The polypeptide backbone of recombinant human zona pellucida glycoprotein-3 initiates acrosomal exocytosis in human spermatozoa *in vitro*

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Human gamete interaction is of fundamental biological importance, yet the molecular interactions between spermatozoa and the zona pellucida are poorly understood. Surprisingly, the role of the polypeptide backbone of zona pellucida glycoprotein 3 (ZP3), the putative ligand for spermatozoa activation, has been largely overlooked. Purified recombinant human ZP3 was expressed in *Escherichia coli* as a C-terminal fusion to the dimeric glutathione *S*-transferase (GST) from *Schistosoma japonicum*

and was shown to induce acrosomal exocytosis in live, capacitated human spermatozoa. The level of exocytosis is comparable with that obtained using purified, glycosylated, recombinant human ZP3 [van Duin, M., Polman, J. E. M., DeBreet, I. T. M., Van Ginneken, K., Bunschoten, H., Grootenhuys, A., Brindle, J. and Aitken, R. J. (1994). *Biol. Reprod.* **51**, 607–617]. These data imply that the polypeptide chain of human ZP3 contributes to recognition of spermatozoa during acrosomal exocytosis *in vitro*.

INTRODUCTION

Fertilization is the most important cell adhesion event in the life time of any living organism that reproduces by sexual means. Furthermore, in a diverse range of organisms (from echinoderms to mammals), the recognition of carbohydrate epitopes by complementary protein receptors has been proposed to be a critical factor in gamete interaction [1–3].

At the molecular level, the survival of an organism can depend upon the success of interactions between proteins and carbohydrates. For example, lectin-containing adhesion molecules termed selectins, resident on both vascular endothelium and leucocytes, are vital for the phenomenon of leucocyte rolling and extravasation into damaged tissue during the inflammatory response [4]. Conversely, protein–carbohydrate interactions may also bring an untimely end to the organism's existence. Examples of this include the binding of some viral particles (influenza) and bacterial toxins (cholera toxin from *Vibrio cholerae* and pertussis toxin from *Bordetella pertussis*) to the plasma membrane of eukaryotic cells that is mediated by protein–carbohydrate interactions (microbial recognition of target-cell glycoconjugates has been reviewed in [5]). However, protein–protein interactions also provide fundamental triggers for a great number of inter- and intra-cellular signalling processes, perhaps the best characterized example being that of the growth hormone–growth hormone receptor complex [6].

A comprehensive understanding of the molecular mechanism of human gamete interaction is a pre-requisite for the development of novel methods of contraception aimed at blocking fertilization. In mammals such as mice and pigs, it is generally accepted that carbohydrate epitopes displayed by zona pellucida glycoprotein 3 (ZP3) play a pivotal role in spermatozoa–ZP3 interaction [2,7]. However, although both native human zona pellucida and purified, glycosylated recombinant human ZP3 (rhuZP3) secreted by Chinese Hamster Ovary (CHO) cells have been shown to stimulate acrosomal exocytosis in human spermatozoa [8–11], it has yet to be firmly established whether the ability

to initiate this exocytotic event resides in the carbohydrate moieties of the ZP3 glycoprotein alone, the polypeptide backbone of ZP3 or elements of both. One method to examine the role of the protein backbone in gamete interaction has been to deglycosylate zona proteins either enzymatically or chemically [2,7]. Although both approaches successfully remove carbohydrate from the polypeptide, there are a number of limitations in such experiments. For example, enzymatic removal of carbohydrate can often be incomplete [12] and moreover chemical deglycosylation employs harsh conditions which may modify the polypeptide backbone itself [2]. Recombinant proteins expressed in *Escherichia coli* are free from carbohydrate modifications and therefore provide a homogenous molecular population for the experimental analysis of ZP3 interacting with spermatozoa.

MATERIALS AND METHODS

General reagents

The reagents used for the following procedures were of the highest standard commercially available and were obtained from Sigma (Poole, Dorset, U.K.), Fisher (Loughborough, Leics., U.K.) and BDH (Lutterworth, Leics., U.K.). Restriction enzymes were from New England Biolabs (Beverly, MA, U.S.A.) or MBI Fermentas (Vilnius, Lithuania). Glutathione–agarose affinity chromatography resins, polyclonal rabbit anti-*S. japonicum* GST antiserum and FITC-labelled goat anti-mouse IgG antiserum were purchased from Sigma. Hybond-ECLTM nitrocellulose membranes, horseradish peroxidase conjugated to donkey-anti-rabbit immunoglobulins and ECL Western blotting detection reagents were supplied by Amersham International (Slough, Bucks., U.K.). *E. coli* culture media were supplied by Oxoid and Difco Laboratories. Mouse monoclonal antibody ab 18.6 was a kind gift from Professor Harry Moore (University of Sheffield, U.K.). Rabbit anti-porcine ZP3 antiserum was a kind gift of Professor A. G. Sacco (University of Michigan, U.S.A.); this antiserum had been raised against purified porcine ZP3 and had

Abbreviations used: IPTG, isopropyl- β -D-thiogalactopyranoside; OD₅₉₅, optical density at 595 nm; rhuZP3, recombinant human ZP3; GST-HuZP3, glutathione *S*-transferase-recombinant human ZP3 fusion protein; TTB, Towbin Transfer Buffer.

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Figure 1. pHuZP3-GEX, the plasmid used for expression of recombinant human ZP3

Expression of GST-HuZP3 is under the control of the IPTG-inducible *tac* promoter. Positions in base pairs (position 1 is 118 bp upstream from the start of the *tac* sequence) of unique restriction sites are given.

been shown to cross-react strongly with both intact human zonae pellucidae [13] and purified recombinant human ZP3 [14]. The Hamilton–Thorne motility analyser (HTM model 2030; Hamilton–Thorne Research Inc, Danvers, MA, U.S.A.) was used to assess spermatozoa motility before and after the following experimental procedures (see Clements et al. [15] for HTM set up and quality control measures).

Expression and purification of GST-HuZP3 in *E. coli*

The cDNA encoding the full length human ZP3 [16] was a generous gift from Professor Jurrien Dean (NIDDK). The Polymerase Chain Reaction (PCR) was employed to engineer convenient restriction sites at the 5' and 3' ends of ZP3 open reading frame to facilitate insertion of the PCR product into the expression vector pGEX-KG [17] forming the expression plasmid pHuZP3-GEX. Oligonucleotide primers were synthesized on an Applied Biosystems 392 DNA synthesizer (Perkin–Elmer, Warrington, Cheshire, U.K.). Primer sequences were as follows:

HuZP3.1:

5'-CGCGAATTCGGATGGAGCTGAGCTATTA-3'
(*EcoRI* site bold-italicized).

HuZP3.2:

5'-GCGCCAAGCTTTTATTCGGAAGCAGAC-3'
(*HindIII* site bold-italicized).

PCR reactions were carried out for 30 cycles of: 94 °C for 45 s; 52 °C for 2 min and 72 °C for 2.5 min on a Techne PHC-2 thermal cycler. The PCR products were digested with 10 U of both *EcoRI* and *HindIII*, purified from an agarose gel using GeneClean (Anachem, Luton, U.K.) and were ligated into pGEX-KG (cut with the same enzymes) using 40 U of T4 DNA ligase at 16 °C overnight. pHuZP3-GEX is shown schematically in Figure 1.

pHuZP3-GEX was used to transform *E. coli* DH5αMCR (Genotype: F[−] *endA1 supE44 thi-1λ[−] recA1 gyrA96 relA1 deoR Δ(lacZYA-argF)U169 φ80dlacZM15 mcrA Δ[mrr hsdRMS mcrBC]*) [18,19].

Single colonies of *E. coli* DH5αMCR harbouring pHuZP3-GEX were then used to inoculate 5 ml M9 medium which was then incubated overnight at 37 °C. M9 minimal medium was

prepared as follows: M9 salt solution: 49 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.5 mM NaCl and 7.57 mM (NH₄)₂SO₄. The pH of the salt solution was adjusted to 7.35 with 10 M NaOH and then autoclaved. Stock solutions of MgSO₄ (1.0 M) and CaCl₂ (0.5 M) were autoclaved separately and were used at working concentrations of 1 mM and 0.5 mM, respectively. Thiamine (vitamin B₁) was prepared as a stock solution of 10 mg/ml, filter sterilized through a 0.22 μm sterile acrodisc filter (Gelman Sciences) and then used at a working concentration of 0.05 mg/ml. D-Glucose was used as the carbon source at a working concentration of 1.5% (w/v). Ampicillin at a final concentration of 200 μg/ml was added to all cultures. All solutions were made up using MQ water. The following day an overnight 5 ml culture was used to inoculate 50 ml of M9 medium. This culture was again grown overnight at 37 °C, then subsequently used to inoculate 1 l of M9 medium. This culture was allowed to attain an OD₅₉₅ of 0.35 before induction of glutathione *S*-transferase-recombinant human ZP3 fusion protein (GST-HuZP3) expression was initiated by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The cells were then grown for a further 3.5 h at 37 °C. Control cultures of non-transformed *E. coli* DH5αMCR were grown in parallel. IPTG was also added to these cultures at the same stage of growth.

Cells were harvested by centrifugation at 17000 *g* for 10 min at 4 °C in a Beckman J2-HS centrifuge (JA-10 rotor), spent medium was aspirated and the cell pellet re-suspended in 6 ml phosphate buffered saline (PBS pH 7.4; 136 mM NaCl, 2.68 mM KCl, 1.76 mM KH₂PO₄ and 10.14 mM Na₂HPO₄). A cocktail of protease inhibitors was then added (final concentrations given in parentheses) to this cell suspension; these included leupeptin (100 μM), PMSF (200 μM) and EDTA (1 mM). The cell suspension was then lysed by sonication and Triton X-100 was added at a final concentration of 1% (v/v). The mixture was then gently vortexed and incubated for a further 25 min on ice. Soluble and insoluble fractions were then separated by centrifugation at 12100 *g* for 10 min at 4 °C in a Beckman J2-HS centrifuge (JA-20 rotor). The soluble fraction was then applied to a glutathione–agarose affinity matrix to purify GST-HuZP3 as detailed in [20].

SDS/PAGE and Western analysis

The purity of GST-HuZP3 eluted from the affinity matrix was determined by 10% SDS/PAGE [18] and the identity of the protein determined by Western analysis. The gels used a discontinuous buffer system with a 5% stacking gel and 10% resolving gel. Protein samples (usually 5 μl) from crude cell lysates (both transformed and non-transformed) were diluted 1:6 with SDS loading buffer (prepared to a final volume of 24.0 ml: 3.0 ml 500 mM Tris pH 6.8, 2.4 ml glycerol, 4.8 ml 10% (w/v) SDS, 1.2 ml β-mercaptoethanol, 600 μl 0.05% (w/v) bromophenol blue and 12.0 ml MQ water). Column samples were diluted with SDS loading buffer to obtain equivalent protein concentrations and were then boiled for 10 min, briefly centrifuged (in a benchtop microcentrifuge) to collect condensed water and then between 10 and 40 μl were electrophoresed at 100 V (40 mA) until the dye front had migrated from the resolving gel. Gels were then either stained immediately in Coomassie blue (450 ml methanol/distilled water (1:1 (v/v)), 50 ml glacial acetic acid and 1.25 g Coomassie brilliant blue R250) or electroblotted on to Hybond-ECL[®] nitrocellulose as described below. Proteins were subsequently visualized after destaining the gel overnight in destain (30:10:60 methanol:glacial acetic acid:water). Gels were calibrated with high molecular mass standards obtained from Sigma.

The amount of purified recombinant protein recovered after each induction was determined using a Bio-Rad Protein Assay kit. The protocol was based on the method of Bradford [21] and was supplied by the manufacturer. A standard curve was prepared using BSA as the standard. The yield of GST-HuZP3 was $\sim 120 \mu\text{g/l}$ of culture medium.

For Western analysis both the gels and nitro-cellulose membranes were first equilibrated in Towbin Transfer Buffer (TTB; 25 mM Tris pH 7.4, 192 mM glycine and 20% (v/v) methanol) for 15 min. Proteins were then transferred to nitro-cellulose membrane (Hybond-ECLTM; Amersham International, U.K.) for 30 min at 9 V (constant current) using a Trans Blot SDTM Semi-Dry Transfer Cell (Bio-rad). Once the gel had been blotted, the membrane was washed (all subsequent washes and incubations were carried out at room temperature) for 15 min in Tris Buffered Saline (TBS; 20 mM Tris pH 7.4 and 500 mM NaCl) and then blocked in 10% blocking buffer (TTBSM; TBS containing 0.05% (v/v) Tween-20 and 10% (w/v) low fat dried milk powder (Boots pharmaceuticals)) for two 30 min sessions. Excess TTBSM was removed from the membrane with thorough washing in TTBS (TTBSM without the milk powder) for a total time of 35 min (15 min plus 4 $\times$ 5 min).

The membrane was then probed for the presence of GST-HuZP3 with either a rabbit antiserum raised against porcine ZP3 (1:1000 dilution in TTBS for 1 h) or rabbit anti-*Schistosoma japonicum* GST antiserum (1:1000 dilution in TTBS for 1 h) (hereafter these antisera are referred to as the 'primary ab'). Excess primary ab was then removed by washing in TTBS as described above. The membrane was probed for the presence of rabbit immunoglobulins with a 1:2000 dilution of secondary ab (a horseradish peroxidase-linked donkey antiserum raised against rabbit IgG) in TTBS for 45 min. Excess secondary ab was then removed by washing in TTBS as described above. The membrane was then quickly rinsed in TBS and then developed according to the ECL detection protocol described by the manufacturer (Amersham).

Preparation of spermatozoa

Human semen samples were obtained from healthy, fertile donors attending the University Clinic at the Jessop Hospital for Women, Sheffield, U.K. Motile spermatozoa were isolated using a direct swim-up technique into Earle's balanced salt solution (EBSS; Gibco-BRL Paisley, Scotland) supplemented with 3.5% (w/v) human serum albumin (HSA) as described in [14]. The motility and concentration of the spermatozoa in the swim-up sample and throughout the experiments described in this paper were examined on a Hamilton-Thorne motility analyser. The percentage of motile spermatozoa in the swim-up sample was 94 ± 2 (mean $\pm$ S.E.M.). The spermatozoa were then capacitated *in vitro* for 3 h at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. After this time, spermatozoa were centrifuged at 500 g for 6 min at room temperature in a MSE Mistral 2000 centrifuge. Spent medium was discarded and the pellet then resuspended in fresh EBSS supplemented with 0.3% (w/v) HSA and the spermatozoa concentration adjusted to $10 \times 10^6/\text{ml}$. These spermatozoa were then used to assess the biological activity of GST-HuZP3.

Induction of acrosomal exocytosis in human spermatozoa by GST-HuZP3

Separate 200 μl aliquots of spermatozoa (prepared as detailed above) were challenged with either EBSS (supplemented with 0.3% (w/v) HSA), or 10 μM A23187 (a bivalent Ca²⁺ ionophore

which was prepared fresh each day as follows: A frozen stock solution of 10 mM A23187 in dimethyl sulphoxide (DMSO) was diluted to 1 mM with EBSS. The latter would then be diluted to a final concentration of 10 μM upon addition to the spermatozoa-containing medium), or GST-HuZP3 (5 ng/ μl ; final concentration; the amount of ZP3 resident in a human zona pellucida has been estimated at $\sim 5 \text{ ng/zona}$ [11]) or GST-M.*MspI* (5 ng/ μl ; final concentration) for 3 h and 18 h at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Affinity-purified GST-M.*MspI* (prepared as detailed for GST-HuZP3 but using the M.*MspI* expression vector pGEX-M.*MspI*_{cas} supplied by Dr. Paul Hurd [22]; data not shown) was an ideal control fusion protein for use in the following experiments to determine the level of non-specific acrosomal exocytosis induced by a non-physiological agonist (GST-M.*MspI* is of similar molecular mass to GST-HuZP3; $\sim 72 \text{ kDa}$). Spermatozoa were also challenged with heat-denatured (samples incubated at 100 °C for 15 min) GST-HuZP3 and GST-M.*MspI* to investigate whether the tertiary structure of GST-HuZP3 was necessary for induction of acrosomal exocytosis in human spermatozoa.

Viability and acrosomal status of spermatozoa

Viability and acrosomal status of spermatozoa was evaluated simultaneously using the hypo-osmotic swelling (HOS) test and indirect immunofluorescence using a mouse anti-hamster primary monoclonal antiserum (mab 18.6). This was essentially as detailed in reference [23], with the sole modification being that mab 18.6 was used neat in the present study and not as a 1:4 dilution. The HOS test facilitates discrimination between live and moribund spermatozoa on the basis that live cells have swollen, coiled tails, dead spermatozoa have straight tails [23]. Mab 18.6 is an antiserum that specifically binds both to an antigen within the inner face of the outer acrosomal membrane and acrosomal content. In fixed spermatozoa one observes a decrease in fluorescence intensity over the spermatozoon acrosome due to the loss of the antigen-mab 18.6 complex as acrosomal exocytosis proceeds [24]. Using a fluorescence microscope (Laborlux S, Leitz) at $\times 1000$ magnification spermatozoa were scored as follows: (1) viable, acrosome-intact (uniform fluorescence over the acrosome region with coiled tails); (2) viable acrosome-reacted (fluorescence over the equatorial segment or no fluorescence with coiled tails); (3) non-viable, acrosome-intact (uniform fluorescence over the acrosome region with straight tails); (4) non-viable acrosome-reacted (fluorescence over the equatorial segment or no fluorescence with straight tails).

Viable spermatozoa exhibiting patchy fluorescence were scored as partially acrosome-reacted but were discounted from the data analysis in this study. At least 100 spermatozoa were scored for each agonist and all experiments were done blind with the slides being coded to avoid any bias in data interpretation. The percentages of motile spermatozoa at the end of the 3 and 18 h incubation periods were 92 ± 2 and 76 ± 9 (mean $\pm$ S.E.M.), respectively.

Statistical analysis

Results from at least seven experiments using different fertile donors are presented as mean percentage of live acrosome-reacted spermatozoa $\pm$ S.E.M. To calculate the significance of any differences observed between the means of each spermatozoa/agonist sample at each time point, paired *t* tests were performed after the data had firstly been transformed using the following equation: $\log(x+0.5)/(500-x)$ to normalize percentage data (such as the acrosome reaction data) [23]. In this equation *x* represents the mean percentage of live, acrosome-reacted sperma-

tozoa observed with a given agonist after a set time course. A result was considered significant when $P < 0.05$.

RESULTS

Expression and purification of GST-HuZP3

Figure 2A shows an SDS gel analysis of expression and purification of GST-HuZP3 in *E. coli* DH5 α MCR grown in M9 medium. A protein corresponding to the M_r of GST-HuZP3 (72000) can clearly be seen in lane 8 following purification on glutathione-agarose. In addition, a second band of ~ 26 kDa co-purifies with this species. This band was identified as *S. japonicum* GST by protein sequence analysis. The results of this sequence analysis are given in Table 1. The sequence obtained is identical to the N-terminal sequence of *S. japonicum* GST (M-S-P-I-L-G-Y-W-K).

To verify that the molecular species observed in lane 8 (Figure 2A) was GST-HuZP3, Western analysis was performed as described in materials and methods. Figure 2B illustrates the Western blot probed with polyclonal anti-GST antiserum as the primary ab. Two bands show strong cross-reactivity with this antibody in lane 8. The high molecular weight band is GST-HuZP3, while the stronger reacting, lower molecular weight species corresponds to GST. Figure 2C shows an identical Western blot probed with anti-porcine ZP3 antiserum. A cross-reacting species corresponding to GST-HuZP3 is observed in lane 8 which is not present in the control fractions, see lanes 2 and 3. No cross-reactivity was observed against GST with this antiserum. It can be clearly seen that the vast majority of GST-HuZP3 expressed in *E. coli* partitions into the insoluble phase as judged by the presence of a prominent band of ~ 72 kDa in lane 5 (the induced, insoluble fraction from *E. coli* DH5 α MCR harbouring pHuZP3-GEX) and not the control insoluble fraction (lane 3). The inherent insolubility of human ZP3 has proved to be a major obstacle in purifying milligram quantities of this protein. A single band of ~ 60 kDa is present in lanes 2–6 of Figure 2C. This is an *E. coli* protein that cross-reacts with this anti-porcine antibody. The identity of this protein was not determined in the present study.

It is clear that there is strong cross reactivity between both the rabbit anti-porcine ZP3 and rabbit anti-*S. japonicum* GST

Table 1 N-terminal amino acid composition of the 26 kDa band as determined by automated Edman degradation

Two bands have been sequenced (labelled major and minor). The sequence of the former is in strong agreement with the database N-terminal sequence for the first nine amino acid residues of *S. japonicum* GST (M-S-P-I-L-G-Y-W-K). The latter is thought to represent an N-terminally truncated GST polypeptide since it lacks both the Met and Ser residues present at positions 1 and 2 in the major band sequence. N.A., not available; ? = identity of residue not determined at all or (when with identity letter) not determined unambiguously.

Major band			Minor band		
Residue	Amino acid	Amount (pmol)	Residue	Amino acid	Amount (pmol)
1	M	3.07	1	?	N.A.
2	S	1.82	2	P?	1.04
3	P	2.19	3	I	0.75
4	I	1.87	4	L	1.18
5	L	2.30	5	G	2.75
6	G	3.87	6	Y	1.06
7	Y	1.95	7	?	N.A.
8	W?	0.57	8	K	0.66

Figure 2. Analysis of expression of and purification of ZP3 by SDS/PAGE and Western blotting

A. Lane 1, Molecular mass markers; lane 2, induced, soluble extract from non-transformed *E. coli* DH5MCR; lane 3, as (2) but the insoluble fraction; lane 4, induced, soluble extract from *E. coli* DH5MCR harbouring pHuZP3-GEX; lane 5, as (4) but the insoluble fraction; lane 6, flow-through from glutathione-agarose column; lane 7, column wash in 50 mM Tris/HCl pH 8.0; lane 8, specific elution from glutathione-agarose affinity column using 50 mM Tris/HCl per 10 mM reduced glutathione pH 8.0. Identical gels to that presented in **A** were electroblotted and the blots probed for the presence of GST-HuZP3 with either a rabbit anti-*S. japonicum* GST antiserum (1:1000 dilution in TTBS; **B**) or a rabbit anti-porcine ZP3 antiserum (1:1000 dilution in TTBS; **C**) as detailed in materials and methods. The upper arrow on the right of each figure indicates the position of GST-HuZP3 (~ 72 kDa); the lower arrow on the right of **A** and **B** indicates the position of recombinant *S. japonicum* GST (~ 26 kDa). The molecular mass standards are indicated where visible.

antisera and a number of lower molecular weight species which may derive from GST-HuZP3 (Figure 2B and 2C, lanes 4 and 5). The evidence for this is that these cross reacting species are exclusive to lanes containing the insoluble fractions from strains

Table 2 Ability of GST-HuZP3 to induce acrosomal exocytosis in alive, capacitated human spermatozoa

Spermatozoa were capacitated for 3 h and then incubated for a further 3 h with EBSS, 10 μ M A23187, 5 ng/ μ l GST-HuZP3 or 5 ng/ μ l GST-M.*MspI* as described in materials and methods. Values given are means $\pm$ S.E.M. n = number of semen samples from fertile donors; one ejaculate from each donor. No significant difference was observed in the level of acrosomal exocytosis in spermatozoa challenged with GST-HuZP3 compared to EBSS and GST-M.*MspI* ($P > 0.05$).

Agonist	n	% live acrosome-reacted
EBSS	10	8.9 $\pm$ 2.29
10 μ M A23187	10	58.3 $\pm$ 4.72
5 ng/ μ l GST-HuZP3	10	11.8 $\pm$ 2.4
5 ng/ μ l GST-M. <i>MspI</i>	10	7.0 $\pm$ 1.15

Table 3 Ability of GST-HuZP3 to induce acrosomal exocytosis in live, capacitated human spermatozoa

Spermatozoa were capacitated for 3 h and then incubated for a further 18 h with EBSS, 10 μ M A23187, 5 ng/ μ l GST-HuZP3 or 5 ng/ μ l GST-M.*MspI* as described in materials and methods. Values given are means $\pm$ S.E.M. n = number of semen samples from fertile donors; one ejaculate from each donor. Significantly greater levels of acrosomal exocytosis were observed in spermatozoa challenged with GST-HuZP3 compared to EBSS ($*P < 0.001$) and GST-M.*MspI* ($**P < 0.01$).

Agonist	n	% live acrosome-reacted
EBSS	10	16.5 $\pm$ 4*
10 μ M A23187	10	65.1 $\pm$ 4.4
5 ng/ μ l GST-HuZP3	10	58.3 $\pm$ 4*, **
5 ng/ μ l GST-M. <i>MspI</i>	10	30.6 $\pm$ 7.74**

harbouring the pHuZP3-GEX plasmid (but not strains that had not been transformed).

Induction of acrosomal exocytosis in human spermatozoa by GST-HuZP3

No significant difference in the level of acrosomal exocytosis was observed between spermatozoa challenged with EBSS, GST-HuZP3 or GST-M.*MspI* for three hours (Table 2; 8.9 $\pm$ 2.29, 11.8 $\pm$ 2.40 and 7.0 $\pm$ 1.15%, respectively, $P > 0.05$; $n = 10$). This suggests that the HuZP3 moiety of the GST fusion protein was unable to initiate a physiological acrosome reaction above control levels within this time course. Furthermore, acrosome reactions induced with GST-HuZP3 were significantly less than those induced using the ionophore A23187 (Table 2; 11.8 $\pm$ 2.40 vs. 58.3 $\pm$ 4.72%; $P < 0.01$; $n = 10$). Studies using either purified recombinant glycosylated human ZP3 or purified non-glycosylated human ZP3 indicate that prolonged periods of incubation with ZP3 are necessary to induce significant levels of acrosomal exocytosis [11,14]. In order to determine if longer incubation times with the GST-HuZP3 were necessary to induce maximal stimulation (similar to levels obtained using the ionophore) spermatozoa were incubated for 18 h with GST-HuZP3 (5 ng/ μ l; final concentration); the amounts and nature of control agonists were as for the 3 h incubation. Spermatozoa incubated with GST-HuZP3 for 18 h were seen to have undergone significantly higher levels of acrosomal exocytosis to those observed for both the EBSS control (Table 3; 58.3 $\pm$ 4.0 vs. 16.5 $\pm$ 3.22%; $P < 0.01$;

Table 4 Ability of boiled GST-HuZP3 to induce acrosomal exocytosis in live, capacitated human spermatozoa

Spermatozoa were capacitated for 3 h and then incubated for a further 18 h with 5 ng/ μ l GST-HuZP3, 5 ng/ μ l boiled GST-HuZP3, 5 ng/ μ l GST-M.*MspI* or 5 ng/ μ l boiled GST-M.*MspI* as described in materials and methods. Values given are means $\pm$ S.E.M. n = number of semen samples from fertile donors; one ejaculate from each donor. A significantly reduced level of acrosomal exocytosis was observed in spermatozoa challenged with boiled GST-HuZP3 compared to non-boiled GST-HuZP3 ($*P < 0.05$). No significant difference in the number of acrosome reactions was recorded in live, capacitated human spermatozoa challenged with boiled GST-HuZP3, boiled GST-M.*MspI* or non-boiled GST-M.*MspI* ($P > 0.05$).

Agonist	n	% live acrosome-reacted
5 ng/ μ l GST-HuZP3	7	60.14 $\pm$ 5.68
5 ng/ μ l boiled GST-HuZP3	7	34.57 $\pm$ 7.69*
5 ng/ μ l GST-M. <i>MspI</i>	7	39.7 $\pm$ 7.74
5 ng/ μ l boiled GST-M. <i>MspI</i>	7	32.43 $\pm$ 8.06

$n = 10$) and the GST-M.*MspI* control (Table 3; 58.3 $\pm$ 4.0 vs. 30.6 $\pm$ 7.74%; $P < 0.01$; $n = 10$). Together these data strongly suggest that the polypeptide backbone of the HuZP3 moiety in the GST-HuZP3 fusion protein is biologically active and able to elicit acrosomal exocytosis in live, capacitated human spermatozoa.

The response of live, capacitated spermatozoa challenged with GST-HuZP3 for 18 h could be reduced to control levels (i.e. that observed for either heat inactivated GST-HuZP3, heat inactivated GST-M.*MspI* or GST-M.*MspI*) if this fusion protein was heat-denatured (Table 4; 34.57 $\pm$ 7.69% for boiled GST-HuZP3, 32.43 $\pm$ 8.06% for boiled GST-M.*MspI*, 39.7 $\pm$ 7.74% for GST-M.*MspI* and 60.14 $\pm$ 5.68% for GST-HuZP3, $P < 0.05$; $n = 7$).

DISCUSSION

Expression of GST-HuZP3 in M9 minimal medium

This study demonstrates that for the first time that human ZP3 can be expressed and purified from *E. coli*. A number of low molecular weight fragments which may derive from GST-HuZP3 are seen only in the lysate of *E. coli* DH5 α MCR harbouring pHuZP3-GEX. The presence of multiple gene products from pHuZP3-GEX could arise for three reasons: (a) multiple initiations of translation on the mRNA, (b) partial proteolysis of the GST-HuZP3 and (c) contributions from both (a) and (b) [25].

The generation of multiple gene products associated with expression of heterologous genes in *E. coli* has been previously documented [25,26]. The reason(s) for this phenomenon are unclear but it is thought that the small sections of the nucleotide sequence within the mRNA of a given eukaryotic protein resemble those sequences utilized by prokaryotes when initiating synthesis of nascent polypeptides [25]. Such a sequence is the purine-rich Shine–Delgarno (SD) sequence (which is found within the ribosome binding site (RBS)) located approx. 5–13 nucleotides upstream of the initiating methionine codon [27]. This stretch of nucleotides is complementary to the 3' end of the 16S rRNA that makes up the ribosomes within a prokaryotic cell. Examination of the HuZP3 cDNA reveals that a SD sequence (5'-AGGAGG-3') occurs at two locations throughout the HuZP3 mRNA (positions 1108–1113; 5'-AGGAGGCCACUCUGAUG-3' and 1968–1973; 5'-AGGAGGCCAGCCUCAUGUCAUG-3'). Both stretches of nucleotides are complementary to the 3' end of the 16S rRNA (3'-AUUCCUCCACCA-5') and are located 8 and

12 nucleotides upstream of a methionine codon respectively. Attachment to one or both of these sequences by the ribosome could result in the initiation of translation at incorrect sites. This would give rise to truncated proteins of molecular masses of ~ 41 and ~ 9.3 kDa respectively. Products around the size of the former are observed in Figure 2B and 2C. However, molecular species of other sizes are also present suggesting that partial proteolytic degradation of GST-HuZP3 cannot be completely discounted as reasons for the presence of multiple gene products from pHuZP3-GEX.

Non-specific proteolysis of the GST-HuZP3 fusion protein probably also accounts for the observed co-purification of free GST. Non-specific cleavage at the fusion joint between GST and the γ chain of the high affinity IgE receptor (Fc ϵ RI γ) has been documented even in the presence of a broad range of protease inhibitors [28], suggesting that a non-specific bacterial protease is cleaving GST-HuZP3 at the fusion joint. Attempts to express GST-HuZP3 in protease deficient strains of *E. coli* are presently underway.

Biological activity of GST-HuZP3 *in vitro*

In a number of non-human mammals such as mice and pigs, it is generally held that glycosylation of ZP3 is required for successful gamete interaction to occur [2,7]. However, contrasting data concerning the role of carbohydrate in spermatozoa-ZP3 (or ZP3 α) binding in both mice and pigs have been presented. For example, it has been documented that carbohydrates associated with mouse ZP3 or pig ZP3 α mediate spermatozoa-zona binding in these mammals *in vitro* [2,7] yet fertilization in both mice and pigs can be blocked by specific monoclonal antisera directed against synthetic peptides based on the native ZP3 primary amino acid sequences. However, although such observations indicate a central role for the polypeptide backbone of ZP3 in the binding of spermatozoa to the zona in these species [29,30], one should exercise caution with such interpretations. The inhibitory activity of such antibodies could arise through non-specific steric hindrance of the target epitopes due to the large molecular size of the immunoglobulins. However, such complications, which have been reported previously during investigations into spermatozoa-zona binding in the mouse, can be circumvented by using Fab fragments as opposed to intact antibodies [31].

Similar data regarding the role of ZP3-associated carbohydrate in human gamete interaction are scarce. However, it has recently been documented that anti-peptide antibodies generated against ZP3 fail to cross-react with ZP3 from certain oocytes that failed to fertilize at IVF [32], suggesting a more significant role for the ZP3 polypeptide backbone in human fertilization.

The data presented above demonstrate that GST-HuZP3 stimulates significantly higher levels of acrosomal exocytosis in live human spermatozoa compared to a series of non-specific controls. However, this level of stimulation could only be attained after the spermatozoa had been challenged with GST-HuZP3 for a period of 18 h. A number of authors have commented that native, intact human zona pellucida can stimulate acrosomal exocytosis within 1–2 h in $> 45\%$ of a given population of live human spermatozoa [8,9]. Furthermore, recombinant mouse ZP3 (used at a final concentration of $5 \text{ ng}/\mu\text{l}$) can initiate acrosomal exocytosis in 51% of mouse spermatozoa after only 20 min [33]. These data are in contrast to those observations made in the present study in that maximal acrosomal exocytosis was attained after spermatozoa had been incubated with GST-HuZP3 for a period of 18 h. However, our data are entirely consistent with those observed for spermatozoa incubated for 18 h with non-glycosylated rhuZP3 produced using an *in vitro*

transcription and translation system [14]. Furthermore, the level of acrosomal exocytosis observed with our non-glycosylated GST-HuZP3 is considerably higher than studies using purified glycosylated rhuZP3 even though such studies used final concentrations of ZP3 of $15\text{--}20 \text{ ng}/\mu\text{l}$ [11].

It is possible that the HuZP3 moiety of the GST-HuZP3 fusion protein has not adopted its native tertiary structure after expression in *E. coli*, thereby reducing its specific activity. The cytoplasm of *E. coli* does not favour the formation of di-sulphide bonds [34]; human ZP3 contains 15 Cys residues, two of which are located in the signal sequence [15]. Fourteen of the 15 Cys residues found in the human ZP3 polypeptide are conserved in ZP3 primary sequences from a number of mammals, suggesting they may be involved in maintaining the structure of ZP3 [35]. At the present time, it is not clear if any of these Cys residues participate in either inter- and/or intra-molecular di-sulphide bridge formation, both of which could potentially act to stabilize and maintain the tertiary structure of ZP3 in the intact zona. These interactions may be missing in the recombinant protein.

The spermatozoa used in this study may not have been capacitated sufficiently and as such were refractory to the effects of the GST-HuZP3 fusion protein. However, the method of capacitation employed in this study has been used on previous occasions and shown not to preclude the reactivity of spermatozoa toward rhuZP3 agonists [14,36].

S. japonicum GST is a homodimer [37], and the fusion proteins produced by the pGEX series of vectors are heterodimeric [38]. Thus, GST-HuZP3 is also probably dimeric. The presentation of a dimeric ZP3 molecule to the spermatozoa may partly mimic that occurring *in vivo* and may offer an explanation for the observation why this form of non-glycosylated HuZP3 can induce acrosomal exocytosis in human spermatozoa but only after 18 h. If the distance between the two juxtaposed HuZP3 moieties within the fusion protein are not in the correct spatial context, then one assumes it would be difficult for the second HuZP3 moiety to bind the spermatozoon in such a way so as to initiate possible receptor aggregation [6,39–42] and subsequently induce both rapid and maximal acrosomal exocytosis. This explanation for the time-lag seems unlikely in view of the fact that purified, non-glycosylated monomeric rhuZP3 is capable of stimulating acrosomal exocytosis in $\sim 53\%$ of capacitated human spermatozoa after a period of 18 h [14], implying that the state of the non-glycosylated HuZP3 polypeptide (monomeric or dimeric) is not a major determinant of whether the recombinant protein will stimulate the acrosome reaction.

The question of whether or not other components of the zona matrix are required either (a) directly or (b) indirectly to stimulate acrosomal exocytosis is not clear. Regarding (a), it is presently thought that in a number of mammals such as humans, mice and hamsters, only ZP3 possesses acrosomal exocytosis-inducing activity, not ZP1 or ZP2 [3,8]. However, it is possible that these other major zona components (namely ZP1 and ZP2) indirectly contribute to the bioactivity of ZP3 by maintaining the latter in a specific, three-dimensional conformation that facilitates induction of maximal exocytosis. This hypothesis has yet to be addressed experimentally. To our knowledge, minor zona-associated glycoproteins such as hyaluronic acid, have yet to be ascribed a role in human spermatozoa-zona interactions. This is in contrast to recent data, presented in reference [43], that demonstrates hyaluronic acid enhances the zona pellucida-induced acrosome reaction of cynomolgus macaque spermatozoa [43].

However, it is clear that authentic glycosylation of the HuZP3 polypeptide is required for this protein to possess full biological activity. As stated above, previously it has been shown that

glycosylated rhuZP3 purified from CHO cells could only initiate maximal acrosomal exocytosis in human spermatozoa after a period of ~20 h [11]. Glycosylation therefore appears to influence the kinetics but not the outcome of the ZP3-induced acrosome reaction.

In other glycoproteins, the presence of carbohydrate has a number of roles in protein function ranging from ensuring the correct folding of the α -subunit of the nicotinic acetylcholine receptor from *Torpedo californica* [44] to the secretion and dimerization of human interferon- γ [45]. More recently, Wang et al. [46] have suggested that a general function of protein glycosylation is firstly to stabilize the glycoprotein structure and secondly to ensure the glycoprotein adopts its native tertiary structure during its transit through the endoplasmic reticulum (ER) and Golgi body of the cell.

Non-specific induction of acrosomal exocytosis

Why a non-specific fusion protein such as GST-M.*MspI* was able to elicit acrosomal exocytosis was not determined. It is unlikely that the size of GST-M.*MspI* was sufficient to stimulate these cells to exocytose since one would have then expected to observe a comparable number of acrosome reactions in spermatozoa challenged with EBSS containing 0.3% (w/v) HSA (molecular mass = 66 kDa); this was not seen. One can also rule out the possibility that the GST moiety of both fusion proteins was responsible for initiating acrosomal exocytosis. This is because one would expect to observe similar levels of exocytosis in spermatozoa challenged with either GST-HuZP3 or GST-M.*MspI*; again this was not seen. Presently it is believed that the ability of GST-M.*MspI* to initiate acrosomal exocytosis reflects the inherent poor agonist specificity of the acrosome reaction assay. A number of other, non-physiological agonists can elicit the acrosomal exocytosis in spermatozoa, e.g. corticosteroid-binding protein [47], Epidermal Growth Factor [48] and immunoglobulins [49].

As detailed above, non-glycosylated HuZP3 has been shown to be capable of stimulating acrosomal exocytosis in live human spermatozoa after 18 h. This time course is similar to that reported for purified glycosylated rhuZP3 [11]. A detailed kinetic analysis of ZP3-induced acrosomal exocytosis is now required to clarify whether this is the case. Moreover, it would be interesting to compare the rates of acrosomal exocytosis induced by both CHO- and *E. coli*-derived ZP3 side by side. Until the nature of the carbohydrate in human ZP3 is established, bacterial-expressed ZP3 remains the best defined inducer of the acrosome reaction for the study of this process in humans.

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