP3 was increased beyond 40 pmol/reaction. A time kinetics study revealed that significant induction of acrosomal exocytosis by pBg-ZP3$_{(214–348\ aa)}$ can be seen as early as 5 min after exposure of capacitated spermatozoa to recombinant protein and that acrosomal exocytosis plateaus at 60 min (Fig. 3B).

Delineation of Downstream Signaling Events Associated with Recombinant pBg-ZP3$_{(214–348\ aa)}$-Mediated Induction of Acrosomal Exocytosis

Induction of acrosomal exocytosis mediated by recombinant pBg-ZP3$_{(214–348\ aa)}$ required extracellular Ca^{2+}, as prior incubation of capacitated sperm in BWW medium with EGTA (8 mM) before exposure to recombinant protein led to a significant decrease in acrosomal exocytosis (Table 3). Acrosomal exocytosis mediated by pBg-ZP3$_{(214–348\ aa)}$ was inhibited by prior incubation of capacitated sperm with the T-type CCB pimozide (10 μM), whereas the L-type CCBs verapamil (10 μM) and diltiazem (10 μM) failed to do so. The GABA(A) receptor antagonist picrotoxin (100 μM) also inhibited induction of acrosomal exocytosis mediated by pBg-ZP3$_{(214–348\ aa)}$. Induction of acrosomal exocytosis by recombinant pBg-ZP3$_{(214–348\ aa)}$ is also dependent on activation of the G_i pathway, as pretreatment with PTX (0.1 μg/ml) led to a significant decrease in induction of the acrosome reaction (52.6% ± 0.7% to 21.4% ± 0.5%, $P < 0.01$).

TABLE 3. Induction of acrosome reaction in capacitated human sperm by recombinant human pBg-ZP3$_{(214–348\ aa)}$ in presence or absence of various pharmacological inhibitors.

Treatment[a]	Percentage induction of acrosomal exocytosis (mean ± SEM)[b]	Statistical significance
Group I		
Control (BWW + 0.3% BSA)	15.5 ± 1.6	
pBg-ZP3$_{(214–348\ aa)}$	50.0 ± 1.6	$P = 0.0002^{*c}$
pBg-ZP3$_{(214–348\ aa)}$ + ethanol	49.9 ± 1.1	$P = 0.0001^{*c}$, $P = 0.9369^{d}$
pBg-ZP3$_{(214–348\ aa)}$ + PTX (0.1 μg/ml)	17.9 ± 0.8	$P = 0.2597^{c}$, $P = 0.0001^{*d}$
pBg-ZP3$_{(214–348\ aa)}$ + EGTA (8 mM)	26.5 ± 1.1	$P = 0.0045^{*c}$, $P = 0.0004^{*d}$
pBg-ZP3$_{(214–348\ aa)}$ + picrotoxin (10 μM)	20.3 ± 1.8	$P = 0.1182^{c}$, $P = 0.0003^{*d}$
Group II		
pBg-ZP3$_{(214–348\ aa)}$ + water	49.9 ± 0.5	$P = 0.0001^{*c}$, $P = 0.9006^{d}$
pBg-ZP3$_{(214–348\ aa)}$ + DMSO	48.6 ± 0.4	$P = 0.0001^{*c}$, $P = 0.4658^{d}$
pBg-ZP3$_{(214–348\ aa)}$ + pimozide (10 μM)	20.9 ± 1.5	$P = 0.2597^{c}$, $P = 0.0001^{*d}$
pBg-ZP3$_{(214–348\ aa)}$ + amiloride (10 μM)	28.2 ± 2.1	$P = 0.0078^{*c}$, $P = 0.0011^{*d}$
pBg-ZP3$_{(214–348\ aa)}$ + verapamil (10 μM)	49.6 ± 1.6	$P = 0.0002^{*c}$, $P = 0.8382^{d}$
pBg-ZP3$_{(214–348\ aa)}$ + diltiazem (10 μM)	48.9 ± 1.1	$P = 0.0001^{*c}$, $P = 0.6194^{d}$
pBg-ZP3$_{(214–348\ aa)}$ + nifedipine (10 μM)	35.6 ± 1.6	$P = 0.0009^{*c}$, $P = 0.0032^{*d}$

[a] Capacitated sperm pretreated with or without respective inhibitor were incubated with pBg-ZP3$_{(214–348\ aa)}$ at a concentration of 10 pmol/100 μl for 60 min.

[b] Percentage induction of acrosomal exocytosis was calculated by dividing the number of acrosome reacted sperm by total number of sperm counted and multiplying by 100; values represent mean ± SEM of at least three independent experiments.

[c] Statistical significance (*P* values) with respect to the medium control (calculated by one-way ANOVA followed by Newman-Keuls multiple comparisons test).

[d] Statistical significance (*P* values) with respect to the pBg-ZP3$_{(214–348\ aa)}$ (calculated by one-way ANOVA followed by Newman-Keuls multiple comparisons test).

* Values are statistically significant.

Binding Characteristics of Recombinant Human ZP3 Fragments to Spermatozoa

The fluorescein:recombinant protein molar ratios of the FITC-labeled recombinant proteins were as follows: 1.2 for *E. coli*-expressed ZP3$_{(214–305\ aa)}$; 1.8, 1.3, and 1.0 for pAc-ZP3$_{(1–370\ aa)}$, pBg-ZP3$_{(23–175\ aa)}$, and pBg-ZP3$_{(214–348\ aa)}$; and 1.8 for fetuin. These FITC-labeled recombinant human ZP3 fragments were analyzed for their ability to bind to human spermatozoa using a direct binding assay. The acrosomal status of the spermatozoa showing binding of the recombinant proteins was simultaneously assessed using TRITC-PSA. Binding studies revealed that all four recombinant ZP3 fragments bind to the anterior head or equatorial segment of the capacitated spermatozoa. In acrosome-reacted sperm, no binding of the recombinant proteins was observed to the anterior head of the spermatozoa, whereas binding to the equatorial segment was observed. A representative binding profile of pBg-ZP3$_{(23–175\ aa)}$ and pBg-ZP3$_{(214–348\ aa)}$ with capacitated human spermatozoa is shown in Figure 4. Under similar experimental conditions, FITC-labeled fetuin did not show any binding to the capacitated or acrosome-reacted spermatozoa. Computation of the binding characteristics for each recombinant ZP3 fragment revealed that 14.9% ± 4.1%, 21.9% ± 3.2%, and 24.3% ± 2.1% of capacitated sperm exhibited binding of baculovirus-expressed pBg-ZP3$_{(23–175\ aa)}$, pBg-ZP3$_{(214–348\ aa)}$, and pAc-ZP3$_{(1–370\ aa)}$, respectively. Also, 15.2% ± 2.7% of capacitated sperm showed binding with *E. coli*-expressed ZP3$_{(214–305\ aa)}$ vs. 2.7% ± 0.7% with fetuin. The binding patterns for each ZP3 protein are shown in Supplemental Table S1. Binding of FITC-labeled pAc-ZP3$_{(1–370\ aa)}$ (10 pmol) with capacitated spermatozoa was investigated in the presence of a 1:10 molar excess of cold pAc-ZP3$_{(1–370\ aa)}$, pBg-ZP3$_{(23–175\ aa)}$, and pBg-ZP3$_{(214–348\ aa)}$. A reduction of 67.9% ± 2.2% in the binding of FITC-labeled pAc-ZP3$_{(1–370\ aa)}$ was observed in the presence of cold pAc-ZP3$_{(1–37\ aa)}$, whereas cold pBg-ZP3$_{(23–175\ aa)}$ and pBg-ZP3$_{(214–348\ aa)}$ resulted in 49.2% ± 2.9% and 66.3% ± 4.1% reductions, respectively.

DISCUSSION

Human ZP3 and other human zona proteins share a sequence motif referred to as the ZP domain comprising ~260 aa with conserved polar and hydrophobic patterns [15]. The ZP domain of human ZP3 has eight conserved Cys residues, whereas the ZP domains of ZP1, ZP2, and ZP4 have 10 Cys residues each [21]. The first four conserved Cys residues of the ZP domain are engaged in identical intramolecular disulfide bonds in all ZP proteins, but the presence of two extra Cys residues within the C-terminal portion of the ZP domains of ZP1, ZP2, and ZP4 renders disulfide bonds of conserved Cys 5–8 different from those of ZP3. This disulfide linkage pattern of the ZP domain suggests that it is a bipartite structure with two independently folding subdomains [15]. Following murine investigations and the elucidation of disulfide linkages and *N*- and *O*-linked glycosylation sites in human ZP3 using Liquid Chromatography Quadrupole time-of-flight (LC-QTOF) tandem mass spectrometry [14], we herein attempted to delineate the functional domain of human ZP3 using N- and C- terminal fragments. To assess the importance of signal peptide for proper glycosylation of the ZP3 polypeptide, native (heterologous for insect cell host machinery) and gp64 secretory signal peptide-harboring constructs were expressed in *Sf21* insect cells using a baculovirus expression system. To delineate the importance of Cys^{319}, Cys^{321}, Cys^{322}, and Cys^{327} residues, the ZP3 C-terminus was further truncated to the end of the ZP domain (214–305 aa).

Expression of human ZP3 fragments as polyhistidine-tagged fusion proteins allowed their purification by Ni-NTA affinity column. Minor differences in the apparent molecular weights of purified recombinant ZP3 fragments in SDS-PAGE vs. Western blot may have been observed because prestained markers were used in Western blot, whereas unstained markers were used in SDS-PAGE. A higher increase in apparent molecular weight in the case of pAc-ZP3$_{(1–175\ aa)}$ and pBg-ZP3$_{(23–175\ aa)}$ compared with pBg-ZP3$_{(214–348\ aa)}$ may be because there are two *N*-linked glycosylation sites in the N-terminal fragment of human ZP3

(Asn^{125} and Asn^{147}) compared with one *N*-linked glycosylation site in the C-terminal fragment (Asn^{272}) [14]. The presence of more than one band in the SDS-PAGE and Western blot profiles for constructs expressed using the baculovirus expression system may be due to differential glycosylation of the polypeptide. Failure of expression of pAc-ZP3$_{(1–175\ aa)}$ and pAc-ZP3$_{(1–370\ aa)}$ under the influence of native human ZP3 signal peptide (1–22 aa) in secreted form can be attributed to failure of the host cell machinery to recognize foreign signal peptide and inability to direct the polypeptide into the secretory pathway. In contrast, pBg-ZP3$_{(23–175\ aa)}$ expressed under the influence of gp64 signal peptide (present in pBacGus-6 vector) was secreted in culture supernatant of insect host cells. A similar expression strategy was used to obtain ZP3 C-terminal fragments in properly glycosylated and soluble form in culture supernatant of the respective recombinant virus-infected host insect cells.

The predominant glycosylation in the baculovirus-expressed human ZP3 fragments was characterized by the binding of lectins such as Con A and jacalin. Using immunocytochemistry, native human ZP has been shown to bind with both lectins [13]. Purified baculovirus-expressed recombinant human ZP3 fragments after lectin-binding characterization were assessed for their ability to induce acrosomal exocytosis in capacitated human sperm. The acrosome reaction refers to the sequential process of fusion and fenestration of the outer acrosomal membrane and its overlying plasma membrane, followed by release of the acrosomal contents that facilitate penetration of the sperm through the ZP [22]. Baculovirus-expressed human ZP3 C-terminal fragments pBg-ZP3$_{(214–348\ aa)}$ and pBg-ZP3$_{(214–305\ aa)}$ in addition to pAc-ZP3$_{(1–370\ aa)}$ were able to induce the acrosome reaction in capacitated human sperm, whereas human ZP3 N-terminal fragments expressed with or without native signal peptide failed to do so. Baculovirus-expressed human pAc-ZP2$_{(1–745\ aa)}$ and fetuin (used as negative controls) failed to significantly induce an increase in the acrosome reaction in capacitated human sperm compared with control medium ($P > 0.05$), suggesting the importance of the ZP3 polypeptide backbone from its C-terminal region. In murine models using papain or V8 protease-digested ZP3, the mechanism to inhibit binding of sperm to the egg and to induce acrosomal exocytosis in capacitated spermatozoa was mapped to the C-terminus of ZP3 [23, 24]. Exon swapping and site-directed mutagenesis investigations revealed that the sperm-combining site of ZP3 is located in its carboxy terminal region encoded by exon 7 [25]. Mutation of Ser^{332} and Ser^{334} resulted in complete inactivation of mouse ZP3 as a sperm receptor [26]. Fusion constructs encoding human IgG(Fc) and mouse ZP3 exon 7 or 8 were expressed in an embryonal carcinoma cell line [27]. The recombinant human IgG(Fc)/mouse ZP3(exon 7) bound specifically to plasma membrane overlying the sperm head, whereas human IgG(Fc)/mouse ZP3(exon 8) failed to do so [27]. Furthermore, using recombinant overlapping ZP3 fragments, the inhibitory activity for sperm-oocyte binding was mapped to a fragment encompassing exon 7 [28]. Collectively, these observations strongly suggest that the mouse spermatozoa recognize and bind to a region of mouse ZP3 polypeptide that is encoded by exon 7 and is immediately downstream of its ZP domain. As already mentioned, site-directed mutagenesis experiments revealed that glycosylation of Ser^{332} and Ser^{334} may be critical in determining the biological activity of mouse ZP3 [26]. In contrast to murine model observations, LC-QTOF mass spectrometric analysis of recombinant human ZP3 expressed in CHO-Lec3.2.8.1 cells failed to demonstrate glycosylation of these Ser residues [14]. The observed reduced induction of the acrosome reaction mediated by pBg-ZP3$_{(214–305\ aa)}$ vs. by pBg-ZP3$_{(214–348\ aa)}$ may be due to the lack of these Ser residues and in addition the four Cys residues (Cys^{319}, Cys^{321}, Cys^{322}, and Cys^{327}). The glycosylation pattern observed in recombinant human ZP3 (expressed in CHO-Lec3.2.8.1 cells) may or may not correlate with that of native human ZP3. Hence, the relevance of the aforementioned Ser and Cys residues in imparting biological activity of human ZP3 needs further elucidation. To assess the importance of glycosylation in functional activity of ZP3 C-terminal fragments, their *E. coli*-expressed counterparts were used, and it was found that they were unable to induce the acrosome reaction in capacitated human sperm in a statistically significant manner. Moreover, chemical deglycosylation of pBg-ZP3$_{(214–348\ aa)}$ resulted in a significant decrease in the acrosome reaction-inducing ability. The failure by *E. coli*-expressed ZP3 C-terminal fragments to induce the acrosome reaction may not be due to their nonbinding to capacitated sperm, as the binding profile of ZP3$_{(214–305\ aa)}$ revealed characteristics similar to those observed with baculovirus-expressed C-terminal fragments. These observations are in agreement with recently published data from our laboratory demonstrating that glycosylation of zona proteins per se is not critical for binding to spermatozoa but is critical for induction of acrosomal exocytosis [9]. However, the role of glycosylation remains controversial in imparting biological activity to the zona proteins [29].

At least two different receptor-mediated signaling pathways in sperm plasma membrane have been shown to be responsible for ZP-induced acrosomal exocytosis. One is a G_i protein-coupled receptor that activates the phospholipase C (PLCB1)-mediated signaling pathway, and the other is a tyrosine kinase receptor coupled to PLCG [10, 30–32]. Both pathways culminate in release of Ca^{2+} from the interior of the acrosome to the cytosol. The increase in $[Ca^{2+}]_i$ and pH subsequently leads to membrane fusion, resulting in acrosomal exocytosis. As an inhibitor of the G_i protein-mediated signaling pathway, PTX is known to inhibit human ZP-mediated acrosomal exocytosis, whereas it fails to do so in human ZP4 [8, 10]. In the present study, the inhibition of pBg-ZP3$_{(214–348\ aa)}$-mediated induction of acrosomal exocytosis by PTX suggests that it is a specific event following a downstream signaling pathway similar to that of full-length ZP3 polypeptide [8, 33, 34]. Requirement of extracellular Ca^{2+} to bring out pBg-ZP3$_{(214–348\ aa)}$-mediated induction of the acrosome reaction corroborates with earlier investigations using recombinant or native ZP3 [10, 35]. The inhibition of pBg-ZP3$_{(214–348\ aa)}$-mediated induction of the acrosome reaction by picrotoxin and pimozide suggests the involvement of the Cl^- channel and the T-type CCB, whereas failure of verapamil and diltiazem to inhibit the same suggests that L-type Ca^{2+} channels may not have an important role in downstream signaling events. These inhibitors have been used to study the role of Ca^{2+} channels in the acrosome reaction of human spermatozoa [36–38]. The results corroborate with earlier reports in the literature [10].

In light of these observations, it can be hypothesized that the C-terminal region of human ZP3 polypeptide, owing to a unique disulfide linkage pattern that is different from that of other human zona proteins, facilitates appropriate disposition of critical glycosides, which are responsible for effecting acrosomal exocytosis after coming in contact with capacitated sperm. Acrosomal exocytosis of capacitated human sperm mediated by pBg-ZP3$_{(214–348\ aa),}$ is as effective as that induced by ZP3$_{(1–370\ aa)}$ and ZP3$_{(1–424\ aa)}$ and is only slightly less than that induced by calcium ionophore ($P > 0.05$). Dose-response results with pBg-ZP3$_{(214–348\ aa)}$ indicate that a dose as low as 600 ng/ml of recombinant protein is sufficient to significantly

induce acrosomal exocytosis in capacitated human sperm. This amount far exceeds the amount of ZP3 required for in vivo induction of acrosomal exocytosis, in which the ZP surrounding a single human oocyte consists of ~5–8 ng of ZP3 [7, 39]. In contrast to recombinant human ZP3 (present in soluble form), native ZP3 is associated with other zona proteins that constitute the ZP matrix, and results of recent studies [8, 10] in humans suggest that ZP4 and ZP3 induce acrosomal exocytosis in human spermatozoa. In vivo, other zona proteins and various physiological agents such as progesterone may act synergistically with ZP3 to effect acrosomal exocytosis.

In conclusion, the present study has delineated for the first time (to our knowledge) a functionally active subdomain of human ZP3 polypeptide that is competent to bind in the acrosomal cap region of human spermatozoa and to induce acrosomal exocytosis in the capacitated human sperm following a downstream signaling pathway similar to that of full-length human $ZP3_{(1–424\ aa)}$. This finding suggests that the functional activity of human ZP3 resides toward its C-terminus.

ACKNOWLEDGMENTS

We gratefully acknowledge the volunteers who donated semen samples for these studies.

REFERENCES

1. Wassarman PM. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. Cell 1999; 96:175–183.
2. Lefievre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, Lenton W, Afnan M, Brewis IA, Monk M, Hughes DC, Barratt CL. Four zona pellucida glycoproteins are expressed in the human. Hum Reprod 2004; 19:1580–1586.
3. Conner SJ, Lefievre L, Hughes DC, Barratt CL. Cracking the egg: increased complexity in the zona pellucida. Hum Reprod 2005; 20:1148–1152.
4. Ganguly A, Sharma SK, Gupta SK. Bonnet monkey (*Macaca radiata*) ovaries, like human oocytes, express four zona pellucida glycoproteins. Mol Reprod Dev 2008; 75:156–166.
5. Bleil JD, Wassarman PM. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse zonae pellucidae possessing receptor activity for sperm. Cell 1980; 20:873–882.
6. 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.
7. van Duin M, Polman J, De Breet IT, van Ginneken K, Bunschoten H, Grootenhuis A, Brindle J, Aitken RJ. Recombinant human zona pellucida protein ZP3 produced by Chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. Biol Reprod 1994; 51:607–617.
8. Chakravarty S, Suraj K, Gupta SK. Baculovirus expressed recombinant human zona pellucida glycoprotein-B induces acrosomal exocytosis in capacitated spermatozoa in addition to zona pellucida glycoprotein-C. Mol Hum Reprod 2005; 11:365–372.
9. Chakravarty S, Kadunganattil S, Bansal P, Sharma RK, Gupta SK. Relevance of glycosylation of human zona pellucida glycoproteins for their binding to capacitated human spermatozoa and subsequent induction of acrosomal exocytosis. Mol Reprod Dev 2008; 75:75–88.
10. Chiu PC, Wong BS, Chung MK, Lam KKW, Pang RT, Lee KF, Sumitro SB, Gupta SK, Yeung WS. Effects of native human zona pellucida glycoprotein-3 and -4 on acrosome reaction and zona pellucida binding of human spermatozoa. Biol Reprod 2008; 79:869–877.
11. Chamberlin ME, Dean J. Human homolog of the mouse sperm receptor. Proc Natl Acad Sci U S A 1990; 87:6014–6018.
12. Jovine L, Qi H, Williams Z, Litscher ES, Wassarman PM. A duplicated motif controls assembly of zona pellucida domain proteins. Proc Natl Acad Sci U S A 2004; 101:5922–5927.
13. Jimenez-Movilla M, Aviles M, Gomez-Torres MJ, Fernandez-Colom PJ, Castells MT, de Juan J, Romeu A, Ballesta J. Carbohydrate analysis of the zona pellucida and cortical granules of human oocytes by means of ultrastructural cytochemistry. Hum Reprod 2004; 19:1842–1855.
14. Zhao M, Boja ES, Hoodbhoy T, Nawrocki J, Kaufman JB, Kresge N, Ghirlando R, Shiloach J, Pannell L, Levine RL, Fales HM, Dean J. Mass spectrometry analysis of recombinant human ZP3 expressed in glycosylation deficient CHO cells. Biochemistry 2004; 43:12090–12104.
15. Jovine L, Qi H, Williams Z, Litscher E, Wassarman PM. The ZP domain is a conserved module for polymerization of extracellular proteins. Nat Cell Biol 2002; 4:457–461.
16. Jovine L, Janssen WG, Litscher ES, Wassarman PM. The PLAC1-homology region of ZP domain is sufficient for protein polymerization. BMC Biochem 2006; 7:11.
17. Schwoebel E, Prasad S, Timmons TM, Cook R, Kimura H, Niu P, Cheung P, Skinner A, Avery SE, Wilkins B, Dunbar BS. Isolation and characterization of a full length cDNA encoding the 55 kDa rabbit zona pellucida protein. J Biol Chem 1991; 266:7214–7219.
18. Suarez SS, Wolf DP, Meizel S. Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid. Gamete Res 1986; 14:107–121.
19. Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In: Daniel JC Jr (ed.), Methods in Mammalian Embryology. San Francisco: WH Freeman and Co.; 1971:86–116.
20. Björndahl L, Söderlund I, Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum Reprod 2003; 18:813–816.
21. Jovine L, Darie CC, Litscher ES, Wassarman PM. Zona pellucida domain proteins. Annu Rev Biochem 2005; 74:83–114.
22. Morales P, Llanos M. Interaction of human spermatozoa with the zona pellucida of oocyte: development of the acrosome reaction. Front Biosci 1996; 1:146–160.
23. Rosiere TK, Wassarman PM. Identification of a region of mouse zona pellucida glycoprotein mZP3 that possesses sperm receptor activity. Dev Biol 1992; 154:309–317.
24. Litscher ES, Wassarman PM. Characterization of mouse ZP3-derived glycopeptide, gp55, that exhibits sperm receptor and acrosome reaction-inducing activity *in vitro*. Biochemistry 1996; 35:3980–3986.
25. Kinloch RA, Sakai Y, Wassarman PM. Mapping the mouse ZP3 combining site for sperm by exon swapping and site-directed mutagenesis. Proc Natl Acad Sci U S A 1995; 92:263–267.
26. Chen J, Litscher ES, Wassarman PM. Inactivation of the mouse sperm receptor, mZP3, by site directed mutagenesis of individual serine residues located at the combining site for sperm. Proc Natl Acad Sci U S A 1998; 95:6193–6197.
27. Williams Z, Litscher ES, Jovine L, Wassarman PM. Polypeptide encoded by mouse ZP3 exon-7 is necessary and sufficient for binding of mouse sperm in vitro. J Cell Physiol 2006; 207:30–39.
28. Li D, Cao S, Xu C. Polypeptide backbone derived from carboxyl terminal of mouse ZP3 inhibits sperm-zona binding. Mol Reprod Dev 2007; 74:1327–1336.
29. Chapman NR, Kessopoulou E, Andrews P, Hornby D, Barratt CR. The polypeptide backbone of recombinant human zona pellucida glycoprotein-3 initiates acrosomal exocytosis in human spermatozoa in vitro. Biochem J 1998; 330:839–845.
30. Baldi E, Luconi M, Bonaccorsi L, Forti G. Signal transduction pathways in human spermatozoa. J Reprod Immunol 2002; 53:121–131.
31. Breitbart H. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. Mol Cell Endocrinol 2002; 187:139–144.
32. Doherty CM, Tarchala SM, Radwanska E, De Jonge CJ. Characterization of two second messenger pathways and their interactions in eliciting the human sperm acrosome reaction. J Androl 1995; 16:36–46.
33. Bastiaan H, Franken D, Wranz P. G-protein regulation of the solubilized human zona pellucida-mediated acrosome reaction and zona pellucida binding. J Assist Reprod Genet 1999; 16:332–336.
34. 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.
35. Schuffner AA, Bastiaan HS, Duran HE, Lin ZY, Morshedi M, Franken DR, Oehninger S. Zona pellucida-induced acrosome reaction in human sperm: dependency on activation of pertussis toxin-sensitive G(i) protein and extracellular calcium, and priming effect of progesterone and follicular fluid. Mol Hum Reprod 2002; 8:722–727.
36. O'Toole CM, Roldan ER, Fraser LR. Role for Ca^{2+} channels in the signal transduction pathway leading to acrosomal exocytosis in human spermatozoa. Mol Reprod Dev 1996; 45:204–211.
37. Garcia MA, Meizel S. Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. Biol Reprod 1999; 60:102–109.
38. Son WY, Lee JH, Han CT. Acrosome reaction of human spermatozoa is mainly mediated by alpha1H T-type calcium channels. Mol Hum Reprod 2000; 6:893–897.
39. Chiu PC, Wong BS, Lee CL, Pang RT, Lee KF, Sumitro SB, Gupta SK, Yeung WSB. Native human zona pellucida glycoproteins: purification and binding properties. Hum Reprod 2008; 23:1385–1393.