Sperm plasma-membrane-associated glutathione S-transferases as gamete recognition molecules

Tummala Hemachand, Bagavathi Gopalakrishnan, Dinakar M. Salunke, Satish M. Totey and Chandrima Shaha*

National Institute of Immunology, Aruna Asaf Ali Road, New Delhi 110067, India

*Author for correspondence (e-mail: cshaha@nii.res.in)

Accepted 12 February 2002

Journal of Cell Science 115, 2053-2065 (2002) © The Company of Biologists Ltd

Summary

Glutathione S-transferases (GSTs) are enzymes that detoxify electrophilic compounds. Earlier studies from our laboratory showed that anti-GST antibodies interfered with the fertilising ability of spermatozoa from *Capra hircus* (goat) in vitro, suggesting that GSTs are localised at the cell surface. In this study, we provide evidence for the presence of GSTs of 24 kDa on the sperm plasma membrane attached by non-covalent interactions. The GST activity associated with the spermatozoal plasma membrane was significantly higher than the activity present in the plasma membranes of brain cells, hepatocytes, spleenocytes and ventriculocytes. Analysis of GST isoforms demonstrates the presence of GST Pi and Mu on the sperm plasma membranes. Both isoforms were able to bind to solubilised as well as intact zona pellucida (ZP) through their N-terminal regions but failed to bind to ZP once the oocytes were fertilised. Solubilised goat ZP separates into three components, one of which, the ZP3-like component, bound to sperm GSTs. High concentrations of anti-GST antibodies or solubilised ZP led to aggregation of sperm GSTs, resulting in the release of acrosin. In contrast, inhibition of sperm GST binding to ZP, by saturation of binding sites for sperm GSTs on the solubilised ZP using peptides designed from the N-terminii of GST Pi or Mu or blocking of binding sites for ZP on sperm GSTs with antibodies raised against the N-terminal GST peptides, inhibited essential prefertilisation changes in sperm.

These data therefore demonstrate the strategic location of catalytically active defensive enzymes on the sperm surface that also act as zona-binding proteins. Therefore, sperm-surface GSTs serve as bifunctional molecules in a transcriptionally inactive cell whose requirement for cellular defense and economy of molecules that it can carry is greater than that of any somatic cell type.

Key words: GST, Plasma membrane, Sperm, Zona binding

Introduction

GSTs are a family of enzymes that catalyze a number of glutathione (GSH)-dependent reactions and have been primarily described as cytosolic or microsomal detoxification enzymes that are also capable of functioning as intracellular binding proteins (Hayes and Pulford, 1995). The origin of the work described in this paper is the synthesis of several earlier findings reported from this laboratory. These observations include the evidence for the presence of glutathione S-transferases (GSTs) on goat spermatozoa (Aravinda et al., 1995), where they function as detoxifying enzymes (Gopalakrishnan and Shaha, 1998a), and the inability of goat sperm to fertilise oocytes when treated with anti-GST antibodies (Gopalakrishnan et al., 1998b; Aravinda et al., 1995). These antibodies did not inhibit the catalytic activity of the sperm GSTs even though they could inhibit fertilisation, and thus a possible role, distinct from the catalytic role, for sperm GSTs in fertilisation-related events was envisaged. Fertilisation is a multiphasic event involving several components from both the gametes (McLeskey et al., 1998). Although the finer details of fertilisation varies enormously from species to species, the event of fertilisation generally consists of (a) contact and recognition between the sperm plasma membrane and the egg coat or the zona pellucida (ZP), (b) binding of ZP to sperm and induction of acrosome reaction (AR), (c) secondary attachment and penetration through the oocyte coat and (d) fusion of the nuclei of the two gametes (Yanagimachi, 1981). Although it was evident from our studies that binding of anti-GST antibodies on specific sites of sperm-surface GSTs could prevent fertilisation, the precise role of sperm GSTs in this process was not clear. Therefore, this study was undertaken to explore the possible functional role of the sperm-surface GSTs in the process of fertilisation.

GSTs are cytosolic proteins, except for in one cell type, namely the hepatocyte, which is reported to express GSTs of 17.2 kDa on the plasma membrane (Sies et al., 1998). We present clear evidence that GSTs of 24 kDa are present on mature cauda epididymal sperm plasma membrane and are attached to it by peripheral non-covalent interactions. Spermatozoal plasma-membrane-associated GST activity is significantly higher than that of plasma membrane from somatic cells such as brain cells, spleenocytes, ventriculocytes and hepatocytes. Two GST isoforms, namely GST Mu and Pi are present on the sperm plasma membrane, and both are capable of binding to the ZP. This binding appears to be mediated primarily via the N-terminii of these molecules. Interestingly, both the isoforms bind to the same site on ZP although GST Pi shows a higher affinity than GST Mu. Goat ZP resolves into three components of which only the ZP3-like component binds to biotinylated sperm plasma membrane GSTs. Solubilised ZP (SZP) or higher concentrations of anti-GST antibodies leads to aggregation of sperm-surface GSTs,

resulting in acrosin release. In contrast, saturation of sperm GSTs by lower concentrations of anti-GST antibodies or their Fab fragments does not induce aggregation and therefore prevents ZP binding to sperm, resulting in inhibition of acrosome reaction. This inhibition appears to be caused by reduction of the intracellular Ca^{2+} increase that normally occur in response to ZP binding. Having a detoxification enzyme as a zona-binding protein would give a great survival advantage to a cell that is highly streamlined, vulnerable to oxidative stress and exposed to a variety of environments en route from the testis to the oocyte in the fallopian tube. Taken together, the data provide a comprehensive view of the functional role of sperm GSTs as ZP-binding proteins that add to the importance of multifunctional molecules in a transcriptionally inactive cell such as sperm.

Materials and Methods

Animals

Goat (*Capra hircus*) testes and ovaries were obtained from the local slaughterhouse along with the cauda epididymides. New Zealand White rabbits were obtained from the animal facilities of the National Institute of Immunology, New Delhi, India.

Materials

Goat anti-rabbit IgG antibodies conjugated to fluorescein isothiocyanate (FITC) were procured from Jackson Laboratories (West Grove, PA). The bicinchoninic acid protein assay reagent was from Pierce Chemical Company (Rockford, IL). The GST purification module and Protein G Sepharose Fast Flow were obtained from Pharmacia Biotech (Uppsala, Sweden). Problott™ membranes and reagents for synthesis and conjugation of peptides were from Applied Biosystems Inc. (Foster City, CA). Other chemicals, unless specified, were purchased from Sigma Chemical Co. (St. Louis, MO). Na[^{125}I] was purchased from NEN Life Science Products (Boston, MA).

Peptides and antibodies

A total of four peptides designed from two different regions of domain I of the GST Pi and Mu were synthesised and characterised as described previously (Gopalakrishnan et al., 1998b). Two peptides representative of the N-terminal sequences of the two GST isoforms were GST PiN (Pro-Pro-Tyr-Thr-Ile-Val-Tyr-Phe-Pro-Val) and GST MuN (Pro-Met-Thr-Leu-Gly-Tyr-Trp-Asp-Ile). Two other peptides representing a region that is involved in glutathione (GSH) binding (Manoharan, 1992) were GST PiC (Gln-Leu-Pro-Lys-Phe-Gln-Asp-Gly-Asp-Leu-Thr-Leu-Tyr) and GST MuC (Asn-Leu-Pro-Tyr-Leu-Ile-Asp-Gly-Ser-His-Lys-Ile-Thr). The peptides were conjugated to diptheria toxoid by the glutaraldehyde method (Avrameas, 1969).

The conjugated peptides were used to raise antisera in rabbits using standard protocols (Vaitukaitis et al., 1971). Characterisation of the antisera was done as described previously (Gopalakrishnan et al., 1998b). The specificity of the antisera was judged by (a) immunoprecipitation of GSTs from sperm extracts, (b) the reactivity of antisera to their respective peptides and the absence of cross reactivity towards other peptides (Gopalakrishnan et al., 1998b), (c) the inability of the antisera to crossreact with any other proteins from total sperm extracts on western blots, (d) the ability of antigen pre-adsorption to abolish immunorecognition and (e) reproducibility of the results with antisera from different animals.

Fab fragments were prepared from IgG purified from 50% ammonium sulfate precipitates of the immune sera prepared using Protein G Sepharose 4B Fast Flow column. Papain (25 μg) activated with β-mercaptoethanol was used to digest 500 μg of purified IgG, and Fab fragments were purified on a Protein Pak column DEAE-5PW [Semiprep 21.5 mm×150 mm (Waters, USA)] equilibrated with 10 mM Tris-HCl (pH 8.0) on a gradient of 0-100% of 10 mM Tris-HCl, 1 M NaCl (pH 8.0) (Jungbauer et al., 1989).

Circular dichroism

Circular dichroism (CD) experiments were carried out as described previously (Kaur et al., 1997) on a JASCO 710 spectropolarimeter with a 2.0 nm band width, 1 nm resolution and 1 second response time. The peptide concentrations of GST PiN and MuN was 30 μM in 10 mM Tris-HCl (pH 7.0).

SDS-PAGE and western blots

Electrophoresis was carried out using the buffer system of Laemmli (Laemmli, 1970) on 10% polyacrylamide gels. Western blotting was performed following a modification of the method of Towbin (Towbin et al., 1979) as described previously (Aravinda et al., 1995).

Preparation of capacitated spermatozoa, gametes, embryos and solubilised zonae

Sperm preparation and capacitation, recovery and maturation of oocytes were carried out as described previously (Gopalakrishnan et al., 1998b). Embryos were allowed to develop in TC199 medium supplemented with 20% fetal calf serum (FCS).

For the preparation of SZP, oocytes matured in vitro with cumulus oophorous were treated with 5 mg/ml hyaluronidase for 5 minutes to remove the cumulus cells. ZP were then removed from cumulus-free eggs mechanically by forcing through a narrow-bore micropipette. Isolated zonae were washed thoroughly to remove any adherent egg cytoplasm and were subsequently heat solubilised. The purity of this SZP preparation was checked routinely by SDS-PAGE. SZPs were drop frozen and stored in liquid nitrogen until use.

Preparation of plasma membranes

Germ cells from goat testes were prepared by digestion with collagenase/dispase in Ham's F-12 media as described previously (Aravinda et al., 1995) and crude membranes prepared according to Scott et al. (Scott et al., 1993). Liver and brain tissues were digested with collagenase/dispase (3 mg/ml) and 0.25% trypsin (Ferreira and Loomis, 1998), respectively. Spleens were mashed between two frosted slides to prepare spleenocytes (Coligan et al., 1992), and ventriculocytes (Springhorn, 1998) were prepared from heart ventricular tissue by trituration in Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose) followed by treatment with 3 mg/ml collagenase and 0.25% trypsin. The resulting tissue masses were filtered through cheesecloth, and cell suspensions were separated on 40% percoll gradients. The cells were homogenised in STM (10 mM Tris-HCl, 1 mM EDTA, 0.25 M sucrose, pH 7.4) in the presence of a protease inhibitor cocktail (1 mM EDTA, 1 mM PMSF, 1 mM EGTA 0.17 units/ml aprotinin, and 1 mM leupeptin). Unbroken cells were removed at low speed and the nuclear pellet collected after spinning at 600 ***g*** for 10 minutes at 4°C. The mitochondrial fraction was separated out by spinning the supernatant at 10,000 ***g*** for 10 minutes. Crude membrane fraction was obtained after centrifugation of the above supernatant at 100,000 ***g*** for 60 minutes in a Beckman TL-100 ultracentrifuge with an SN863 rotor (Beckman Inc. Palo Alto, CA). Crude plasma membrane was further purified on a 25% linear Percoll gradient, and purity was checked by assaying for plasma membrane marker enzyme 5′-nucleotidase (Glastris and Pfieffer, 1974).

Study of mode of membrane attachment

Purified sperm plasma membranes were treated with different concentrations of proteases, trypsin type III and pronase A (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml for different time periods, 10 and 20 minutes), and protease inhibitors were used to stop reactions. To detach peripherally attached proteins, membranes were incubated with 10 mM, 20 mM HCl and 1 M NaCl for 30 minutes at 4°C. To remove GPI-anchored proteins, phosphotidylinositol-specific phospholipase C (PIPLC, 0.05 units/ml, for 10 minutes) (Shur and Neely, 1988) was used. Release of GSTs from the plasma membranes after treatment was checked in the 100,000 ***g*** supernatants by GST activity assay with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. Purification of GSTs from peripherally attached proteins was done as described below and run on SDS PAGE, western blots of which were probed with anti-GST antibodies.

Purification of GSTs, GST assay and identification of isoforms

GSTs from NP-40 extracts of sperm plasma membrane preparations and extracts of total blood cells were purified by GSH affinity chromatography as described earlier (Aravinda et al., 1995). The GST assay was carried out according to Warholm (Warholm et al., 1985) with slight modifications as described previously (Aravinda et al., 1995; Mukherjee et al., 1999).

Reverse-phase high performance liquid chromatography (RP-HPLC) was used to purify GST isoforms from the total sperm plasma membrane GSTs using a Vydac C_{18} column (Type 218TP54, Waters, USA) equilibrated with 60% Solvent 1 (0.08% Trifluoroacetic acid, TFA in water) and 40% Solvent 2 (0.08% TFA in 80% acetonitrile) (Gopalakrishnan et al., 1998b). N-terminal sequencing of the purified peaks was carried out as described in an earlier report (Gopalakrishnan et al., 1998b).

Radiolabelling of cells and proteins

To label the spermatozoal surface, we selectively used a highly motile population of spermatozoa that was purified by centrifugation on Percoll gradient (90-45%). Iodination was carried out according to Spencer and Nicoloff (Spencer and Nicoloff, 1980). Briefly, 10^7 Percoll-purified sperm per vial were mixed with 200 μM lactoperoxidase, 0.5 mCi Na[^{125}I] and 0.03% H_2O_2 on ice. After gentle mixing for 5 minutes, 20 μM lactoperoxidase and 0.03% H_2O_2 were added followed by three additions of 0.03% H_2O_2 on ice. The cells were thoroughly washed with 15 mM NaI and then tested for viability by trypan blue exclusion and the motility pattern of the cells. Vials with cell viability above 95% were homogenised to break open the cells, and plasma membrane fractions were separated as mentioned above. GSTs were purified from 0.1% NP-40 extracts of these fractions, and other remaining fractions followed by autoradiography that was carried out after running these extracts on SDS-PAGE. The sperm GSTs and SZP (50 μg of protein) were iodinated. Iodinated proteins were purified on a Sephadex G-75 column to remove free iodine.

Immunocytochemistry

For visualising the binding of GST MuN and PiN peptides to oocytes and embryos, oocytes were incubated with GST PiN and MuN peptides (10 μg/ml) for 30 minutes at 37°C followed by treatment with rabbit anti-GST PiN and MuN antibodies (1:100) and subsequently treated with anti-rabbit IgG (1:200) conjugated to FITC. Oocytes were mounted in hanging drop preparations and observed under a Nikon Optiphot fluorescence microscope. To detect any staining of oocyte cytoplasmic GSTs, inner cytoplasmic masses were pushed out just before the oocytes were observed. Embryos were also treated similarly.

To visualise the aggregation of sperm-surface GSTs, capacitated sperm were incubated with SZP (10 μg/ml/10^7 sperm), anti PiN, anti MuN antibodies (1:10) or their Fab fragments at 4°C followed by incubation at 37°C. Aliquots of sperm were fixed at different time points in 4% buffered paraformaldehyde. The movement of the GST molecules was visualised by using FITC-labelled second antibody (1:500). *Pisum sativum* agglutinin (PSA) conjugated to rhodamine (1:100) was used to stain the same samples to visualise the status of the acrosome. A minimum of 200 sperm were scored according to the following classification: (a) staining for the total acrosome; (b) patchy staining implying ongoing aggregation - patching; c) aggregation towards the tip of the acrosome - capping and d) unstained sperm.

Sperm GST-SZP binding

To check the binding of sperm GSTs to SZP, 5 μg each of BSA, sperm plasma membrane and blood cell GSTs were dotted on nitrocellulose sheet and blocked with 2% BSA-PBS. The dot blots were probed with ^{125}I-labelled SZP for 16 hours at 4°C and exposed to x-ray films or phosphorimager screens for autoradiography. For specificity studies, dot blots were preincubated with unlabelled SZP followed by incubation with the iodinated material. Quantitation of binding of SZP to the dot blots was done with a Molecular Dynamics Phosphorimager (Sunnyvale, CA) with Imagequant software package.

To check the binding of SZP with the GST PiN and MuN peptides, binding studies were performed with different concentrations of GST PiN and MuN peptides immobilised on microtitre plates and probed with 50 ng of I^{125}-SZP. For competition studies, excess unlabelled SZP (200 ng) or competitive peptides (50 μg) was used.

In addition to these studies, GST Mu and Pi purified by RP-HPLC from plasma membrane GST were radiolabelled and used for binding studies with SZP (1 μg) immobilised onto microtitre plates. Each individual well of the microtitre plate was transferred to a vial, and readings were taken in a 1275 Minigamma counter (Wallac Inc., Turku, Finland).

For equilibrium-binding studies, SZP (1 μg/well) was immobilised on microtitre plates and blocked with 2% BSA in PBS before allowing concentration-dependent equilibrium binding (3 hours at 37°C) of ^{125}I labelled GST (*GST). Unbound *GST was washed out with 0.05% Tween-20 in PBS. Non-specific binding was determined as the binding of *GST to zona after GST sites on zona were blocked with 100 mM cold GST. Affinity constant (K_d) of the GSTs was calculated by using Langmuir and Scatchard plots of specific binding. For competition experiments, 600 nM *GST was used to bind to immobilised SZP in the presence of different concentrations of GST PiN and GST MuN peptides. IC_{50} was calculated from normalised fractional binding curves.

Identification of ZP components binding to sperm GSTs

To determine the specific ZP components that the sperm GSTs bind to, ZP (10 μg) was run on SDS-PAGE and transferred onto nitrocellulose. These blots were probed with biotinylated sperm plasma membrane GSTs (1 μM) for 3 hours at room temperature, and binding was visualised by avidin linked to HRP. The plasma membrane GSTs were biotinylated using NHS-biotin as described earlier (Rao and Shaha, 2001). For identification of the component of the ZP that the biotinylated sperm GSTs were binding to, similar blots were probed with two monoclonal anti-ZP3 antibodies. One was raised against an epitope conserved among species (MA 451) (Kaul et al., 1997), and another was raised against the N-terminus of the porcine ZP (MA 455/467) (Gupta et al., 1995). Further characterisation of the component band was carried out by N-terminal sequencing as described previously (Gopalakrishnan et al., 1998b).

In vitro sperm agglutination, immobilisation and sperm-oocyte binding assay

The agglutination assay was carried out as described previously (Shaha et al., 1988). Briefly, 10^6 sperm were placed in a 50 μl droplet of media under mineral oil, and antibodies were added at dilutions of

1:25, 1:50 and 1:100, following which observations were made under an inverted microscope.

To directly determine the effects of the antibodies bound to sperm on their capability to bind to oocytes, antibody-treated (1:100 dilution, anti-GST-PiN, MuN, PiC, MuC and a mixture of anti-GST MuN and PiN) capacitated spermatozoa were washed and allowed to interact with mature oocytes (10^6 sperm/10 oocytes) in fertilisation media for 30 minutes at 37°C under mineral oil (Aravinda et al., 1995). To check if saturation of the GST-binding sites on oocyte ZP would inhibit sperm-oocyte binding, oocytes were exposed to total sperm plasma membrane GSTs and relevant peptides for 30 minutes at 37°C prior to interaction with capacitated spermatozoa. In both the above experiments, the number of sperm bound to each oocyte was scored in a standard plane of focus that passed approximately through the center of the oocyte as described earlier (Shaha et al., 1991).

Acrosome reaction and acrosin assay

Binding to the multimeric ZP induces acrosome reaction in sperm (Yanagimachi, 1981). To determine the effect of binding of anti-GST antibodies to sperm on zona-induced acrosome reaction, sperm were capacitated in the presence of anti-PiN and anti-MuN antisera (1:100), their Fab fragments (10 μg/ml) and preimmune sera (1:100) for 3 hours and incubated with SZP (10 μg/ml). SZP preadsorbed with GST PiN and MuN was used to induce an acrosome reaction in untreated capacitated spermatozoa to check if saturation of GST-binding sites on SZP would render the SZP unable to induce acrosome reactions. Acrosin release was measured by a spectrophotometric assay using BAEE (Benzoyl Arginine Ethyl Ester) as substrate (Polakoski and Zaneveld, 1977).

To assess specifically if crosslinking of sperm-surface GSTs could initiate intracellular signaling events for acrosome reaction, capacitated sperm were treated with various concentrations of antibodies [1:10, 1:25, 1:50 of anti-GST-MuN and PiN, total IgG or Fab fragments (50 μg/ml)] for 60 minutes at 37°C, and acrosin release was measured as described above. In groups treated with higher dilutions (1:50) of anti-GST antisera, secondary antibody (1:100) was used to precipitate immunoaggregation.

Measurement of intracellular levels of Ca^{2+}

Intracellular Ca^{2+} was measured according to Gorczynska and Handelsman (Gorczynska and Handelsman, 1993) with slight modifications. Briefly, spermatozoa (10^7) capacitated in the presence or absence of anti-GST antibodies were loaded with 1 μM of Ca^{2+}-binding dye Fura 2-AM at 37°C for 40 minutes. Acrosome reaction was induced with SZP (10 μg/ml), and subsequently cells were lysed with 0.1% digitonin. After centrifugation at 750 ***g*** for 30 minutes, fluorescence in the supernatant was measured using a spectrophotofluorimeter (RF-1501, Shimadzu Inc., Tokyo, Japan) keeping the $\lambda_{excitation}$ at 340 nm and $\lambda_{emission}$ at 400-500 nm. For microscopic estimation, the number of Fura 2-AM-positive sperm was scored under a Nikon Optiphot fluorescence microscope.

Statistical analyses

Data from various treatment groups were compared using unpaired two-tailed student's *t*-test. Tadpole III software (Elsevier Biosoft, Cambridge, UK) was used for the analysis.

Results

Sperm plasma membranes contain multiple GST isoforms and show higher GST activity than the somatic cell plasma membranes

GSTs are known as cytosolic enzymes; however, our earlier studies suggested a surface localisation of these proteins on sperm. To determine whether GSTs are associated with the sperm plasma membrane and whether this is a unique feature, we compared GST activities of plasma membrane preparations from sperm with that of hepatocytes, brain cells, spleenocytes and ventriculocytes. Using CDNB as a substrate, it was found that GST activity was significantly higher in the plasma membranes of germ cells and spermatozoa compared with membrane preparations from the somatic cell types (Fig. 1). This activity was saturable with increasing concentrations of CDNB (Fig. 1, inset).

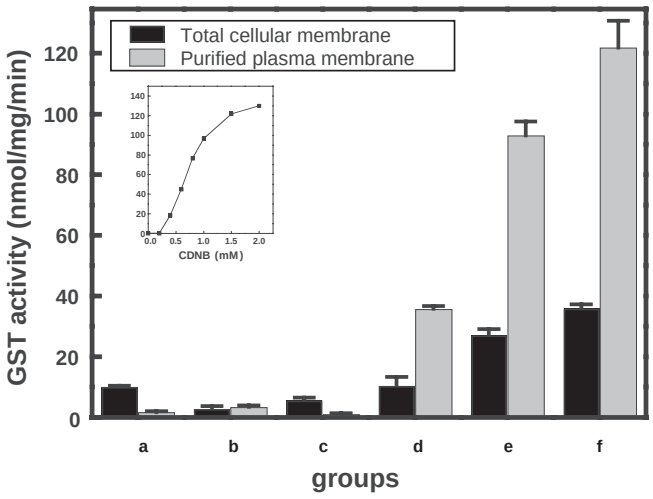

Fig. 1. Comparison of activity of plasma-membrane-associated GSTs in different cell types. This figure represents GST activities measured using 1 mM CDNB and 1 mM GSH at a 2 minute time point with purified plasma membrane preparations from (a) ventriculocytes, (b) spleenocytes, (c) brain cells, (d) hepatocytes, (e) testicular germ cells and (f) epididymal spermatozoa. The inset shows sperm plasma membrane GST activity with increasing concentrations of CDNB in the presence of 1 mM GSH. The activities of total cellular membranes are also represented.

Having established that GSTs are associated with the sperm plasma membrane, we tried to determine whether they are exposed to the extracellular side of the cell. In case of an extracellular side orientation, GSTs should theoretically be labelled when cell surface proteins were radioiodinated. We purified radiolabelled GSTs of apparent molecular mass of 24 kDa from extracts of spermatozoa that were radioiodinated in live conditions (Fig. 2A). Radiolabelling of internal proteins was ruled out by autoradiography of sperm proteins after their plasma membranes have been removed. No significant labelling was observed in these extracts (Fig. 2A, lane a). Therefore, it can be inferred that GSTs are present on the sperm surface. This observation further supports our previous report that spermatozoa excluding propidium iodide stain positive for both GST Pi and Mu when immunostained with anti-GST antibodies (Gopalakrishnan et al., 1998b).

To confirm the molecular mass, purified GSTs from sperm plasma membrane were run on SDS-PAGE, which revealed two protein bands of apparent molecular mass of 25.6 and 23.4 kDa (Fig. 2B). The identities of these bands was further confirmed by immunoprecipitation of plasma membrane extracts with anti GST Pi and Mu antisera that showed

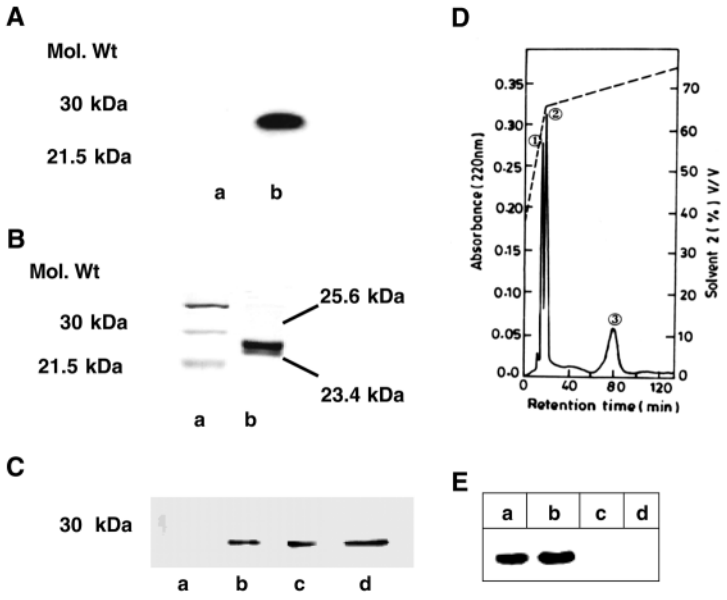

Fig. 2. Plasma-membrane-associated GSTs on sperm. (A) Autoradiogram of ^{125}I-GSTs purified by GSH affinity chromatography from extracts of the plasma membrane of surface-iodinated sperm in live conditions (lane b). Lane a represents extracts of sperm after removal of the plasma membrane. (B) SDS-PAGE (12%) of GSH affinity-purified GSTs from sperm plasma membrane preparations. Lane a, molecular weight marker; lane b, purified GSTs. (C) Non-reducing SDS-PAGE showing immunoprecipitated proteins from sperm plasma membranes using anti-GST antibodies. (lane a) Preimmune serum; (lane b) anti-GST MuN antibodies; (lane c) anti-GST PiN antibodies; (lane d) migration of GSH affinity-purified sperm plasma membrane GSTs used as a control lane. (D) The RP-HPLC elution profile of sperm plasma membrane GSTs purified by GSH affinity chromatography. Peaks 1 and 2 are GST-Mu, Peak 3 is GST-Pi. (E) Western blots of peaks 1 and 2 represented in D probed with anti-GST M1 antibody (lanes a,b) and anti-GST Pi antibody (lanes c,d), respectively.

precipitation of proteins of molecular masses between 23 and 26 kDa (Fig. 2C).

Distinct identification of the GST proteins was established by N-terminal sequencing and western blots. Affinity-purified plasma membrane GSTs resolved into three peaks on RP-HPLC (Fig. 2D). Peak three showed a 100% sequence identity to bovine GST Pi N-terminii over 10 amino acids (Pro-Pro-Tyr-Thr-Ile-Val-Tyr-Phe-Pro-Val). Attempts to sequence the N-terminii of peaks one and two were not successful, implying that the N-terminii may be blocked. The identity of the two peaks was confirmed by western blots with antibodies against GST-M1 that reacted to the first two peaks, whereas anti-GST Pi antisera did not recognise either of them (Fig. 2E). Attempts to sequence endo-protease glu-C digests of peaks one and two were not successful owing to limitations in the starting material. GSTs purified by RP-HPLC from goat blood resolved into one distinct peak that shared 100% sequence identity at the N-terminus with sperm plasma membrane GST Pi (data not shown).

The above results provide evidence that plasma-membrane-associated GSTs are present on spermatozoa. Two GST isoforms, namely GST Pi and Mu, are present on the plasma membrane and have a molecular mass range between 23 and 26 kDa. Plasma-membrane-associated GST activity was highest on sperm - higher than on the four types of somatic cells tested.

GSTs are attached to the sperm surface by non-covalent interactions

To determine the mode of attachment of GSTs, plasma membranes were exposed to high salt, low pH buffer, PIPLC or subjected to controlled trypsinisation. All three treatments except PIPLC yielded GST activity in the supernatants (Fig. 3), indicating non-covalent interactions as the possible mode of attachment. Absence of GST activity in the supernatants of the treatments using PIPLC rules out the possibility of GPI anchoring. No activity could be detected in the pronase digest supernatants. Anti-GST antibodies on western blots of 1M NaCl extract of plasma membranes recognised a 24 kDa band (Fig. 3, inset, lane b). Immunorecognisable ~24 kDa GSTs could be purified from these supernatants by GSH affinity chromatography as well (Fig. 3, inset, lane c). Trypsinisation of live spermatozoa yielded GST activity in the supernatant (data not shown); however, this experiment could not be carried out with 1 M NaCl as the sperm lost membrane integrity, and therefore there was a possibility that cytosolic proteins including sperm GSTs might have leaked out. In trypsinisation experiments, time points and concentrations were chosen

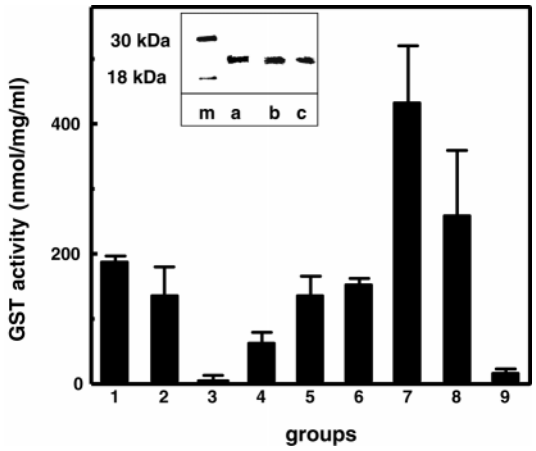

Fig. 3. Attachment of GSTs to the sperm plasma membrane evaluated after treatment of the plasma membranes with different reagents. The relative GST activity in 100,000 ***g*** supernatants of plasma membranes was measured (using 1 mM CDNB and GSH at a 2 minute time point) after treatment with (3) Pronase, 0.5 mg/ml, 10 minutes, (4) Trypsin, 0.5 mg/ml, 10 minutes, (5) Trypsin, 0.2 mg/ml, 10 minutes, (6) 20 mM HCl, 5 minutes, (7) 1 M NaCl, 15 minutes, (8) 0.1% NP 40, (9) PIPLC, 0.05 units/ml, 20 minutes. (1) The GST activity of the cytosol and (2) the GST activity of the plasma membrane. (Inset) Western blots (probed with anti-GST antibody at 1:1000 dilution). Lane a, total plasma membrane proteins; lane b, supernatant of treatment 7; lane c, GSH affinity-purified GSTs from the supernatant of treatment 7; m, molecular weight marker.

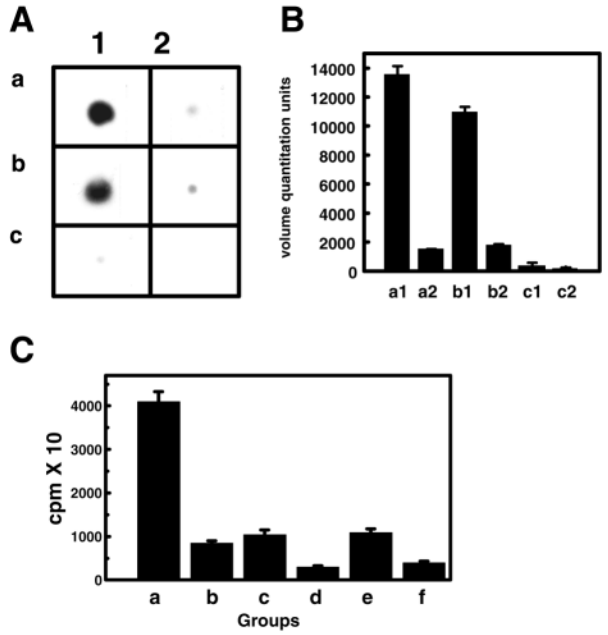

Fig. 4. Binding of sperm plasma membrane and blood cell GSTs to ^{125}I-SZP and of labelled sperm GST-Pi and Mu to SZP. Proteins were blotted onto nitrocellulose membranes and were probed with labelled SZP. (A) Dot blots of (a1) total sperm plasma membrane GSTs probed with ^{125}I-SZP, (a2) total sperm plasma membrane GSTs preadsorbed with cold SZP and subsequently probed with ^{125}I-SZP, (b1) total blood cell GSTs probed with ^{125}I-SZP, (b2) total blood cell GSTs preadsorbed with cold SZP and probed with ^{125}I-SZP, (c1) BSA (5 μg) probed with ^{125}I-SZP and (c2) BSA (5 μg) preadsorbed with cold SZP and subsequently probed with ^{125}I-SZP. (B) Volume quantitation of 'A' using a Molecular Dynamics Phosphorimager with Imagequant software package. Groups are as described in A. (C) Binding of radioiodinated GST Pi and GST Mu purified from sperm plasma membranes to SZP coated on to microtitre plates (1 μg/well). Binding of (a) labelled sperm plasma membrane GSTs; (b) labelled sperm plasma membrane GSTs preadsorbed with SZP (10 μg/ml); (c) labelled sperm plasma membrane GST-Mu; (d) labelled sperm plasma membrane GST Mu preadsorbed with SZP (10 μg/ml); (e) labelled sperm plasma membrane GST-Pi; and (f) labelled GST Pi preadsorbed with SZP (10 μg/ml).

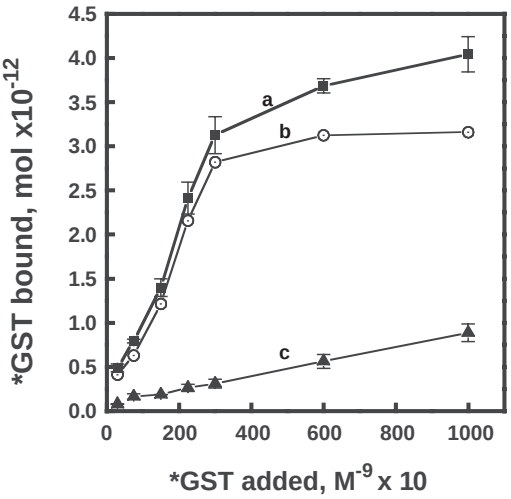

Fig. 5. Equilibrium-saturation binding of [^{125}I]-labelled sperm plasma membrane GSTs (*GST) to immobilised SZP. SZP (1 μg/well) was immobilised on microtitre plates and [^{125} I]-labelled sperm plasma membrane GSTs were allowed to bind under different conditions. (a) Moles of total sperm plasma membrane GSTs bound to SZP; (b) specific binding of GSTs, which equals total binding (a) minus non-specific binding (c); (c) moles of total sperm plasma membrane GSTs bound to SZP after blocking with 100 mM cold GST. Data are means±s.e.m.

where sperm membrane integrity was intact and sperm were viable after treatment.

The above data suggested that the mode of attachment of GSTs to the sperm plasma membrane was primarily through non-covalent interactions.

Sperm GSTs bind to oocyte ZP and are involved in sperm-oocyte binding

Considering that the above data suggested a plasma membrane localisation of sperm GSTs, with them facing the extracellular side, and our earlier data on the ability of anti-GST antibodies to reduce fertilisation, it seemed possible that sperm GSTs might take part in the process of primary binding between the gametes. We therefore, explored several ways to determine if sperm GSTs bound to molecules on the ZP.

Purified sperm plasma membrane GSTs immobilised on the nitrocellulose membrane was able to bind to radiolabelled SZP that could be displaced by preincubation of the blots with unlabelled SZP (Fig. 4Aa1,a2, Fig. 4Ba1,a2). GST Pi derived from goat blood cells was also capable of binding to labelled SZP specifically (Fig. 4Ab1,b2, Fig. 4Bb1,b2). BSA was used as a negative control (Fig. 4Ac1,c2; Fig. 4Bc1,c2). Both GST isoforms, GST Mu and Pi purified from sperm plasma membrane by RP-HPLC and subsequently labelled with [^{125}I] could bind to immobilised SZP on microtitre plates (Fig. 4C). Binding of GST Mu and Pi to SZP (Fig. 4Cc,e) could be successfully competed out if these isoforms were preadsorbed with SZP prior to incubation with immobilised zona (Fig. 4Cd,f). Total sperm plasma membrane GSTs were used as a positive control (Fig. 4Ca) and SZP preadsorption could displace binding (Fig. 4Cb).

Equilibrium saturation binding experiments where SZP was immobilised and subsequently probed with radiolabelled GSTs showed a specific binding (Fig. 5) with an equilibrium constant of (K_d) 11.23 M×10^{-6} calculated from Scatchard plots. When GST PiN and MuN peptides were immobilised and subsequently probed with radiolabelled SZP, peptides could also bind to radiolabelled SZP in a typical Langmuir pattern (Fig. 6A,B). This binding could be competed out with unlabelled SZP, showing the specificity of the binding. Unlabelled PiN could compete out the binding of SZP to MuN and vice versa (Fig. 6A,B). Competitive binding curves (Fig. 6C) show that both peptides can compete for zona binding with sperm GSTs. IC_{50} calculated from normalised binding curves reveals high affinity of GST PiN ($IC_{50,}$ 1.067 μM) for SZP binding compared to MuN peptide ($IC_{50,}$ 6.5 μM) (Fig. 6C). To resolve whether or not the differential affinity of the binding of PiN and MuN was caused by differences in the structure they adopt in solution, circular dichroism (CD) spectra of both peptides were scanned. It showed that the PiN peptide had a broad minima around 206

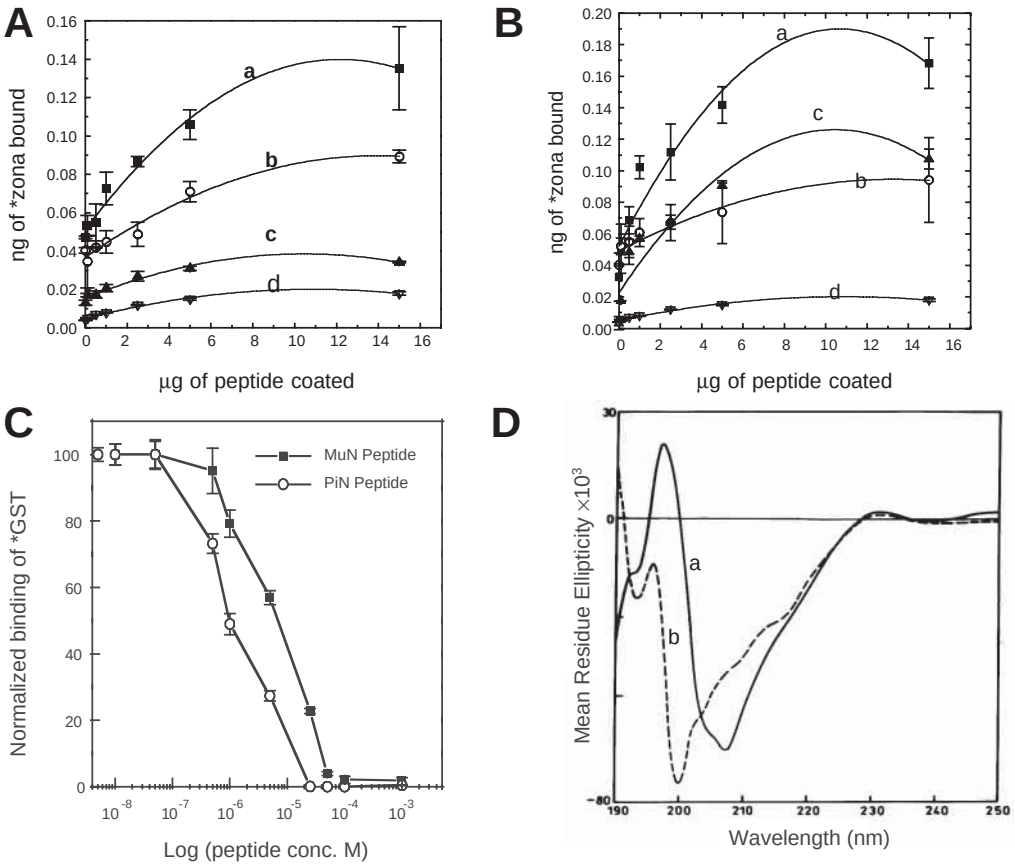

Fig. 6. Binding of SZP to MuN and PiN peptides immobilised on microtitre plates. Different concentrations of GST PiN and MuN peptides were coated onto microtitre plates and probed with 50 ng of ^{125}I-SZP under different conditions. Competition was carried out with unlabelled SZP or peptides. (A) Saturable binding of ^{125}I-SZP to MuN peptide; (a) MuN peptide + ^{125}I-SZP; (b) MuN peptide + ^{125}I-SZP + excess unlabelled SZP (200 ng); (c) MuN peptide + ^{125}I-SZP + PiN peptide (in solution with ^{125}I-SZP); (d) MuC peptide + ^{125}I-SZP. (B) Saturable binding with ^{125}I-SZP to PiN peptide; (a) PiN peptide + ^{125}I SZP; (b) PiN peptide + ^{125}I-SZP + excess unlabelled SZP (200 ng); (c) PiN peptide + ^{125}I-SZP + MuN peptide (in solution with ^{125}I-SZP); (d) PiC peptide + ^{125}I-SZP. (C) Inhibition profile of binding of 600 nM *GST to SZP (1 µg) in the presence of excess peptides. (D) CD spectra of PiN and MuN peptides. CD spectra of the Pi-N peptide (a) has a characteristic minima of polyproline structure II at 206 nm, that shows that majority of the PiN peptide assumes an extended conformation. MuN peptide (b) has a minima below 200 nm and shows a typical pattern of random coil. *GST, radiolabelled GSTs.

nm, a characteristic of polyproline II structure, suggesting that the majority of the PiN peptide assumes an extended conformation. MuN peptide has a minima at around 200 nm by CD, which is closer to representing a random coil structure (Fig. 6D).

Since total SZP bound to sperm GSTs, it was of interest to determine the component of ZP that was primarily involved in ZP-GST binding. Goat ZP from mature oocytes separated predominantly into three bands in reducing SDS-PAGE (Fig. 7, lane a). Densitometric analysis of the bands from several zona preparations showed a slight variation in band density (band 1, 1362±47; band 2, 1874±114; band 3, 1999±103, *n*=6). Biotinylated sperm GSTs could bind to only band three (Fig. 7, lane b). This binding could be displaced by preincubation of the labelled GSTs with excess SZP (data not shown). Anti-ZP3 antibody directed towards a region of porcine ZP that is conserved among species (EEEKLVFSLRLM) recognised band three (Fig. 7, lane c); however, monoclonal antibodies towards a N-terminal peptide of porcine ZP (PQPVWQDQGQRL) did not recognise band three (data not shown). This suggested that sperm GSTs could bind to the ZP3-like component of goat ZP. N-terminal sequencing of this band gave a sequence of DVT/PA/NGPKPQMG. This had extremely low homology with the N-terminus of known ZP3 sequences from other species. It is of interest that the N-terminii of ZP3 is the least conserved region among the species (Zhu and Naz, 1999).

The above experiments suggested that sperm GSTs bound specifically to ZP in a saturable fashion. The next question was if sperm GSTs are able to bind to ZP, can specific blocking of binding sites for GSTs on the ZP or for the ZP on the sperm GSTs reduce the extent of sperm-oocyte binding. Prior to the blocking experiments, we labelled oocytes with the peptides and visually tested peptide binding. Distinct labelling of the oocytes treated with GST PiN and MuN peptides or sperm plasma membrane GSTs was visible (Fig. 8A-C). Inner cell masses of the oocytes were pushed out to rule out any staining

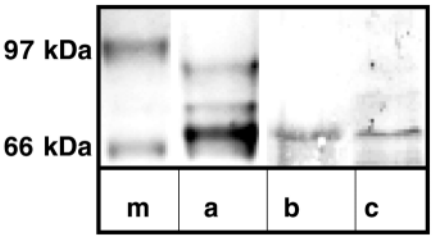

Fig. 7. Binding of biotinylated sperm GSTs to components of goat ZP. Glycoproteins of goat ZP on SDS-PAGE under reducing conditions separate into three bands (lane a). Lane b shows the binding of biotinylated sperm plasma membrane GSTs to band three of goat ZP. Lane c shows the binding of monoclonal antibody MA 451, raised against a ZP 3 peptide from a conserved region, to band three of goat ZP. m, marker.

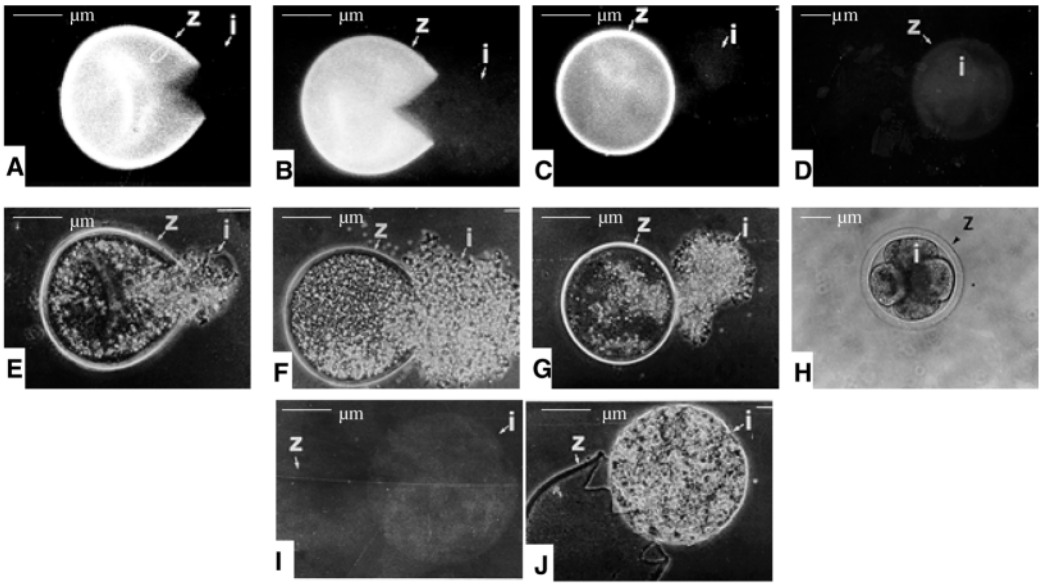

Fig. 8. Binding of GST Mu and Pi peptides to oocyte ZP. Oocytes were incubated with GST PiN and MuN peptides (10 µg/ml) for 30 minutes at 37°C followed by treatment with rabbit anti-GST PiN and MuN antibodies (1:100) and subsequently stained with anti-rabbit IgG (1:200) conjugated to FITC. This figure shows fluorescence photomicrograph of oocytes incubated with (A) total sperm plasma membrane GSTs. (B) GST PiN peptide. (C) GST MuN peptide. (D) Four-cell embryo incubated with total plasma membrane GSTs. (E-H) Phase-contrast photomicrographs of A-D, respectively. (I) A control oocyte treated with unrelated peptide (tritrypticin-VRRFPWWWPFLRR). (J) Phase contrast of I. Inner cytoplasmic masses (i) have been pushed out to rule out any background staining. Z, ZP. Bars, 100 µm.

Table 1. Effect of blocking of sperm GST-zona binding on sperm-oocyte interaction

A. Sperm-oocyte binding in the presence of anti-PiN, MuN, PiC and MuC antibodies bound to sperm

Groups	No. of sperm bound per oocyte (mean±s.e.m.)
1. Control (P.I.)	123±14.76
2. Anti-PiN	16±1.54
3. Anti-MuN	18±1.54
4. Anti-MuN + anti-PiN	15±2.50
5. Anti-PiC	122±12.3
6. Anti-MuC	120±11.0

(1) versus (2), (3) and (4) $P<0.0001$.

Sperm-oocyte binding in vitro in the presence and absence of relevant antibodies (1:100) bound to sperm. Each experiment was a pool of 25 ovaries with 90-100 oocytes per group, $n=6$. P.I., Preimmune sera.

B. Sperm-oocyte binding in the presence of sperm plasma membrane GST, GST PiN, MuN, PiC and MuC peptides bound to the oocyte

Groups	No. of sperm bound/oocyte (mean±s.e.m.)
1. Control	123±14.7
2. Sperm GST	6±0.4
3. PiN	17±1.5
4. MuN	15±1.5
5. PiC	120±8.7
6. MuC	115±15.2

(1) versus (2), (3) and (4), $P<0.0001$.

Sperm-oocyte binding in vitro in the presence and absence of relevant peptides, MuN, PiN, MuC and PiC (50 µg/ml) bound to oocytes. Each experiment was a pool of 25 ovaries with 90-100 oocytes/group, $n=4$. The control group did not contain any peptide in solution.

of oocyte cytoplasmic GSTs contributing to the staining of the oocyte ZP. Both the peptides and plasma membrane GSTs did not bind to the embryos. A representative figure is shown (Fig. 8D).

We blocked sites on the sperm GSTs using antibodies directed against two different regions of GST Pi and Mu. When sperm were treated with anti-GST PiN, MuN, PiC and MuC during capacitation, before incubation with mature oocytes, antibodies against GST PiN and MuN could reduce sperm oocyte binding by 87% and 85%, respectively, in comparison to those treated with preimmune sera (Table 1A). Antisera directed against the peptides (MuC and PiC) designed from the protein region involved in GSH binding (Table 1A) did not interfere with sperm-oocyte binding. No agglutination or immobilisation of the spermatozoa after treatment with the antibodies was observed; hence the reduction in binding was not caused by interference with sperm motility. When oocytes were preincubated with GST PiN and MuN peptides to block sperm GST-binding sites and subsequently incubated with sperm, a decreased level of sperm binding to oocytes (a reduction of 86% and 88%, respectively) was noted (Table 1B). PiC and MuC could not influence sperm-oocyte binding. Preincubation of oocytes with sperm GSTs also blocked sperm-oocyte binding.

Collectively, the above data suggested that reactive sites on the N-terminus of sperm GSTs were able to interact with counterparts in the ZP. Any blocking of the interacting molecules on either of the gametes reduced sperm-oocyte binding. The binding pattern of sperm GSTs to SZP followed typical saturation kinetics. The data also suggested ZP3 as the component on goat ZP required for binding to sperm GSTs.

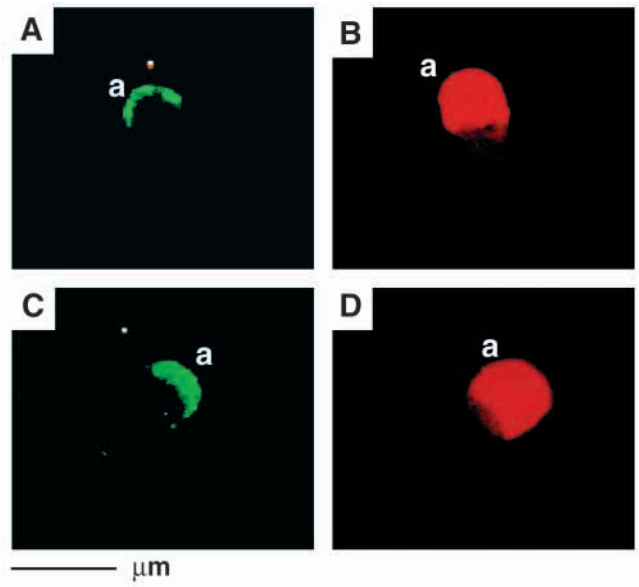

Fig. 9. Acrosomal status of sperm undergoing GST aggregation. To visualise aggregation of sperm-surface GSTs, capacitated sperm were incubated with SZP (10 μg/ml /10^7 sperm) at 37°C, and aliquots of sperm were fixed at different time points in 4% buffered paraformaldehyde. The movement of the GST molecules was visualised by staining with FITC-labelled secondary antibody (1:500). *Pisum sativum* agglutinin (PSA) conjugated to rhodamine (1:100) was used to stain the same samples to visualise the status of the acrosome. (A) Aggregation of sperm GST Pi on goat sperm surface after treatment with SZP at a 60 minute time point. B shows the same spermatozoa as in microphotograph A stained with rhodamine-labelled *Pisum sativum* agglutinin showing the intactness of the entire acrosome at 1 hour. (C) Aggregation of sperm GST Mu on goat sperm surface after treatment with SZP at a 60 minute time point. (D) The same spermatozoa as in microphotograph C stained with rhodamine-labelled *Pisum sativum* agglutinin showing the intactness of the entire acrosome at 1 hour. Bar, 10 μm.

GST aggregation either by SZP or anti-GST antibodies induce acrosin release

Multimeric ZP functionally activates the sperm by crosslinking its receptors on the surface of spermatozoa (Leyton and Saling, 1989). Since the above experiments indicate that sperm GSTs are involved in ZP binding, we checked whether crosslinking of GSTs on sperm could be precipitated by SZP. When capacitated sperm were incubated with SZP, aggregation of both GST-Pi and Mu occurred in a time-dependent manner (Table 2). Increase in the number of unlabelled sperm at the end of 2 hours shows the completion of acrosome reaction. The intactness of the acrosome during the process of patching and capping was checked by double labelling the same sperm with *Pisum sativum* agglutinin linked to rhodamine (Fig. 9). Treatment with anti-GST antibodies mimics the action of ZP and induces the aggregation of sperm membrane GSTs to form a cap-like structure on the acrosomal tip (Fig. 10A-D). Aggregation induced by anti-GST Pi antibodies is represented in Fig. 10. Anti-GST Mu shows similar pattern (data not shown). The aggregation of GSTs by anti-GST antibodies also induced the release of acrosin (Fig. 10E). No crosslinking and aggregation was observed with Fab fragments of the antibodies (data not shown). At higher dilutions (1:50), where there was no aggregation of GSTs, no acrosin release was observed (Fig. 10Ee,i). In these groups, crosslinking was accomplished with the help of secondary antibodies and thus the release of acrosin was observed (Fig. 10Ef,j). Acrosin release by SZP was used as a positive control. Insignificant acrosin release was observed in second antibody control - the data being similar to group ‘a’ (data not shown).

When SZP was used to induce acrosin release in sperm treated with antibodies (above 1:100 dilution) or the Fab fragments, acrosin release was inhibited as compared with the controls (Table 3). If SZP was preincubated with the GST peptides and then allowed to interact with the sperm, it could not induce an acrosin release (Table 3) presumably because of occupancy of GST-binding sites on zona showing that

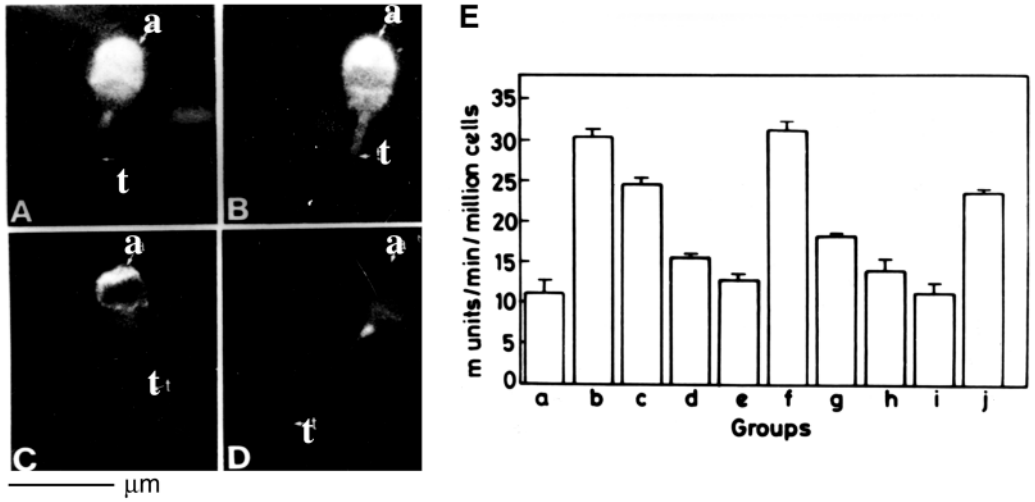

Fig. 10. Antibody-induced aggregation of sperm plasma membrane GSTs. Capacitated sperm were exposed to anti-GST antibodies and fixed at different time points in 4% buffered formaldehyde and immunostained using anti-GST Pi antibodies. (A) 0 minutes, (B) 15 minutes, (C) 30 minutes and (D) 60 minutes incubation of live spermatozoa with anti GST PiN antibodies (1:25) at 37°C. (E) The effect of antibody crosslinking of sperm plasma membrane GSTs on acrosin release. Acrosin release was measured after aggregation of GSTs on capacitated spermatozoa and was induced by anti-GST PiN antibodies. The bar shows mu/minute of acrosin released/10^6 sperm. Capacitated sperm were treated with (a) preimmune serum (1:25), (b) SZP (10 μg/ml), (c) anti-GST-Pi (1:10), (d) anti-GST-Pi (1:25), (e) anti-GST-Pi (1:50), (f) anti-GST-Pi (1:50) + antirabbit IgG (1:100), (g) anti-GSTMu (1:10), (h), Anti-GSTMu (1:25), (i) anti-GSTMu (1:50), and (j) anti-GSTMu (1:50) + antirabbit IgG (1:100). Anti-rabbit IgG (1:50) did not have any effect on acrosin release (data not shown). a versus b,c,f,g,j; e versus f; i versus j: $P<0.0001$. a versus d: $P<0.005$. Bar, 20 μm.

Table 2. Aggregation of GST Pi and Mu on sperm surface after exposure to SZP

Time (min)	Full staining (% sperm)	Patching (% sperm)	Capping (% sperm)	Unlabeled (% sperm)
GST Mu				
0	70.20	15.00	6.00	8.80
15	50.00	23.80	9.98	16.22
30	30.50	25.00	25.00	19.50
45	20.00	16.98	45.00	18.02
60	15.50	15.00	50.50	19.00
120	17.94	7.69	12.82	61.53
GST Pi				
0	74.12	12.62	6.98	6.28
15	50.62	20.38	15.25	13.75
30	33.33	20.83	20.38	25.46
45	19.25	15.00	40.00	25.75
60	20.00	15.95	45.05	19.00
120	15.00	8.25	20.75	56.00

Capacitated sperm were fixed and stained with anti-GST PiN and anti-GST MuN antisera (1:500) followed by labelling with FITC-conjugated secondary antibody at different time points after exposure to SZP (10 μg/ml). Sperm were scored for the type of staining as incubation progressed to visualise GST aggregation. This table is representative of one typical experiment out of four.

Table 3. Acrosin release under various treatments

Groups		Acrosin released (m.u acrosin/ minutes/10^6 sperm) (mean±s.e.m.)
1.	0 h uncapacitated sperm	3.8±0.6
2.	3 h capacitated sperm	12.3±0.44
3.	3 h capacitated sperm +SZP	23.4±1.07
4.	3 h capacitated sperm + SZP+ anti-PiN	10.5±1.37
5.	3 h capacitated sperm + anti-PiN (Fab) + SZP	12.3±1.01
6.	3 h capacitated sperm + SZP + anti-MuN	10.3±0.75
7.	3 h capacitated sperm + anti-MuN (Fab) + SZP	12.9±0.4
8.	3 h capacitated sperm + SZP adsorbed with PiN	12.9±2.4
9.	3 h capacitated sperm + SZP adsorbed with MuN	17.1±2.6

(2) versus (3), (3) versus (6), $P<0.0001$.
(3) versus (7), (4) and (5), $P<0.0003$.
(3) versus (8), $P<0.003$, (3) versus (9) $P<0.02$.

This table shows the comparative rate of (10 μg/ml) SZP-induced acrosin release by sperm treated with antibodies (1:100) or their Fab fragments (10 μg/ml) during capacitation. Acrosin release by capacitated sperm treated with SZP adsorbed with MuN and PiN peptides (50 μg/ml) (groups 8 and 9) is also shown (n=6, each sample is a pool of sperm from at least two cauda epididymides, each from a different animal).

the acrosin release is very much dependent on ZP-GST binding.

Ca^{2+} increase is affected by the inhibition of GST-zona binding

One of the prerequisites for the acrosome reaction to occur is the increase in intracellular Ca^{2+} (Fraser, 1998). When sperm capacitated in the presence or absence of anti-GST PiN and MuN were loaded with the Ca^{2+}-binding dye Fura-2AM and subsequently treated with SZP, it was observed that the post zona binding increase in intracellular Ca^{2+} was significantly less in the antibody-treated groups than in the controls (Fig. 11A). Groups where antibodies were adsorbed with excess peptide (GST PiN) prior to incubation with sperm, had antibodies that did not affect Ca^{2+} accumulation. Saturation of GST-binding sites on SZP with PiN and MuN peptides also inhibited the zona-induced increase in intracellular Ca^{2+} in capacitated sperm. Data obtained microscopically showed a similar pattern (Fig. 11B).

Discussion

The first phase of mammalian fertilisation is a recognition event that involves primary binding of receptor molecules associated with the plasma membrane of the respective gametes. Recent studies indicate that this process is complex and may involve multiple receptors on sperm that bind to ZP3, a component of the oocyte coat - the zona pellucida (McLeskey et al., 1998; Brewis and Wong, 1999).

In this paper, we make five conclusions: (1) GST activity is associated with the sperm plasma membrane, and this membrane has significantly higher associated GST activity than other somatic cell plasma membranes; (2) the sperm plasma membrane GSTs bind to the ZP3-like component of goat ZP; (3) the GST pool of sperm plasma membrane contain two isoforms, namely GST Pi and Mu; (4) both isoforms show saturable binding to the ZP; and (5) interference with sperm GST-ZP binding leads to functional impairment of spermatozoa, resulting in reduced sperm-oocyte binding.

GSTs are conventionally known as cytosolic enzymes; therefore, the observation pertaining to the presence of catalytically active GSTs on the sperm plasma membrane is of great importance. We provide evidence for the plasma membrane localisation of GSTs by several observations: (1)

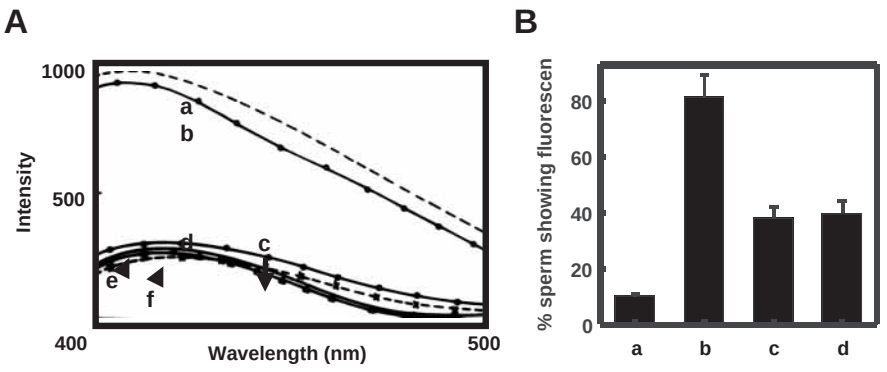

Fig. 11. Intracellular Ca^{2+} concentration of sperm after SZP-induced acrosome reaction. Capacitated spermatozoa (CSP) (10^7) were incubated with 1 μM of Ca^{2+}-binding dye Fura 2-AM at 37°C for 40 minutes and was incubated with SZP. The fluorescence pattern was subsequently measured. (A) a, CSP + SZP; b, CSP + anti-PiN adsorbed with PiN + SZP; c, (CSP + anti-PiN) + SZP; d, (CSP + anti-MuN) + SZP; e, CSP + SZP adsorbed with PiN; f, CSP + SZP adsorbed with MuN. (B) For microscopic estimation, the number of Fura 2-AM-positive sperm was scored under a Nikon Optiphot fluorescence microscope. a, CSP, b, CSP + SZP, c, (CSP + anti-PiN) + SZP, d, (CSP + anti-MuN) + SZP.

immunolocalisation of surface GSTs on live spermatozoa shown by immunostaining and FACS analysis (Gopalakrishnan et al., 1998b); (2) recovery of labelled GSTs from the surface iodinated live spermatozoa (Fig. 2A); (3) the sperm plasma-membrane had associated GST activities (Fig. 1); (4) inhibition of in vitro sperm-zona interaction by anti-GST antibodies (Table 1); (5) successful removal of sperm-surface GSTs by controlled trypsinisation of live spermatozoa (data not shown); (6) the presence of GSTs in high salt buffer extracts of sperm plasma membrane preparations (Fig. 3); and (7) translocation of around 10% of freshly synthesised GSTs to plasma membrane shown by ^{35}S-methionine incorporation in germ cells (data not shown).

The above data provide a clear indication of the plasma membrane association of GSTs on sperm. This association is particularly valuable to a cell such as sperm from the point of view of cellular defense. A detoxification enzyme on the sperm surface would be a great asset, as this particular cell is exposed to a variety of environments during its passage from the testis to the fallopian tube (Yanagimachi, 1981). In addition, the sperm plasma membrane is rich in oxidation-prone polyunsaturated fatty acids owing to its fusible nature (Aitken, 1999). This feature in combination with the deficiency of defensive enzymes in sperm owing to the presence of meagre cytoplasmic space (Russell et al., 1990) makes this cell more vulnerable to oxidative stress in comparison with any other cell type. It is an established fact that functional incompetence of sperm owing to lipid peroxidation of their membranes is a major cause of infertility in the male (Aitken, 1999). Since GSTs detoxify products of lipid peroxidation, their association with the plasma membrane is strategic, as the process of lipid peroxidation is initiated in lipid-containing structures such as membranes. One report identifies plasma-membrane-associated GSTs with a molecular mass of ~17 kDa (Horbach et al., 1994) in hepatocytes, a mass that is close to that of microsomal GSTs. However, the two isoforms of GSTs that we identify on the sperm plasma membrane have molecular masses of around 23.4 and 25.6 kDa. It is noteworthy that in our studies the activity of sperm plasma-membrane-associated GSTs was found to be around threefold higher than that of hepatic cell plasma membrane GSTs.

Since the mode of attachment of GSTs to the sperm plasma membrane was found to be peripheral, with no covalent interactions, it would be reasonable to speculate that the GSTs are anchored to the plasma membrane through an integral membrane protein. If this is true then the role of GSTs in sperm-oocyte interaction would be solely to recognise and bind to the ZP and help the integral protein to initiate signal transduction. Once bound to the ZP, the hypothetical integral membrane protein may transmit the signal required to trigger membrane fusion and acrosome reaction. Unpublished data from this laboratory with testicular germ cells that are progenitor cells of sperm show that around 10% of total cellular GSTs synthesised are transported to the plasma membrane. Sperm would have acquired the proteins during earlier stages of differentiation, as sperm themselves are transcriptionally inactive.

The orientation of the GSTs on the extracellular side of the plasma membrane, as shown by the recovery of labelled GSTs from live surface iodinated spermatozoa, together with our earlier observations of a reduced fertilisation rate when sperm were treated with anti-GST antibodies, prompted us to hypothesise that ZP has binding sites for sperm GSTs. Sperm GSTs fulfilled the essential criteria for any ZP-binding molecule, such as orientation towards the extracellular side of the apical region of the sperm head plasma membrane. A quantitative assessment of binding with steady-state equilibrium binding studies showed that both GST isoforms were able to bind to ZP, and, since one could compete out the binding of the other, it appears that both isoforms bind to the same site. The higher affinity of PiN peptides compared with MuN peptides for the ZP could be explained by their CD profiles. MuN peptides assume no distinct secondary structure and are randomly coiled or highly flexible, therefore, binding in this case would be an induced-fit type. In contrast, PiN peptide has an extended structure (polyproline II like) that is similar to the secondary structure of this peptide sequence in the original GST crystal structure (Oakley et al., 1997). In other words, PiN has a definitive structure, unlike MuN peptide, and that is possibly the reason for the higher binding affinity of PiN to ZP compared with MuN. Since the N-terminal sequences are well conserved within the GST classes and we found that the N-terminal sequence of goat blood cell GST is similar to goat sperm GSTPi, it was not surprising that blood cell GSTs could also bind to ZP, although this is biologically inappropriate. Therefore, GSTs have the structural requirements to bind to the oocyte ZP. It is possible that during evolution the system might have used this property to its advantage to meet the unusual demands of the spermatozoa.

We used total SZP in binding studies, as it is known that components other than the primary binding component assist in ZP binding to sperm receptors (Yurewicz et al., 1998); however, it was of interest to identify the primary component on ZP that binds to sperm GSTs. The binding of sperm GST to the ZP3 component of the goat ZP indicates similarity with other species, such as mice, where ZP3 acts as the primary sperm receptor (McLeskey et al., 1998). Unpublished data from this laboratory shows that mannose and N-oligosaccharides do not play a role in ZP-GST binding unlike in the bovine system, which is close to goat in phylogeny, where N-oligosaccharides are involved in sperm-ZP interactions (Nakano et al., 1996). This however, does not rule out the possibility of sperm binding to ZP through mannose residues that are involved with other sperm proteins. Since the characterisation of goat ZP is yet to be done, the types of oligosaccharide present in goat zona and their role in GST binding have to be carefully investigated.

Given the importance of ZP binding to sperm, which brings about functional changes in the male gamete (Breitbart et al., 1997), the inhibition of ZP-sperm GST binding should theoretically interfere with the required changes in sperm and consequently disrupt the sperm-oocyte interaction. Since the in vitro fertilisation system provides an excellent tool to dissect the phases of fertilisation (Frayne and Hall, 1999), we used this system and several reagents that either bound to GSTs on sperm or GST recognition sites on the ZP to decipher the precise phase of fertilisation in which sperm GSTs were involved. Blocking of GST-zona binding by either anti-GST antibodies or N-terminal peptides could functionally impair spermatozoa in terms of acrosin release and binding to the oocyte, showing that sperm GST-ZP binding was a necessary event for successful fertilisation. This was in contrast to the

inability of peptides (GST PiC and MuC), designed from a region of the GST Pi and Mu involved in GSH binding or antibodies against them, to block the gamete interaction. The inability of these antibodies to block such events was not caused by inaccessibility of the antibodies to the desired region as they recognised live sperm in indirect immunofluorescence (data not shown). Therefore, it is possible that the GSH binding site has no role in sperm-oocyte binding. The above observations have clearly demonstrated the importance of the N-terminal regions of GST Pi and Mu in the gamete recognition process. The inability of the antibodies to induce agglutination or immobilisation of spermatozoa after treatment with antibodies confirms that reduced binding is not caused by lack of motility or cell agglutination. It is known that once the oocyte is fertilised, embryos become refractory to molecules involved in primary binding (Yanagimachi, 1981); therefore, binding of the GSTs and peptides to the oocyte but not the embryos further confirm the possibility of sperm GSTs as primary recognition molecules in sperm-oocyte interactions.

As SZP could aggregate sperm GSTs and prevention of binding of SZP to sperm led to the inhibition of acrosin release, sperm GSTs must have a role in post zona binding functions of sperm. This was further corroborated by the observed inhibition of the intracellular Ca^{2+} increase that is a prerequisite for acrosome reaction (Publicover and Barratt, 1999) when anti-GST PiN and MuN antibodies were used to block the sperm GST-zona interaction. An interpretation of this data would be that initiation of acrosome reaction was inhibited owing to the inability of the cell to acquire the required level of intracellular Ca^{2+}, which is caused by inhibition of ZP binding to sperm GSTs.

In the past decade, there has been a considerable effort to identify the proteins and pathways utilised during gamete recognition, and the molecular bases of this process are beginning to be understood. Zona-binding candidates identified include beta 1, 4-galactosyltransferase (Miller et al., 1992; Miller et al., 1993) and sperm adhesins (Topfer-Petersen and Calvete, 1995), in addition to some others (McLeskey et al., 1998). The existence of multiple receptors indicate that sperm-zona binding may involve either a complex of receptors, consisting of different members, or redundancy built into the system because of the important nature of the event. It is also possible that a species-specific preference for a particular molecule may exist that justifies the presence of a molecule on sperm of several species but may be functionally important only in selected groups. One such example is beta galactosyltransferase, which is present on the surface of sperm of multiple species, but it is functionally important in only a few (Rebeiz and Miller, 1999). The multiple sperm-surface components that have been implicated in zona binding may or may not function in concert with sperm GSTs.

The ability of the cells to make multifunctional proteins is of fundamental importance to the course of evolution. As sperm cells are terminally differentiated and possess a variety of functional domains, it is possible that GSTs have evolved to function in the very unique structural environment of this cell. Therefore, the ability of GSTs to serve as receptors for cell-cell interactions, which is evident from this study, and as protective enzymes observed in our earlier studies might reflect the unusual demands the transcriptionally inactive sperm meets in different parts of the reproductive tract to reach the oocyte. It is possible that the presence of two isoforms on the surface gives the cell a larger flexibility in terms of dealing with toxins. The delineation of dual function of GSTs in sperm opens an interesting prospect for future studies into the mechanisms of infertility. Defects in either zona-binding capacity or the catalytic function of these molecules may be responsible for certain forms of infertility.

In summary, our studies clearly demonstrate that GSTs on sperm membranes serve very important functions. In addition to being enzymatically active, they mediate the binding of ZP to sperm, which brings about important prefertilisation changes.

This work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India. We are grateful to S.K. Gupta of the National Institute of Immunology for providing the monoclonal antibodies against ZP3.

References

Aitken, R. J. (1999). The Amoroso Lecture. The human spermatozoon–a cell in crisis? *J. Reprod. Fertil.* **115**, 1-7.

Aravinda, S., Gopalakrishnan, B., Dey, C. S., Totey, S. M., Pawshe, C. H., Salunke, D., Kaur, K. and Shaha, C. (1995). A testicular protein important for fertility has glutathione S-transferase activity and is localised extracellularly in the seminiferous tubules. *J. Biol. Chem.* **270**, 15675-15685.

Avrameas, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochemistry* **6**, 43-52.

Breitbart, H., Rubinstein, S. and Lax, Y. (1997). Regulatory mechanisms in acrosomal exocytosis. *Rev. Reprod.* **2**, 165-174.

Brewis, I. A. and Wong, C. H. (1999). Gamete recognition: sperm proteins that interact with the egg zona pellucida. *Rev. Reprod*. **4**, 135-142.

Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W. (1992). In vitro assays for mouse lymphocyte function, I. Isolation and fractionation of mononuclear cell population: Isolation of mouse mononuclear cells. *In Current Protocols in Immunology*, Vol. 1 (ed. J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach and W. Strober), pp. 3.1.2-3.1.5. New York: John Wiley and Sons.

Ferreira, A. and Loomis, P. (1998). Isolation and culture of primary neuronal cells. In *Cells, A Laboratory Manual*, Vol I (D. L. Spector, R. D. Goldman and L. A. Leinwand eds), pp 9.1-9.13. New York: Cold Spring Harbor Laboratory Press.

Fraser, L. R. (1998). Sperm capacitation and the acrosome reaction. *Hum. Reprod.* **13 Suppl. 1**, 9-19.

Frayne, J. and Hall, L. (1999). Mammalian sperm-egg recognition: does fertilin beta have a major role to play? *BioEssays* **21**, 183-187.

Glastris, B. and Pfieffer, S. B. (1974). Mammalian membrane marker enzymes: sensitive assay for 5′-nucleotidase and assay for mammalian 2′,3′-cyclic-nucleotide-3′-phosphohydrolase. *Methods Enzymol.* **32**, 124-131.

Gopalakrishnan, B. and Shaha, C. (1998a). Inhibition of sperm glutathione S-transferase leads to functional impairment due to membrane damage. *FEBS Lett.* **422**, 296-300.

Gopalakrishnan, B., Aravinda, S., Pawshe, C. H., Totey, S. M., Nagpal, S., Salunke, D. M. and Shaha, C. (1998b). Studies on glutathione S-transferases important for sperm function: evidence of catalytic activity-independent functions. *Biochem. J.* **329**, 231-241.

Gorczynska, E. and Handelsman, D. J. (1993). Requirement for transmembrane sodium flux in maintenance of cytosolic calcium levels in rat Sertoli cells. *Am. J. Physiol.* **264**, E863-E867.

Gupta, S. K., Yurewicz, E. C., Afzalpurkar, A., Rao, K. V., Gage, D. A., Wu, H. and Sacco, A. G. (1995). Localisation of epitopes for monoclonal antibodies at the N-terminus of the porcine zona pellucida glycoprotein pZPC. *Mol. Reprod. Dev.* **42**, 220-225.

Hayes, J. D. and Pulford, D. J. (1995). The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* **30**, 445-600.

Horbach, M. E., Sies, H. and Akerboom, T. P. (1994). Purification of rat

liver plasma membrane glutathione transferase. *Eur. J. Biochem.* **222**, 91-96.

Jungbauer, A., Tauer, C., Reiter, M., Purtscher, M., Wenisch, E., Steindl, F., Buchacher, A. and Katinger, H. (1989). Comparison of protein A, protein G and copolymerised hydroxyapatite for the purification of human monoclonal antibodies. *J. Chromatograph.* **476**, 257-268.

Kaul, R., Afzalpurkar, A. and Gupta, S. K. (1997). Expression of bonnet monkey (Macaca radiata) zona pellucida-3 (ZP3) in a prokaryotic system and its immunogenicity. *Mol. Reprod. Dev.* **47**, 140-147.

Kaur, K. J., Khurana, S. and Salunke, D. M. (1997). Topological analysis of the functional mimicry between a peptide and a carbohydrate moiety. *J. Biol. Chem.* **272**, 5539-5543.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **15**, 680-685.

Leyton, L. and Saling, P. (1989). Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. *J. Cell Biol.* **108**, 2163-2168.

Manoharan, T. H. (1992). Structural studies on human glutathione S-transferase. *J. Biol. Chem.* **267**, 18940-18945.

McLeskey, S. B., Dowds, C., Carballada, R., White, R. R. and Saling, P. M. (1998). Molecules involved in mammalian sperm-egg interaction. *Int. Rev. Cytol.* **177**, 57-113.

Miller, D. J., Macek, M. B. and Shur, B. D. (1992). Complementarity between sperm surface beta-1,4 galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. *Nature* **357**, 589-593.

Miller, D. J., Gong, X. and Shur, B. D. (1993). Sperm require beta-N-acetylglucosaminidase to penetrate through the egg zona pellucida. *Development* **118**, 1279-1289.

Mukherjee, S. B., Aravinda, S., Gopalakrishnan, B., Nagpal, S., Salunke, D. M. and Shaha, C. (1999). Secretion of glutathione S-transferase isoforms in the seminiferous tubular fluid, tissue distribution and sex steroid binding by rat GSTM1. *Biochem. J.* **340**, 309-320.

Nakano, M., Yonezawa, N., Hatanaka, Y. and Noguchi, S. (1996). Structure and function of the N-linked carbohydrate chains of pig zona pellucida glycoproteins. *J. Reprod. Fertil. Suppl.* **50**, 25-34.

Oakley, A. J., Bello, M. L., Battistoni, A., Ricci, G., Rossjohn, J., Villar, H. O. and Parker, M. W. (1997). The structures of human glutathione transferase P1-1 in complex with glutathione and various inhibitors at high resolution. *J. Mol. Biol.* **274**, 84-100.

Polakoski, K. L. and Zaneveld, L. J. D. (1977). Biochemical examination of the human ejaculate. *In Techniques of Human Andrology* (ed. E. S. E. Hafez), pp. 265-286. Amsterdam: Elsevier/North Holland Press.

Publicover, S. J. and Barratt, C. L. (1999). Voltage-operated Ca2+ channels and the acrosome reaction: which channels are present and what do they do? *Hum. Reprod.* **14**, 873-879.

Rao, A. V. and Shaha, C. (2001). Multiple glutathione S-transferase isoforms are present on male germ cell plasma membrane. *FEBS Lett.* **507**, 174-180.

Rebeiz, M. and Miller, D. J. (1999). Porcine sperm surface beta1,4 galactosyltransferase binds to the zona pellucida but is not necessary or sufficient to mediate sperm-zona pellucida binding. *Mol. Reprod. Dev.* **54**, 379-387.

Russell, L. D., Ettlin, R. A., Hikim, A. P. and Clegg, E. D. (1990). Histopathology of the testis. *In Histological and Histopathological Evaluaton of the Testis* (eds L. D. Russell, R. A. Ettlin, A. P. Hikim and E. D. Clegg), pp 210-239. Clearwater, FL: Cache River Press.

Scott, L., Schell, M. J. and Hubbard, A. L. (1993). Isolation of plasma membrane sheets and plasma membrane domains from rat liver. In *Biomembrane Protocols. I. Isolation and Analysis* (*Methods in Molecular Biology*) (eds J. M. Graham and J. A. Higgins), pp 51-58. Totowa, NJ: Humana Press, Inc.

Shaha, C., Sheshadri, T., Suri, A. and Talwar, G. P. (1991). Characterization of 24-kD proteins from rat testes using polyclonal sera reactive to human sperm antigens. *Mol. Reprod. Dev.* **29**, 302-311.

Shaha, C., Suri, A. and Talwar, G. P. (1988). Identification of sperm antigens that regulate fertility. *Int. J. Androl.* **11**, 479-491.

Shur, B. D. and Neely, C. A. (1988). Plasma membrane association, purification, and partial characterization of mouse sperm beta 1,4-galactosyltransferase. *J. Biol. Chem.* **263,** 17706-17714.

Sies, H., Dafre, A. L., Ji, Y. and Akerboom, T. P. (1998). Protein S-thiolation and redox regulation of membrane-bound glutathione transferase. *Chem. Biol. Interact.* **24**, 177-185.

Spencer, C. A. and Nicoloff, J. T. (1980). Improved radioimmunoassay for human TSH. *Clin. Chim. Acta.* **108**, 415-424.

Springhorn, J. P. (1998). Isolation and culture of adult rat ventriculocytes. *In Cells, A Laboratory Manual*, Vol. 1 (eds D. L. Spector., R. D Goldman and L. A. Leinwand), pp. 12.1-12.12. New York: Cold Spring Harbor Laboratory Press.

Topfer-Petersen, E. and Calvete, J. J. (1995). Molecular mechanisms of the interaction between sperm and the zona pellucida in mammals: studies on the pig. *Int. J. Androl.* **18 Suppl. 2**, 20-26.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Vaitukaitis, J. L., Robbins, J. B., Neischlag, E. and Ross, G. T. (1971). A method for producing specific antisera with small doses of immunogen. *J. Clin. Endocrinol. Metab.* **33**, 988-991.

Warholm, M., Guthenberg, C., Bahr, C. and Mannervik, B. (1985). Glutathione transferases from human liver. *Methods Enzymol.* **113**, 499-504.

Yanagimachi, R. (1981). Mechanisms of fertilisation in mammals. *In Fertilisation and Embryonic Development in vitro* (eds L. Mastorianni and J. D Biggers), pp. 81-182. New York: Plenum Press.

Yurewicz, E. C., Sacco, A. G., Gupta, S. K., Xu, N. and Gage, D. A. (1998). Hetero-oligomerization-dependent binding of pig oocyte zona pellucida glycoproteins ZPB and ZPC to boar sperm membrane vesicles. *J. Biol. Chem.* **273**, 7488-7494.

Zhu, X. and Naz, R. K. (1999). Comparison of ZP3 protein sequences among vertebrate species: to obtain a consensus sequence for immunocontraception. *Front. Biosci.* **4**, d212-d215.