Baculovirus-expressed recombinant human zona pellucida glycoprotein-B induces acrosomal exocytosis in capacitated spermatozoa in addition to zona pellucida glycoprotein-C

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To facilitate our understanding of the role of zona pellucida glycoproteins during fertilization in humans, recombinant human zona pellucida glycoprotein-A (hZPA), -B (hZPB) and -C (hZPC) were obtained by using Escherichia coli and baculovirus expression systems. Analysis by SDS-PAGE and Western blot of the Ni-NTA affinity purified recombinant proteins revealed that the baculovirus-expressed hZPA, hZPB and hZPC have an apparent molecular weight of ~110, ~70–75 and ~65 kDa, respectively, as compared to ~80, ~65 and ~50 kDa of the respective E. coli-expressed proteins. Lectin binding studies revealed that the baculovirus-expressed recombinant zona proteins were glycosylated. Major oligosaccharides were represented by strong reactivity with Concanavalin A (mannose α 1–3 or mannose α 1–6 residues) and Jacalin (α-O glyc osides of Gal or GalNAc moieties). A significant increase in acrosomal exocytosis was observed when capacitated human sperm were incubated in vitro with baculovirus-expressed hZPB (P = 0.0005) and hZPC (P = 0.0005) The E. coli-expressed hZPB, hZPC and baculovirus-expressed hZPA failed to induce any significant increase (P > 0.05) in acrosome reaction. In contrast to hZPC, the acrosome reaction induced by recombinant hZPB was not inhibited by pertussis toxin. These studies, for the first time, have demonstrated that in humans, ZPB also induces acrosomal exocytosis through a G₁ independent pathway.

Key words: acrosome reaction/recombinant human zona pellucida glycoproteins/spermatozoa

Introduction

During mammalian fertilization, zona pellucida matrix surrounding the oocyte, mediates the initial recognition and binding of the spermatozoa to the oocyte, induces acrosome reaction in the zona bound spermatozoa and also plays a role in avoidance of polyspermy. In various species, zona pellucida is primarily composed of three glycoproteins (Harris et al., 1994). These are designated as zona pellucida-1 (ZP1), -2 (ZP2) and 3 (ZP3) based on their mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively, based on the size of their mRNA transcripts, these have also been designated as zona pellucida glycoprotein-A (ZPA), -B (ZPB) and -C (ZPC), where ZPA has the longest transcript and ZPC the smallest (Harris et al., 1994). Recent studies, however revealed that human zona pellucida is composed of four glycoproteins (Lefievre et al., 2004) and Xenopus laevis oocyte vitelline envelope is composed of five glycoproteins (Vo et al., 2003). To avoid ambiguity, in the present manuscript, we have followed the ZPA, ZPB and ZPC classification of the zona pellucida glycoproteins.

Various studies suggest that in mouse, ZPC serves as the putative primary sperm receptor and is also responsible for inducing acrosomal exocytosis (Blekil and Wassarman, 1980; Beebe et al., 1992). In this process, O-linked oligosaccharides play a critical role (Florman and Wassarman, 1985; Blekil and Wassarman, 1988). However, in the porcine system both O-linked oligosaccharides (Yurewicz et al., 1991) and the tri- and tetra-antennary neutral complex type of N-linked oligosaccharides (Nakano et al., 1996; Yonezawa et al., 1999) of ZPC have been implicated for its sperm receptor activity. The critical appraisal of the role of ZPC and other zona pellucida glycoproteins during fertilization in humans was hampere due to their non-availability in highly purified form from native source. Subsequently, employing recombinant human ZPC (hZPC) expressed in Chinese hamster ovary cells, it was demonstrated that incubation of capacitated human spermatozoa with hZPC...
leads to induction of acrosomal exocytosis (van Duin et al., 1994). Further, employing recombinant hZPC expressed in Escherichia coli and presumably lacking glycosylation also induced acrosomal exocytosis suggesting that the presence of carbohydrates on ZPC polypeptide backbone may not be an absolute requirement for ZPC to induce acrosome reaction (Chapman et al., 1998).

ZPA, in the mouse model has been shown to serve as the secondary receptor that maintains the binding of the acrosome-reacted spermatozoa to the zona pellucida, while the ZPB acts as a cross-linker of the ZPA–ZPC heterodimeric filaments (Greve and Wassarman, 1985; Bleil et al., 1988). However, in the rabbit model, rec55 (homologue of ZPB) binds to the spermatozoa in a dose-dependent manner (Prasad et al., 1996). Rabbit ZPB also binds to recombinant Sp17 (a family of sperm autoantigens), further reiterating its importance during sperm–oocyte interaction (Yamasaki et al., 1995). The porcine ZP3β (homologue of ZPC) fails to bind to the sperm receptors whereas ZP3α (homologue of ZPB)–ZP3β heterocomplexes bind with high affinity to boar sperm membrane vesicles, suggesting involvement of more than one zona pellucida protein in sperm recognition (Yurewicz et al., 1998). In Xenopus laevis, the envelope glycoproteins, ZPB and ZPC possessed independent sperm binding activity yet, ZPC was the major ligand for sperm binding (Vo and Hedrick, 2000). However, when all the three oocyte proteins were mixed in a ratio of 1:4:4 (ZPA:ZPB:ZPC), they acted in synergy to increase sperm binding (Vo and Hedrick, 2000). Additional studies have also demonstrated that recombinant bonnet monkey (Macaca radiata) ZPB (bmZPB) expressed in E. coli binds to the head region of the capacitated spermatozoa and the binding shifts to the equatorial segment, post-acrosomal domain and mid-piece of the acrosome-reacted spermatozoa, indicating a role for ZPB in sperm binding (Govind et al., 2001).

The above described observations prompted us to revisit the role of zona pellucida glycoproteins during fertilization in humans and to investigate the importance of glycosylation in this process. In this manuscript, we describe the cloning and expression of human ZPA (hZPA), ZPB (hZPB) and ZPC (hZPC) both in E. coli as well as baculovirus expression systems. The efficacy of recombinant proteins to induce in vitro acrosomal exocytosis in capacitated human spermatozoa has been evaluated.

**Materials and methods**

**Expression of hZPA, hZPB and hZPC in E. coli and baculovirus and their purification**

To clone hZPB in E. coli, an internal cDNA fragment devoid of signal sequence and transmembrane-like domain [amino acid (aa) residues 22–463], was amplified by PCR from its pBluescript clone (kindly made available by Dr S. V. Prasad, Baylor College of Medicine, Houston, TX, USA) following manufacturer’s instructions. The hZPA was detected by using monoclonal antibody (MA 925) generated against recombinant bonnet monkey (Macaca radiata) ZPB (bmZPB) expressed in E. coli. The hZPA was detected by using monoclonal antibody (MA 451) generated against porcine ZP3 (homologue of ZPC)–ZP3 heterodimeric filaments (Greve and Wassarman, 1985; Bleil et al., 1988). The hZPA was detected by employing a monoclonal antibody (MA 451) generated against porcine ZP3β (homologue of ZPC), and cross-reactive with hZPC (Afzalpurkar and Gupta, 1997). All three monoclonal antibodies were used to detect the respective recombinant protein at a concentration of 500 ng/well in 50 mM phosphate-buffered saline (PBS), pH 7.4 for 1 h at 37°C followed by overnight at 4°C. All subsequent washings were done three times in 50 mM PBS with 0.05% Tween-20 (PBST). The plates were blocked with 1% BSA in PBS (200 μl/well) for 1.5 h at 37°C followed by incubation with 21 biotinylated lectins (1 μg/ml; 100 μl/well) at 37°C for 1 h. The bound lectins were revealed by incubating with HRP-conjugated streptavidin (1:3000; Pierce; 100 μl/well) at 37°C for 1 h. The enzyme activity was detected by adding 100 μl/well of 0.05% orthophenylene diamine and 0.06% H2O2 in 50 mM citrate-phosphate buffer, pH 5 and the reaction was stopped by adding 50 μl/well of 5N H2SO4.
The absorbance was read at 492 nm with 620 nm as the reference filter. The biotinylated lectins available in the Lectin kit-I, -II and -III (Vector Laboratories, Burlingame, CA, USA) were used in the lectin binding assay.

To confirm the specificity of the lectins binding to the recombinant proteins, lectin blots were also performed. \textit{E. coli} and baculovirus-expressed hZPA, hZPB and hZPC (1 \mu g/\text{lane}) were resolved on SDS-PAGE and transferred onto a 0.45 \mu m nitrocellulose membrane as described above. The membrane was blocked with PBS containing 3% BSA for 1 h at room temperature. Post-blocking, the membrane was incubated with 20 \mu g/ml of the respective biotinylated lectins at room temperature for 1.5 h followed by incubation with HRP-conjugated streptavidin (1:2000; Pierce) at room temperature for 1 h. The blot was developed with 0.6% (w/v) 4-chloro-1-naphthol (Amresco, Solon, OH, USA) in 50 mM PBS containing 25% methanol and 0.06% H2O2.

**Induction of acrosome reaction by the recombinant zona proteins in capacitated human spermatozoa**

All experiments using human spermatozoa were carried out under informed consent and following the clearance from the Institutional Bio-safety and Ethical Committee. Semen samples were collected from healthy donors and subjected to liquefaction at room temperature for 30 min. Aliquots (1–2 ml) of semen from a single donor were layered over a two-step Percoll density gradient, centrifuged at 500 \text{g} for 30 min at room temperature (Suarez et al., 1986). The pellet comprising of >90% motile spermatozoa was washed with Biggers-Whitten-Whittingham (BWW) medium (Biggers et al., 1971) supplemented with 0.3% BSA (cell culture grade; Sigma Chemical Co., St Louis, MO, USA).

The sperm (10,000 cells/ml) were capacitated in BWW medium supplemented with 2.6% BSA for 16 h at 37 \degree C with 5% CO2 in humidified air in aliquots of 500 \mu l. Capacitated sperm (1 \times 10^6 in BWW and 0.3% BSA) were incubated at 37 \degree C with 5% CO2 in humidified air for varying times and in the presence of a range of concentrations of respective recombinant proteins in a total reaction volume of 100 \mu l. In order to account for the spontaneous induction of acrosome reaction, sperm were also incubated with BWW and 0.3% BSA alone. Post-incubation, the sperm were washed with 50 mM PBS pH 7.4, fixed in chilled methanol for 5 min and 25 \mu l aliquots were spotted on slides in duplicates. The spots were air-dried, stained with 5 \mu g/ml trimethylrhodamine isothiocyanate isothiocyanate conjugated \textit{Pisum sativum} agglutinin (TRITC–PSA; Vector Laboratories) for 30 min at room temperature. Any spermatozoa that demonstrated complete loss of PSA staining in the acrosome or revealed staining at the equatorial region was classified as acro-some-reacted. Sperm staining TRITC fluorescence in the acrosomal region of the head were classified as acrosome intact. All slides were read ‘blind’ with coded samples. Two hundred sperm were scored for every spot and the percentage of acrosome reaction was calculated by dividing the number of acrosome-reacted sperm by the total number of sperm counted and multiplying by 100. Progesterone (1 \mu g/ml; Sigma) was also assessed for its ability to induce acrosome reaction in capacitated spermatozoa. Calcium ionophore (10 \mu M, A23187; Sigma) served as a positive control in all the experiments. Each experiment was repeated at least three times by collecting semen samples from three individual donors. To understand the mechanism of action of recombinant proteins, capacitated spermatozoa were treated with Pertussis toxin (PTX; 1 \mu g/ml; Sigma), an inhibitor of G protein, for 30 min at 37 \degree C with 5% CO2 in humidified air prior to the addition of recombinant protein for the induction of acrosome reaction (Lee et al., 1992).

**Statistical analysis**

In the experiments pertaining to the induction of acrosome reaction, the results are expressed as mean \pm SEM of 3–4 different experiments using three male donors. The statistical analysis was done by comparing the means of the medium control (BWW and 0.3% BSA) and experimental sets or within two experimental groups by using one way analysis of variance (ANOVA) followed by Newman–Keuls Multiple Comparison Test. A P value of <0.05 was considered to be statistically significant.

**Results**

**Cloning, expression and purification of recombinant hZPA, hZPB and hZPC**

The hZPA, hZPB and hZPC were cloned in appropriate expression vectors and expressed as polylamda tagged fusion proteins, both in \textit{E. coli} as well as baculovirus-expression systems. The expressed proteins were purified using Ni-NTA affinity chromatography as described elsewhere (Kaul et al., 1997; Gahlay and Gupta, 2003). SDS-PAGE analysis of the purified \textit{E. coli}-expressed hZPA, hZPB and hZPC revealed bands of \sim 80, \sim 65 and \sim 50 kDa, respectively (Figure 1). Analysis of the expression of recombinant hZPA, hZPB and hZPC in baculovirus revealed that these are absent in the supernatant and are present only in the cell lysate (data not shown). The SDS-PAGE analysis and Western blots of the purified baculovirus-expressed hZPA, hZPB and hZPC reveal bands corresponding to \sim 110, \sim 70–75 and \sim 65 kDa, respectively (Figure 2). The \textit{E. coli} as well as baculovirus-expressed recombinant human zona proteins were analysed by an \textit{in vitro} lectin binding assay as well as by lectin blots to determine the nature of carbohydrate residues present on them. The baculovirus-expressed hZPB exhibited strong reactivity with Concanavalin A (Con A) and Jacalin, and weak reactivity with PSA as well as \textit{Dolichos biflorus} agglutinin (DBA) (Figure 3B). hZPA also showed a similar profile with additional binding to Soyabean agglutinin (SBA; Figure 3A). hZPC bound strongly to Con A and Jacalin, and weakly to Wheat germ agglutinin (WGA) and PSA (Figure 3C). While Con A, PSA and WGA have oligosaccharide specificity towards N-linked sugar residues, Jacalin, DBA and SBA detect the presence of O-linked carbohydrate moieties. A representative lectin blot of the recombinant proteins with Con A is shown in Figure 3D.

**Induction of acrosomal exocytosis by recombinant human zona proteins**

The \textit{E. coli}- and baculovirus-expressed recombinant hZPA, hZPB and hZPC were evaluated for their ability to induce acrosomal exocytosis in the human capacitated spermatozoa. Dose–response studies revealed that as low as 1 \mu g/ml (100 ng/reaction) of baculovirus-expressed hZPB as well as hZPC induced a significant increase in acrosome reaction (Figure 4A). No further increase in the acrosomal exocytosis was observed if the concentrations of...
hZPB and hZPC were increased beyond 20 μg/ml. Time-kinetics studies revealed that the significant induction of acrosomal exocytosis by hZPB and hZPC can be seen as early as 15 min after exposure of the capacitated spermatozoa to the recombinant protein (Figure 4B). The maximum acrosomal exocytosis was observed at 60 min, which shows a decline at 120 and 240 min. The results obtained in the presence of 20 μg/ml of the respective recombinant protein, when incubated with capacitated sperm for 60 min, are shown in Table I. Incubation of the capacitated sperm with E. coli-expressed hZPC did not induce significant increase (P > 0.05) in acrosomal exocytosis (16.01 ± 0.39%) as compared to the respective medium control (12.50 ± 1.70%; Table I). However, when capacitated sperm were incubated with baculovirus-expressed hZPC, a significant increase (38.99 ± 4.54%; P = 0.0005) in the acrosomal exocytosis was observed. 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 acrosome reaction (52.11 ± 2.75%). Baculovirus-expressed hZPC also induced a significant increase in the acrosome reaction (19.08 ± 1.70%; P = 0.0005) as compared to control (7.81 ± 0.83%; Table I). E. coli-expressed hZPB and baculovirus-expressed hZPA failed to induce any significant increase in the acrosomal exocytosis in capacitated spermatozoa (Table I). When the capacitated spermatozoa were incubated with a mixture of baculovirus-expressed hZPB (1 μg/100 μl) and hZPC (1 μg/100 μl), the induction of acrosome reaction was found to be 29.25 ± 4.42%, which is not statistically significant as compared to 22.06 ± 2.67% with hZPB (2 μg/100 μl) alone (P = 0.012) and 34.73 ± 4.14% with hZPC (2 μg/100 μl) alone (P = 0.020; Table I). However, induction of acrosome reaction with the combination of hZPB and hZPC was statistically significant as compared to the medium control (P = 0.0017).

**Effect of PTX on recombinant zona pellucida glycoprotein induced acrosome reaction**

The pre-incubation of capacitated human spermatozoa with PTX, at a concentration of 1 μg/ml for 30 min completely inhibited the induction of acrosome reaction mediated by hZPC (Table II). However, capacitated spermatozoa, when pre-incubated with PTX, failed to significantly inhibit the acrosomal exocytosis induced by either progesterone (P = 0.06535) or baculovirus-expressed hZPB (P = 0.6336; Table II).

**Discussion**

In mammals, acrosome reaction of the capacitated spermatozoa is critical for successful fertilization. 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 zona pellucida (Morales and Llanos, 1996). Both physiological and pharmacological agents have been implicated as inducers of acrosome reaction. While zona pellucida has been shown to be the physiological agonist of acrosome reaction in vivo in different species including human (Cross et al., 1988), progesterone and follicular fluid have also been shown to induce acrosome reaction (Tesarik, 1985; Osman et al., 1989). Among the pharmacological agents, calcium ionophore and ionomycin bring about acrosomal exocytosis in spermatozoa (Tesarik, 1985).

The zona pellucida glycoproteins from various species have been studied in great detail to delineate the component that serves as the primary sperm receptor and the pathway involved in acrosomal

Figure 4. Dose–response and time kinetics of baculovirus-expressed hZPB and hZPC induced acrosome reaction in capacitated human spermatozoa. Capacitated sperm (1 × 10^6/100 µl) were incubated with the respective recombinant protein at varying concentrations (Panel A) for 60 min, and subsequently analysed for acrosomal status by TRITC–PSA staining as described in Materials and methods. To optimize the time required for induction of acrosomal exocytosis, capacitated sperm (1 × 10^6/100 µl) were incubated with or without 2 µg/100 µl of hZPB or hZPC for varying time points (Panel B). The y-axis in Panels A and B represent effective induction of acrosome reaction in presence of the respective recombinant protein minus the percent of acrosome reaction observed in the presence of medium alone. Percent induction was calculated by dividing the number of acrosome reacted sperm by total number of sperm counted and multiplying by 100. Values are mean ± SEM of 3–4 different experiments using semen samples from at least three different male donors.

Table I. Ability to induce acrosome reaction in capacitated human sperm by recombinant human zona pellucida proteins

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Percent induction of acrosomal exocytosisa (mean ± SEM)</th>
<th>Statistical significanceb</th>
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<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (BWW and 0.3% BSA)</td>
<td>12.50 ± 1.70</td>
<td>P = 0.1145</td>
</tr>
<tr>
<td>E. coli-expressed hZPC</td>
<td>16.01 ± 0.39</td>
<td>P = 0.0055</td>
</tr>
<tr>
<td>Baculovirus-expressed hZPC</td>
<td>38.99 ± 4.54</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Calcium ionophore</td>
<td>52.11 ± 2.75</td>
<td></td>
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<tr>
<td>Group II</td>
<td></td>
<td></td>
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<tr>
<td>Control (BWW and 0.3% BSA)</td>
<td>7.81 ± 0.83</td>
<td>P = 0.0883</td>
</tr>
<tr>
<td>E. coli-expressed hZPB</td>
<td>10.26 ± 0.79</td>
<td>P = 0.0005</td>
</tr>
<tr>
<td>Baculovirus-expressed hZPB</td>
<td>19.08 ± 1.70</td>
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<tr>
<td>Group III</td>
<td></td>
<td></td>
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<tr>
<td>Control (BWW and 0.3% BSA)</td>
<td>5.42 ± 0.26</td>
<td>P = 0.1502</td>
</tr>
<tr>
<td>Baculovirus-expressed hZPA</td>
<td>6.47 ± 0.27</td>
<td></td>
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<tr>
<td>Group IV</td>
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</tr>
<tr>
<td>Control (BWW and 0.3% BSA)</td>
<td>9.04 ± 1.55</td>
<td></td>
</tr>
<tr>
<td>Baculovirus-expressed hZPB</td>
<td>22.06 ± 2.67</td>
<td>P = 0.0019</td>
</tr>
<tr>
<td>Baculovirus-expressed hZPC</td>
<td>34.73 ± 4.14</td>
<td>P = 0.0005</td>
</tr>
<tr>
<td>and hZPB</td>
<td>29.25 ± 4.42</td>
<td>P = 0.0017</td>
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</table>

^aCapacitated sperm were incubated with the recombinant proteins at a concentration of 2 µg/100 µl for 60 min. The concentration of baculovirus-expressed hZPB and hZPC used in the co-incubation experiments was 1 µg/100 µl. Calcium ionophore was used at a concentration of 10 µM.

^bPercent 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. *Statistical significance with respect to the medium control was calculated by one way ANOVA followed by Newmans–Keuls Multiple Comparison Test.

*Values are statistically significant.

Table II. Effect of PTX on the induction of acrosome reaction by hZPB, hZPC and progesterone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent induction of acrosomal exocytosis (mean ± SEM)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (BWW and 0.3% BSA)</td>
<td>12.42 ± 3.77</td>
<td>P = 0.0027*b</td>
</tr>
<tr>
<td>Baculovirus-expressed hZPC</td>
<td>43.03 ± 6.00</td>
<td>P = 0.1171*b</td>
</tr>
<tr>
<td>PTX and baculovirus-expressed hZPC</td>
<td>16.20 ± 3.22</td>
<td>P = 0.0024*c</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (BWW and 0.3% BSA)</td>
<td>9.19 ± 1.42</td>
<td>P = 0.0212*b</td>
</tr>
<tr>
<td>Baculovirus-expressed hZPB</td>
<td>20.82 ± 3.83</td>
<td>P = 0.0321*b</td>
</tr>
<tr>
<td>PTX and baculovirus-expressed hZPB</td>
<td>19.16 ± 4.06</td>
<td>P = 0.6336**</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
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</tr>
<tr>
<td>Control (BWW and 0.3% BSA)</td>
<td>5.36 ± 0.36</td>
<td>P = 0.047*b</td>
</tr>
<tr>
<td>Progesterone</td>
<td>14.58 ± 2.73</td>
<td>P = 0.043*b</td>
</tr>
<tr>
<td>PTX and progesterone</td>
<td>15.81 ± 3.45</td>
<td>P = 0.6535**</td>
</tr>
</tbody>
</table>

^aPercent 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.

*Statistical significance with respect to the medium control was calculated by one way ANOVA followed by Newmans–Keuls Multiple Comparison Test.

*Statistical significance with respect to the baculovirus-expressed hZPC, hZPB and progesterone in Group I, II and III, respectively.

*Values are statistically significant.
sexotyosis that follows this initial recognition event (Bleil and Wassarman, 1980; Beebe et al., 1992; van Duin et al., 1994; Prasad et al., 1996; Chapman et al., 1998; Yurewicz et al., 1998; Govind et al., 2001). To gain more insight into the role of zona pellucida glycoproteins during fertilization in humans, hZPA, hZPB and hZPC were cloned and expressed as polyhistidine-tagged fusion proteins in E. coli as well as baculovirus expression system. Expression of recombinant hZPA, hZPB and hZPC as polyhistidine-tagged fusion proteins allowed their convenient purification by Ni-NTA affinity column. In contrast to mammalian cells, failure to observe the secretion of baculovirus-expressed human zona proteins in the medium is in agreement with that observed by other investigators (Harris et al., 1999). The plausible reason for this phenomenon is not clear. It may be possible that the S2/1 cells lack furin-like enzymes and fail to cleave the transmembrane-like domain and hence, failure to secrete the expressed zona pellucida glycoproteins. On the SDS-PAGE, the E. coli- as well as baculovirus-expressed hZPA, hZPB and hZPC fusion proteins showed a slightly retarded mobility as is evident from their apparent molecular weights as compared to the theoretical values, which is sometimes the case with His<sub>6</sub>-fusion proteins (The QIAexpressionist, Qiagen, GmbH, Hilden, Germany). In case of the baculovirus expressed recombinant proteins, it may also be due to glycosylation. Lectin binding analysis indeed revealed the presence of both N- and O-linked glycosylation in the baculovirus-expressed recombinant proteins, which was absent in the E. coli-expressed proteins. All three recombinant human zona pellucida proteins expressed in baculovirus, revealed strong reactivity with Con A and Jacalin. While Con A has oligosaccharide specificity towards mannose α-1-3 or mannose α-1-6 residues, Jacalin binds to α-O glycosides of Gal or GaINAc moieties. In addition to Con A and Jacalin, baculovirus-expressed hZPB and hZPC showed weak binding to PSA and DBA while hZPC showed weak binding to WGA. Oligosaccharide specificity of PSA is α-linked mannose containing oligosaccharides, with an N-acetyllactosamino-linked α-fucose residue included in the receptor sequence. While DBA binds to α-N-acetylgalactosamine residues, WGA has oligosaccharide specificity towards GlcNAc and neuraminic acid residues. In earlier studies, mannose has been implicated to play an important role in the sperm receptor activity (Mori et al., 1989; Cornwall et al., 1991; McLeskey et al., 1998; Amari et al., 2001). Glycosides of galactose have also been suggested to be critical for imparting zona pellucida with the sperm receptor function (Bleil and Wassarman, 1988). In the present study, induction of acrosome reaction by baculovirus-expressed hZPC and its failure by E. coli-expressed hZPC, reiterate that glycosylation of the hZPC is essential for induction of acrosome reaction. However, this is in contrast to an earlier report where hZPC expressed in E. coli has been shown to induce acrosome reaction in capacitated spermatozoa (Chapman et al., 1998). However, the E. coli-expressed hZPC mediated induction of acrosome reaction was observed only after 18 h of exposure of sperm to the recombinant protein (Chapman et al., 1998), in contrast to 1 h in our studies.

The studies so far have shown that glycosylated hZPC obtained by the mammalian expression system induces acrosome reaction in human spermatozoa (van Duin et al., 1994; Brewis et al., 1996; Dong et al., 2001; Bray et al., 2002). The present study demonstrates that the hZPC produced by the insect cells can also induce acrosomal exocytosis in capacitated human spermatozoa. Further investigations are needed to understand the differences in the nature and extent of glycosylation of hZPC produced in the baculovirus and by mammalian expression system.

The dose–response results in the present study indicate that at little as 1 μg/ml of recombinant hZPB as well as hZPC are sufficient to induce a significant acrosome reaction in capacitated human sperm, though the maximum induction of acrosome reaction was observed at 20 μg/ml. Earlier reports using recombinant hZPC have also demonstrated that 5–20 μg/ml of the recombinant protein is required to induce significant acrosomal exocytosis (van Duin et al., 1994; Chapman et al., 1998). The amount of recombinant hZPC needed for inducing acrosome reaction in human sperm in an in vitro system far exceeds the amount that is present in vivo where the zona pellucida surrounding a single human oocyte consists of about 5 ng of ZPC (van Duin et al., 1994). The reason for this difference may be the presence of other factors in the milieu of the female reproductive tract that act in synergy with the zona proteins to bring about acrosomal exocytosis in the sperm. The observed ability of the recombinant hZPB and hZPC to induce acrosome reaction is not due to post-mortem acrosomal loss as no change either in the sperm motility or in the sperm viability was observed when the sperm were incubated with the recombinant proteins (unpublished observations).

The time-kinetics studies revealed that the acrosome reaction in human sperm can be observed as early as 15 min post-exposure to recombinant hZPB and hZPC and reaches a maximum at about 60 min. Subsequently, an increase in the spontaneous acrosome reaction in the presence of medium alone was responsible for decrease in the effective induction of acrosome reaction observed at 120 and 240 min in the presence of hZPB as well as hZPC. The tendency of spermatozoa to undergo increased spontaneous acrosome reaction, when they have been incubated for prolonged periods in vitro has been documented (van Duin et al., 1994).

The baculovirus-expressed hZPA expressed in baculovirus failed to induce acrosomal exocytosis in capacitated spermatozoa in spite of sharing the nature of glycosylation with baculovirus-expressed hZPC and hZPB. This observation suggests that the polypeptide backbone of the protein may not be completely dispensable for induction of acrosome reaction. It may be possible that the polypeptide backbone of hZPC or hZPB facilitates appropriate disposition of critical sugar residues that are important for induction of acrosome reaction. It has also been shown that E. coli-expressed recombinant hZPA binds to acrosome-reacted but not to capacitated human spermatozoa (Tsabamoto et al., 1999). In the mouse model also, ZPA has been assigned the secondary sperm receptor function which maintains the binding of acrosome-reacted sperm to oocytes after the primary receptor recognition on the sperm surface has been established by ZPC (Bleil et al., 1988).

In the present study, for the first time, it has been shown that hZPB expressed in baculovirus also has the ability to induce acrosomal exocytosis in capacitated human spermatozoa whereas the E. coli-expressed protein fails to do so. In mouse, ZPB has been assigned only a structural role (Greve and Wassarman, 1985). However, in rabbit, porcine and bonnet monkey, ZPB has been shown to bind to homologous spermatozoa (Prasad et al., 1996; Yurewicz et al., 1998; Govind et al., 2001). The results of this study show that ZPB may also have a role to play in acrosomal exocytosis in humans. The observations that hZPB does not act synergistically with hZPC may be due to the possibility that both the proteins either recognize same receptor on the spermatozoa or soluble hZPB and hZPC cannot bind simultaneously to spermatozoa due to steric hindrance. Observations that PTX, an inhibitor of G<sub>i</sub> protein mediated signalling pathway, did not inhibit the hZPB induced acrosome reaction while the hZPC mediated induction was completely abolished as also shown in earlier studies (McLeskey et al., 1998), suggest that it is unlikely that both the proteins bind to the same receptor. The induction of acrosome reaction mediated by recombinant hZPB, thus, seems to follow a similar mechanism as that of...
progesterone, which has a G protein independent pathway (Tesarak et al., 1993). These observations suggest that hZPB and hZPC may follow different downstream signalling mechanisms to bring about the induction of acrosome reaction. Additional studies need to be carried out to investigate the precise mechanism involved in hZPB mediated acrosomal exocytosis in human spermatozoa. The present finding that hZPB can also induce capacitated spermatozoa to undergo acrosome reaction opens new avenues with respect to the functions of the zona pellucida glycoproteins at different stages of sperm–oocyte interaction, and of the interplay between the various glycoproteins present in the zona matrix.

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References


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