Studies on glutathione S-transferases important for sperm function: evidence of catalytic activity-independent functions

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Our earlier studies reported the identification of a rat testicular protein of 24 kDa with significant similarity at the N-terminus with Mu class glutathione S-transferases (GSTs). Treatment of goat sperm with antisera against this protein identified immunoreactive sites on the spermatozoa and inhibited *in vitro* fertilization of goat oocytes by the antibody-treated sperm. The above observations indicated the presence of GST-like molecule(s) important for fertility related events on goat spermatozoa. In this study, we report the purification of goat sperm GSTs (GSP1) which were purified by glutathione affinity chromatography and were enzymically active towards 1-chloro-2,4,-dinitrobenzene, a general GST substrate, and ethacrynic acid, a substrate for Pi class GSTs. GSP1 resolved into three major components on reverse-phase HPLC: peaks 1 and 2 with molecular masses of 26.5 kDa and peak 3 with a molecular mass of 25.5 kDa, as determined by SDS/PAGE. Multiple attempts to obtain N-terminal sequences of the first two peaks failed, indicating N-terminal block; however, they reacted to specific anti-Mu-GST antisera on Western blots and ELISA, and not to anti-Pi-GST antisera, which provides evidence for the presence of Mu-GST-reactive sites on peaks 1 and 2. The third component showed 80 % N-terminal similarity with human and rat GSTP1-1 over an overlap of 15 amino acids, and reacted to anti-Pi-specific antisera in ELISA. Sperm labelled with antibodies against a 10-mer and an 11-mer peptide, designed from the N-terminal sequences of Mu and Pi class GSTs respectively, showed the presence of both Mu- and Pi-GST on goat sperm surface at distinct cellular domains. Selective inhibition of Pi class GST by the Pi-specific antisera, either at 0 h or at 3 h after initiation of sperm capacitation, leads to a reduction in fertilization rates. In contrast, the inhibition of Mu class GST by specific antisera at 0 h does not inhibit fertilization, although such treatment at 3 h after the initiation of capacitation reduces fertilization rates. The results indicate that both Pi- and Mu-GSTs are involved in fertilization, but the Mu-GST sites essential for fertilization are exposed only after 3 h of capacitation. The enzymic activity of GSP1 or live spermatozoa is not inhibited by the two antisera. The inability of the antibodies to cause such inhibition indicates that the reduction in fertilization rates and acrosome reaction caused by the antibodies is through a mechanism which does not interfere with the catalytic activity of the molecule. Therefore we established the presence of Pi and Mu class GST on goat sperm, their localization and their possible function in fertility-related events.

INTRODUCTION

Earlier reports from this laboratory identified a 24 kDa rat testicular protein with glutathione S-transferase (GST) activity and significant sequence similarity at the N-terminus to Mu class GSTs [1]. Our initial findings on the 24 kDa protein showed it to be glycosylated [2] and present on sperm acrosomes of multiple species [3]. Antibodies against the 24 kDa protein, which is a potent immunogen in mice and rats [4–6], interfered with fertility *in vivo* [5,6] and inhibited mouse sperm–oocyte interactions *in vitro* [7]. This GST-like protein was shown to be important for fertility-related events and was most abundant in the seminiferous tubular fluid of the rat testis. A time-dependent accumulation of the same protein was observed in spent media of rat seminiferous tubule cultures, suggesting secretion *in vitro* by tubular cells. Antibodies against the protein identified immunoreactive epitopes on goat spermatozoa, indicating the presence of common antibody-reactive sites in both species. The fertilizing ability of goat sperm was reduced when they were treated with the same antisera [1]. It was evident from the above findings that the 24 kDa protein was a member of the GST family and was important for sperm function. GST activity has been reported on human sperm [8] and a recent report detailed the identification of a unique Mu class GST in mouse spermatogenic cells [9]. No information regarding localization of GSTs on sperm or their function is available. Known forms of GSTs include cytosolic [10] and microsomal [11] forms serving as detoxification enzymes or intracellular binding proteins [12]. Interest was therefore generated in how a molecular species of cytosolic or microsomal origin was involved in the event of sperm–oocyte interaction.

In the present paper we provide evidence that both Pi-GST and Mu-GST classes are present on goat sperm, although the distribution pattern of the two molecules during epididymal maturation and capacitation *in vitro* are different. Evidence for a potential role(s) of this molecule in fertilization-related events is provided by studies where antibodies directed against the N-termini of Pi-GST and Mu-GST when present on goat sperm are able to inhibit *in vitro* fertilization of goat oocytes. The same antibodies are capable of interfering with acrosome reaction *in vitro*. Interestingly, we find that this inhibition of acrosome reaction and fertilization by the antibodies is through a mechanism which does not interfere with the catalytic activity of the

Abbreviations used: GST, glutathione S-transferase; NP-40, Nonidet P-40; GSP1, purified goat-sperm protein; MuN, 10-mer peptide synthesized against N-terminus of Mu-GST; PiN, 11-mer peptide synthesized against N-terminus of GSP1; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid; IIF, indirect immunofluorescence; CDNB, 1-chloro-2,4-dinitrobenzene; SPTL, sperm Tyrode's lactate medium.

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molecule. To our knowledge, this is the first report of the identification of two different classes of GSTs on sperm, their localization pattern and evidence for their involvement in sperm function through a mechanism which is independent of the catalytic activity of the molecule.

EXPERIMENTAL

Materials

Testes of goat (*Capra hircus*) were obtained from the local slaughterhouse along with the cauda epididymides, and sperm were collected by puncturing the cauda in PBS (50 mM, pH 7.0).

Goat anti-rabbit and anti-mouse IgG antibodies conjugated to horseradish peroxidase and fluorescein isothiocyanate were procured from Jackson Laboratories (West Grove, PA, U.S.A.). GSH was purchased from SISCO Research Laboratories (Bombay, India). The bicinchoninic acid protein assay reagent was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). The GST purification module and Protein G–Sepharose Fast Flow kit were obtained from Pharmacia Biotech (Uppsala, Sweden). The peroxidase-staining kit and *Pisum sativum* agglutinin conjugated to FITC was procured from Vector Laboratories (Burlingame, CA, U.S.A.). Problott™ membranes and reagents for synthesis and conjugation of peptides were from Applied Biosystems Inc. (Foster City, CA, U.S.A.). Rat liver GST and other chemicals, unless otherwise specified, were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.).

Percoll purification of spermatozoa

Percoll purification of sperm was carried out according to the method of Totey et al. [13]. Briefly, goat cauda epididymal sperm were washed in sperm Tyrode's lactate medium (SPTL; 120 mM NaCl/3.2 mM KCl/0.34 mM NaH_2PO_4/10 mM sodium lactate/0.5 mM $MgCl_2$/2 mM $CaCl_2$/2 mM $NaHCO_3$) and 2 ml of sperm suspension at a concentration of 10^6 per ml was loaded on to a 45–90 % Percoll gradient with the 90 % Percoll at the bottom. The tubes were spun at 400 ***g*** for 40 min and viable motile sperm, which settled at the bottom of the tubes, were collected and resuspended in SPTL.

Measurement of sperm viability and integrity of acrosomes

The viability of the sperm was measured by staining the cells with 0.1 % Trypan Blue after excision from the epididymis, following Percoll purification and during *in vitro* capacitation. The integrity of the acrosomes was checked by staining with *P. sativum* linked to FITC [14]. Briefly, sperm were stained with a 1:100 dilution of *P. sativum* agglutinin for 1 h at room temperature after Percoll purification and after calcium ionophore treatment to check the status of the acrosomes.

Cell extracts and membranes

Sperm extracts were prepared by treatment of cauda sperm with 0.1 % Nonidet P-40 (NP-40) for 30 min in lysis buffer (0.14 M NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4, pH 7.4) in the presence of protease inhibitors (1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.17 units/ml aprotinin, 1 μg/ml pepstatin and 1 μg/ml parahydroxymercuric benzoic acid) on ice, centrifuged at 750 ***g*** for 30 min at 4 °C and stored at −20 °C until used.

Sperm membranes were prepared with slight modification by the method of Luzio and Bailyes [15]. Briefly, sperm recovered from the epididymis were homogenized on ice in 50 mM Tris/HCl/1 mM EDTA, pH 7.4, containing 0.25 M sucrose, using a hand-operated loose fitting homogenizer (10 strokes). The homogenate was centrifuged at 1000 ***g*** for 10 min at 4 °C and the resulting supernatant was centrifuged at 100000 ***g*** for 90 min in a Beckman TL-1000 ultracentrifuge with an SN863 rotor. Pellets were resuspended in 50 mM Tris/HCl/1 mM EDTA, pH 7.4, and 1 % sodium deoxycholate and dialysed against the same buffer without sodium deoxycholate. After protein estimation this preparation was subjected to GSH affinity chromatography.

Analytical techniques

GSTs from cell extracts were purified by GSH affinity chromatography as described previously [1]. The purified sperm protein shall be referred to henceforth as GSP1. Protein concentration was estimated by the method of Bradford [16] or by bicinchoninic acid assay [17] using BSA as the standard.

Reverse-phase (RP)-HPLC analyses with the affinity-purified protein was carried out on a RP Vydac C_{18} column (Type 218TP54) equilibriated with 60 % solvent 1 [0.08 % trifluoroacetic acid (TFA) in water] and 40 % solvent 2 (0.08 % TFA in 80 % acetonitrile). A gradient of 40–65 % solvent was developed over a period of 10 min followed by a gradient of 65–75 % over the next period of 50 min. The elution profile was monitored by UV absorption at 220 nm.

SDS/PAGE analysis was carried out using the buffer system described by Laemmli [18] on 12 % polyacrylamide gels. Silver staining of polyacrylamide gels was done according to the method of Switzer et al. [19]. Western blotting was performed following a modification of the method of Towbin et al. [20], as described previously [1], using primary and secondary antibodies at dilutions of 1:1000 and 1:5000 respectively. Reaction products were revealed with a peroxidase-staining kit. For N-terminal amino acid sequence analysis purified proteins were transferred on to PVDF or Problott™ membranes and were sequenced using an Applied Biosystems 470A pulsed-liquid-phase protein sequencer as previously described [1].

Enzyme assays

GST assay was carried out as described by Warholm et al. [21] with some modifications, as described previously [1]. Analysis of enzyme activity inhibition was carried out in the absence or presence of GST inhibitors [*S*-hexylglutathione, ethacrynic acid and *S*-(*p*-azidophenacyl)glutathione] at varying concentrations. For studying GST inhibition by anti-GST antibodies, GSP1 (250 μg/ml) was preincubated with a 1:50 dilution of anti-PiN, anti-MuN (these are defined in the sections Peptide synthesis and conjugation, and Antibodies, below), anti-GSP1 antisera and preimmune sera for 0, 15, 30 and 60 min at 37 °C before assaying with the antigen–antibody complex for GST activity. To confirm association of the enzyme activity with the antigen–antibody complexes, precipitation was carried out by incubating GSP1 (250 μg/ml) with various antisera (1:50) at 37 °C for 40 min, with subsequent treatment with Protein G in 20 mM phosphate buffer, pH 7.0, for 1 h at room temperature. The antigen–antibody–Protein G complexes were precipitated by centrifugation at 60 ***g***. Enzyme activity was measured in the supernatant as well as in the precipitate.

A GST assay with live spermatozoa was carried out using 10^7 or 10^6 cells with 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid as substrates, either in GST buffer without EDTA or in an isotonic medium of 20 mM PBS [22], in the presence and absence of various antisera at a 1:50 dilution.

Peptide synthesis and conjugation

To raise antisera against two separate GST classes, Mu-GST and Pi-GST, two peptides were designed based on the reported N-terminal sequences of Pi-GST and Mu-GST. A 11-mer peptide was designed with 80 and 90 % similarity to rat and human Pi-GST with the sequence Lys-Pro-Pro-Tyr-Thr-Ile-Val-Tyr-Phe-Pro-Val and designated PiN. A 10-mer peptide with the sequence Lys-Pro-Met-Thr-Leu-Gly-Tyr-Trp-Asp-Ile coding for the N-terminus of the 24 kDa GST-like rat testicular protein [1] similar to rGSTM2/M3 and with 90 % similarity to human Mu, was synthesized and designated MuN. Peptide synthesis was performed using Applied Biosystems model 431A peptide synthesizer and the standard synthesis protocol [23]. The synthesized peptides were conjugated to diptheria toxoid using the glutaraldehyde method [24].

Antibodies

Antisera against GSP1, the two synthetic peptides PiN (anti-PiN) and MuN (anti-MuN) were raised in either Balb/c inbred mice or in at least two female New Zealand white rabbits for each protein using standard methods [25]. Antibody titres were monitored by ELISA and Western blots as described previously [26].

Indirect immunofluorescence (IIF) and flow cytometry

To show surface localization, live suspensions of spermatozoa were stained at 4 °C with primary and secondary antibodies and all washings were carried out in the cold [27]. For flow cytometry, after the third wash following secondary antibody incubations, propidium iodide (1 μg/ml) was added as a vital dye [28]. Sperm fixed in 4 % paraformaldehyde were stained as described by Shaha [29] using all three anti-GST antibodies, with preimmune sera alone or immune sera adsorbed with excess antigen (1 μg/ml). The dilution of primary sera used was 1:100 and that of secondary antibody was 1:250. Each IIF experiment was repeated at least five times.

Flow cytometry of live epididymal sperm and 4 % paraformaldehyde-fixed sperm captured at different time points of *in vitro* capacitation, and also of acrosome-reacted sperm, stained with rabbit anti-PiN and anti-MuN antisera or control antisera, was carried out as described previously [29] on a WinBryte flow cytometer (Bio-Rad, Herts, U.K.) using Winbryte software. Fluorescence data for 10000 cells was collected with logarithmic amplification for green fluorescence. Each experiment was repeated at least three times.

Capacitation and acrosome reaction

Epididymal sperm were Percoll purified as described in the section Percoll purification of spermatozoa. Briefly, around 10^7 cells were capacitated in 1 ml of SPTL with 12 mg/ml fatty acid-free BSA and 1 μg/ml heparin for a total of 6 h under various experimental conditions. The percentage of acrosome-reacted sperm was measured by Naphthol Yellow staining [30].

Acrosome reaction was induced in goat sperm capacitated for 3 or 6 h by adding 10 μg/ml of calcium ionophore A23187 to 1×10^7 cells in Hepes medium (0.14 M NaCl/4 mM KCl/4 mM Hepes, pH 7.4/10 mM glucose/2 mM $CaCl_2$) at 37 °C [31].

To observe the effect of antibodies on acrosome reaction without calcium ionophore, sperm were treated with the antibodies at 0 h and capacitation was allowed to proceed with the acrosome reaction measured at the end of 6 h by Naphthol Yellow staining.

In vitro fertilization studies

To determine the effect of antisera against various GST molecules, the goat *in vitro* fertilization system was used as a model [32]. Briefly, sperm were incubated with anti-GST antisera or control preimmune sera at a dilution of 1:50 for 30 min and washed before incubation with the oocytes. Incubation with the oocytes was carried out for 22 h at 38 °C under 5 % CO_2 in air. At the end of incubation, oocytes were fixed with acetic acid and ethanol (1:3) and stained with Aceto Orcein. Oocytes were classified as normal fertilized (2P) when the female and male pronuclei were visible, and as polyspermic (Poly) if multiple pronuclei were seen. They were considered unfertilized when only one pronucleus (1P) or the metaphase spindle (M II) was observed. Each experiment was repeated at least three times.

Data analysis

The fertilization frequencies and the rate of acrosome reaction were compared between the various treatment groups using one way analysis of variance [33]. Tadpole III software (Elsevier Biosoft, Cambridge, U.K.) was used for the analysis.

RESULTS

GSP1 is similar to GST

GSP1 was purified from goat-sperm extract by GSH affinity chromatography. The GSH column retains about 78 % of the GST that is present in the total sperm extract. This protein showed saturable GST activity with varying concentrations of CDNB and ethacrynic acid, with K_m values of 55.5 μM and

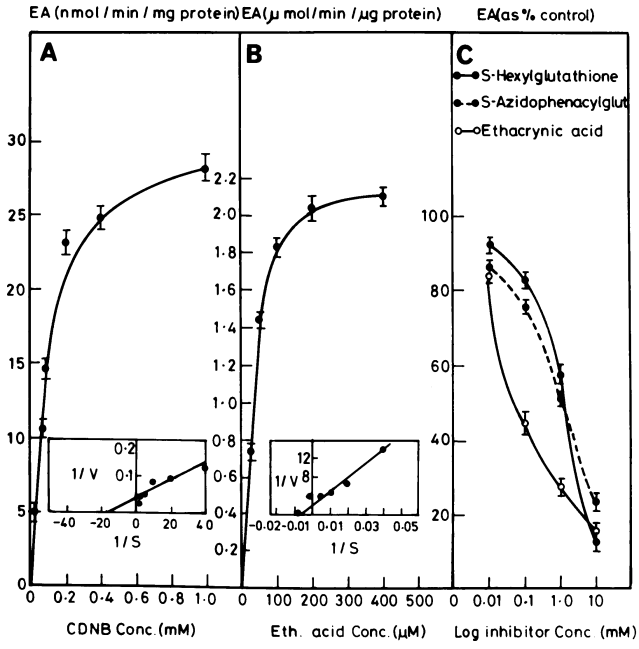

Figure 1 Enzyme kinetics of GSP1

(**A**) Saturable enzyme activity with increasing concentrations of CDNB as substrate. The inset is the Lineweaver–Burk plot for calculation of K_m which is 55 μM. (**B**) GST activity with increasing concentrations of ethacrynic (Eth.) acid as substrate. The inset shows the Lineweaver–Burk plot for calculation of K_m (133 μM). (**C**) Inhibitor profile of GSP1 with CDNB as substrate. IC_{50} values obtained with *S*-hexylglutathione, *S-p*-azidophenacylglutathione and ethacrynic acid with CDNB as substrate are 1.1 mM, 1.18 mM and 0.05 mM respectively.

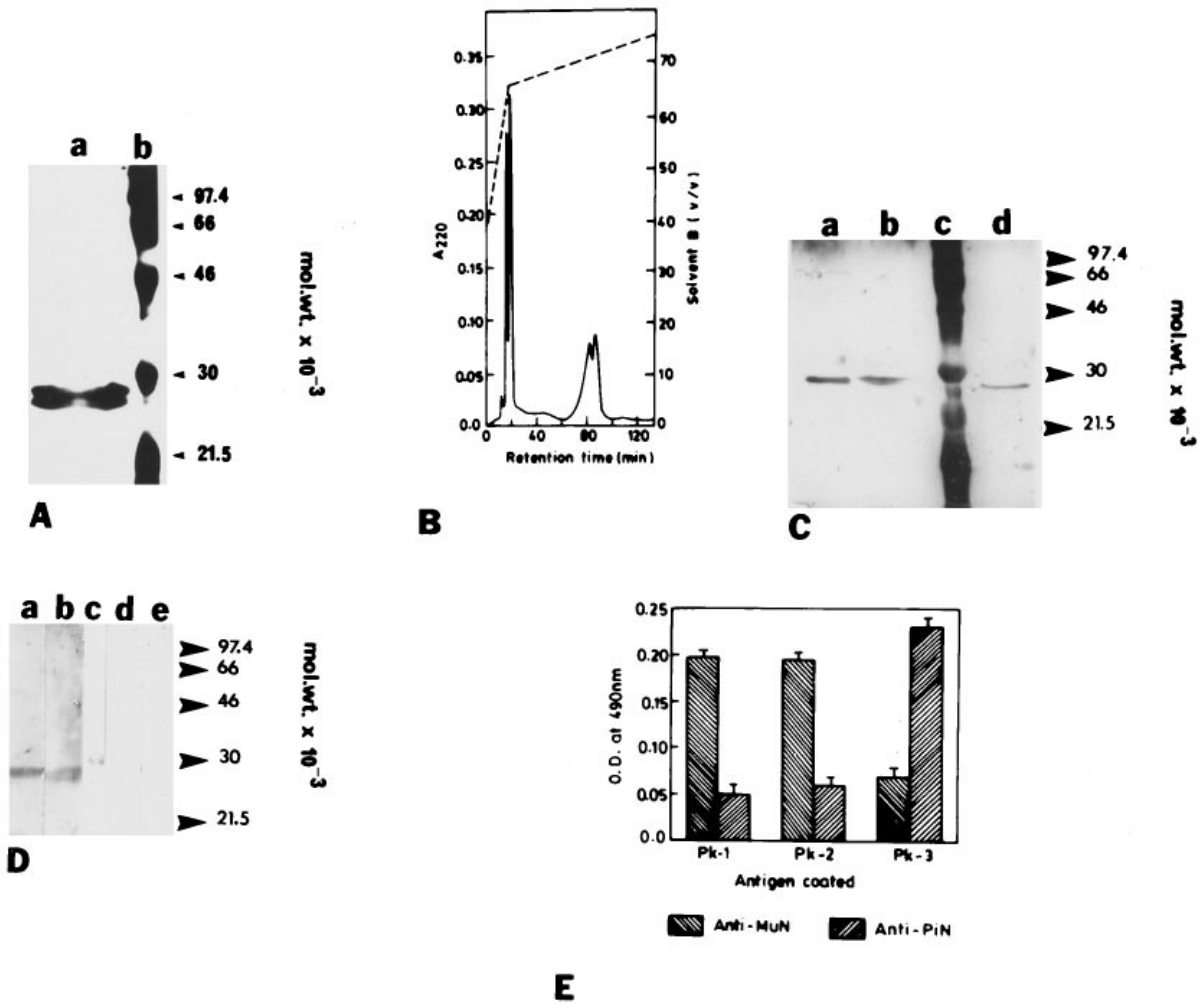

Figure 2 Characterization of GSP1

(**A**) SDS/PAGE of GSH-affinity-purified sperm protein GSP1 on a 12% polyacrylamide gel. Lane a, glutathione affinity-purified GSP1 as shown by silver staining; lane b, molecular-mass markers. (**B**) RP-HPLC profile of GSP1 run on a C_{18} column on a 40–75% gradient using 60% solvent 1 (0.08% TFA in water) and 40% solvent 2 (0.05% TFA in 80% acetonitrile) developed over a period of 70 min. The elution profile was monitored by UV absorption at 220 nm. (**C**) SDS/PAGE profile of the three peaks resolved on RP-HPLC. Lane a, peak 1; lane b, peak 2; lane c, molecular-mass marker; lane d, peak 3. (**D**) Western blots of the eluted peaks with specific antisera. Lane a, and b, reactivity of peaks 1 and 2 with anti-MuN antiserum. Lane c, marker. Lanes d and e, reactivity of peak 1 and 2 with anti-PiN antiserum. (**E**) ELISA reactivity of the eluted peaks to the two antisera.

133 μM for the two substrates respectively (Figures 1A and 1B). The inhibition profiles of GSP1 using various GST inhibitors with CDNB as the substrate are shown in Fig. 1(C). IC_{50} values obtained with *S*-hexylglutathione, *S*-(*p*-azidophenacyl)-glutathione and ethacrynic acid in the presence of CDNB as substrate are 1.1 mM, 1.18 mM and 0.05 mM respectively. On SDS/PAGE, GSP1 separated as two bands with apparent molecular masses of 26–27 kDa and 24–26 kDa respectively (Figure 2A).

GSP1 resolves into three components on RP-HPLC

GSP1 resolved into three components on RP-HPLC (Figure 2B). Three attempts at sequencing the first two peaks with 2 μM protein were unsuccessful. This was apparently due to an N-terminal block. The third peak showed an N-terminal sequence of Pro-Pro-Tyr-Thr-Val-Val-Tyr-Phe-Pro-Val-Arg-Gly-Arg-Asn-Ala, which was 80% similar to human GSTP1-1 and rat GSTP1. On SDS/PAGE, peaks 1 and 2 resolved in the same region (Figure 2C), both showing molecular masses of 26.5 kDa. Peak 3 resolved with a molecular mass of 25.5 kDa. Recoveries of amino acids after Edman degradation are shown in Table 1.

GSP1 is recognized by anti-Mu- and anti-Pi-GSTs

Antisera raised against the N-termini of rat PiN and MuN did not cross react with each other and were specific for their respective proteins (Table 2). GSP1 was reactive to both the antisera, and the mean absorbance readings of anti-MuN and anti-PiN GSP1 on ELISA were 0.767 ± 0.1 and 0.415 ± 0.06 respectively, at a wavelength of 490 nm. Antisera raised against the whole purified GSP1 reacted to GSP1 in ELISA and Western blots (results not shown).

Western blots of peaks 1 and 2 reacted with anti-MuN antisera but not with anti-PiN (Fig. 2D). This was further confirmed by ELISA which showed similar results (Fig. 2E).

Table 1 N-terminal amino acid sequence of peak 3 of GSP1

Yields of amino acids obtained at each step of Edman degradation during N-terminal sequencing of peak 3 of GSP1 after RP-HPLC separation.

Cycle no.	Amino acid	Yield (pmol)
1	Pro	65
2	Pro	97
3	Tyr	70
4	Thr	67
5	Val	78
6	Val	89
7	Tyr	59
8	Phe	33
9	Pro	49
10	Val	57
11	Arg	25
12	Gly	28
13	Arg	30
14	Asn	17
15	Ala	42

Table 2 Specificity of anti-MuN and anti-PiN

Equal amounts of MuN and PiN peptides were coated on ELISA plates and probed with anti-MuN and anti-PiN to check for cross-reactivity. The antisera were absorbed with 10 μg of each antigen to confirm specificity of binding. Results are means $\pm$ S.E.M. for triplicate determinations. Significance: 1 compared with 2, $P < 0.001$; 4 compared with 5, $P < 0.0001$.

		A_{490}	
Antiserum	Antigen ...	MuN	PiN
1. Anti-MuN		0.33 $\pm$ 0.02	0.05 $\pm$ .001
2. Anti-MuN + MuN		0.03 $\pm$ 0.001	–
3. Anti-MuN + PiN		0.32 $\pm$ 0.02	–
4. Anti-PiN		0.02 $\pm$ 0.001	0.28 $\pm$ 0.019
5. Anti-PiN + PiN		–	0.01 $\pm$ 0.0005
6. Anti-PiN + MuN		–	0.32 $\pm$ 0.020

GSP1 is detectable in the membrane fraction

Membrane fractions prepared from goat spermatozoa showed GST activity with both CDNB and ethacrynic acid, the specific activities being 14 nmol/min per mg of protein with CDNB and 1.35 μM/min per mg of protein with ethacrynic acid. GSH affinity-purified material from the membrane fraction showed the same elution profile as that obtained with NP-40 extracts on RP-HPLC (results not shown).

Anti-GST antibodies could not inhibit GST activity of GSP1 or live sperm

The inability of antibodies to inhibit the GST activity of GSP1 towards CDNB and ethacrynic acid was revealed when anti-PiN, anti-MuN and anti-GSP1 were unable to inhibit the enzyme activity after preincubation of the antisera with GSP1 for 0, 15, 30 and 60 min before the enzyme assay (Table 3). Precipitation of GSP1–antibody complexes by Protein G coupled to Sepharose showed that the enzyme activity was confined to the Protein G-precipitable material when precipitation was done with anti-GST antibodies only, whereas no activity was detectable in the supernatant. Enzyme activity was associated with the supernatant

Table 3 Enzymic activity of GSP1 in the presence of different antisera

GSP1 was preincubated with anti-GSP1, anti-MuN and anti-PiN for 0, 15, 30 and 60 min before enzyme assay with (**a**) CDNB and (**b**) ethacrynic acid as substrates. Preimmune sera were used as controls. Results are Data are expressed as means $\pm$ S.E.M. of 3–4 replicates.

(a) CDNB 0.05 mM (GSH 0.025 mM)	GSP1 activity (nmol/min per mg of protein)			
Incubation time (min) ...	0	15	30	60
Antisera				
Pre-immune sera	8.9 $\pm$ 0.2	9.0 $\pm$ 0.2	8.6 $\pm$ 0.3	9.1 $\pm$ 0.4
Anti-GSP1	8.8 $\pm$ 0.2	9.2 $\pm$ 0.2	8.8 $\pm$ 0.2	8.7 $\pm$ 0.2
Anti-MuN	8.8 $\pm$ 0.2	8.6 $\pm$ 0.2	8.4 $\pm$ 0.4	8.7 $\pm$ 0.2
Anti-PiN	8.8 $\pm$ 0.2	8.9 $\pm$ 0.2	8.3 $\pm$ 0.2	8.7 $\pm$ 0.3
(b) Ethacrynic acid 0.05 mM (GSH 0.05 mM)	GSP1 activity (nmol/min per mg of protein)			
Incubation time (min) ...	0	15	30	60
Antisera				
Pre-immune sera	967 $\pm$ 4	963 $\pm$ 18	972 $\pm$ 18	951 $\pm$ 18
anti-GSP1	967 $\pm$ 4	956 $\pm$ 12	969 $\pm$ 10	948 $\pm$ 20
anti-MuN	967 $\pm$ 4	942 $\pm$ 15	959 $\pm$ 10	945 $\pm$ 26
anti-PiN	967 $\pm$ 4	957 $\pm$ 29	953 $\pm$ 11	943 $\pm$ 15

when antisera other than anti-GST were used for precipitation, showing that the GST activity of GSP1 was associated with anti-GST antibody-precipitable material only. GST activity on live sperm also could not be inhibited by the various antibodies, activity on live sperm with CDNB as substrate being 170 nmol/min per 10^7 sperm and within the range 160–240 nmol/min per 10^7 sperm in the presence of anti-GSP1, anti-PiN or anti-MuN antisera.

Sperm viability and acrosomal integrity

After Percoll purification, 99 % of sperm are viable, as revealed by Trypan Blue staining. Staining with *P. sativum* agglutinin linked to FITC showed 80 % of sperm with the acrosome intact (Figure 3A). Various stages of acrosome damage were visible in the remaining 20 % of sperm (Figure 3B). Sperm fixed with paraformaldehyde showed no increase in the number of sperm with acrosome damage (Fig. 3C). After calcium ionophore treatment, 80 % of spermatozoa showed sperm heads devoid of any *P. sativum* staining (Fig. 3D).

Surface localization of Pi- and Mu-GST

Detection of staining on live spermatozoa using anti-PiN and anti-MuN, followed by second antibody conjugated to FITC combined with Propidium Iodide as vital stain, showed a significant channel shift with FITC but not with Propidium Iodide, indicating that the sperm stained were live and the staining was therefore on the surface (Figures 4A and 4B). Staining was clearly visible under the microscope in the same pattern as that of paraformaldehyde-fixed sperm (results not shown).

Immunoreactive PiN and MuN are detectable during different stages of sperm maturation and capacitation

Anti-PiN-reactive sites were confined to the posterior acrosomal region and some parts of the tail on fixed testicular and caput spermatozoa (Figures 5A and 5B), with some additional immuno-

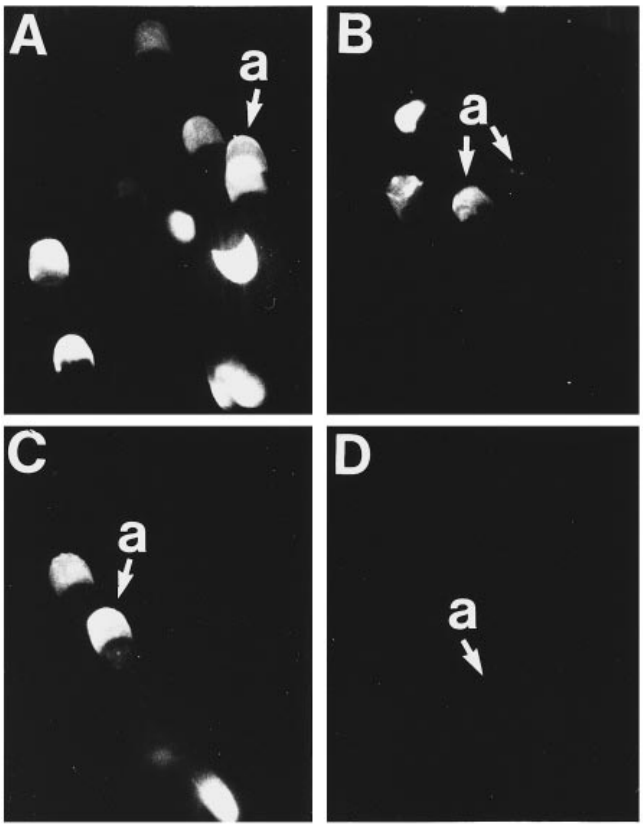

Figure 3 Staining of goat sperm with *P. sativum* agglutinin linked to FITC

(**A**) Representative fraction from Percoll-purified goat sperm with 80% of acrosome-intact sperm stained with *P. sativum* agglutinin linked to FITC, showing uniform staining of acrosomes. (**B**) Representative fraction of remaining 20% of the sperm population at various stages of acrosome disruption. (**C**) Paraformaldehyde-fixed sperm stained with *P. sativum* agglutinin linked to FITC, showing uniform acrosomal staining. (**D**) Sperm treated with calcium ionophore showing no staining on the acrosome, representing total acrosomal disruption.

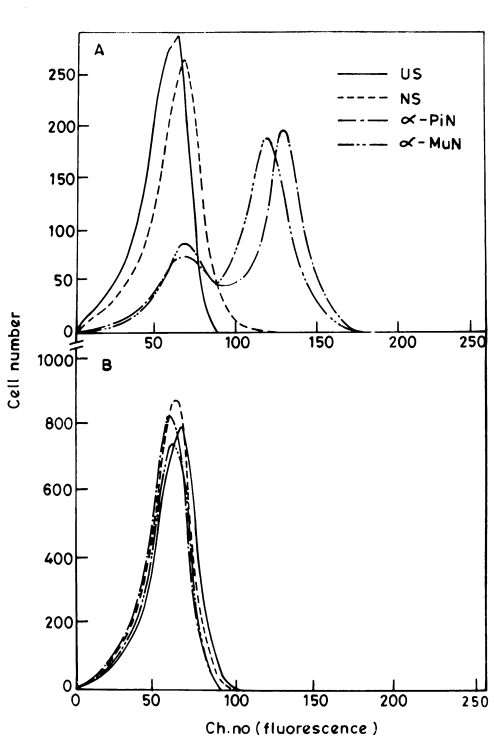

Figure 4 Flow cytometry of live spermatozoa labelled with anti-MuN and anti-PiN at 4 °C

(**A**) Staining of live spermatozoa with anti-PiN and anti-MuN at 4 °C. (**B**) Staining of the same population of spermatozoa with Propidium Iodide as vital dye showing no shift of channels, indicating live population. US, unstained sperm population; NS, sperm population treated with normal rabbit serum; anti-PiN, sperm population treated with anti-PiN; anti-MuN, sperm population treated with anti-MuN; Ch. no, channel number.

reactive sites appearing on the anterior acrosome and on the tail of corpus sperm (Figure 5C). A crescent-like staining pattern over the extreme anterior acrosome, posterior acrosome and main tail piece of cauda sperm was detected with anti-PiN. This pattern of staining remained unaltered during capacitation *in vitro* (Figures 5E–5G). The anterior acrosomes of the testicular, caput, corpus and cauda spermatozoa stained with anti-MuN antisera (Figures 6A–6D), however, there was a change in distribution of anti-MuN-reactive sites during capacitation *in vitro*. At 6 h of capacitation only 30% of sperm showed staining of the anterior acrosome, whereas the remaining 70% of the cells showed immunoreactive sites confined to the equator and the posterior acrosome. The loss of acrosomal staining appeared to be due to loss of acrosomes, as the number of acrosome-reacted sperm was 70% of total sperm after 6 h of capacitation, as visualized by Napthol Yellow staining. When acrosome reaction was induced by calcium ionophore A23187, anti-PiN staining did not show any alteration in staining pattern and anti-MuN staining was lost from the acrosome, staining being confined to equator, posterior acrosome and tail (results not shown). This confirmed that upon acrosome reaction anti-MuN-reactive sites are lost from the acrosome or relocated to some other site.

Flow cytometry of paraformaldehyde-fixed cauda epididymal sperm stained with anti-PiN and anti-MuN showed significant channel shift (Figure 7A) as compared with sperm stained with preimmune sera. After 3 h of capacitation *in vitro* the staining intensity with both anti-PiN and anti-MuN increased, as recorded by channel shift (Figure 7B); however, at 6 h, staining intensity with anti-MuN decreased, as demonstrated by a backward shift of channels, whereas that of anti-PiN did not change significantly (Figure 7C).

GST activity changes during sperm maturation in the epididymis

During epididymal sperm transit an alteration of GST activity on sperm was observed. Activity towards CDNB was significantly higher in the caput spermatozoa as compared with those from corpus or cauda epididymis (Figure 8A). During capacitation there was a progressive decrease in enzyme activity towards CDNB from 0 h onwards, which was not detectable at 6 h after initiation of capacitation (Figure 8B). Activity with ethacrynic acid increased after capacitation, as measured at a single assay time point of 30 s with sperm capacitated for 3 h (1.2 ± 0.1 μmol/min per mg of protein) as compared with non-capacitated sperm (0.2 ± 0.02 μmol/min per mg of protein).

Differential expression of functionally relevant GST during capacitation

Treatment of cauda epididymal spermatozoa with anti-PiN during the first 30 min of *in vitro* capacitation resulted in a decrease in the number of oocytes fertilized as compared with

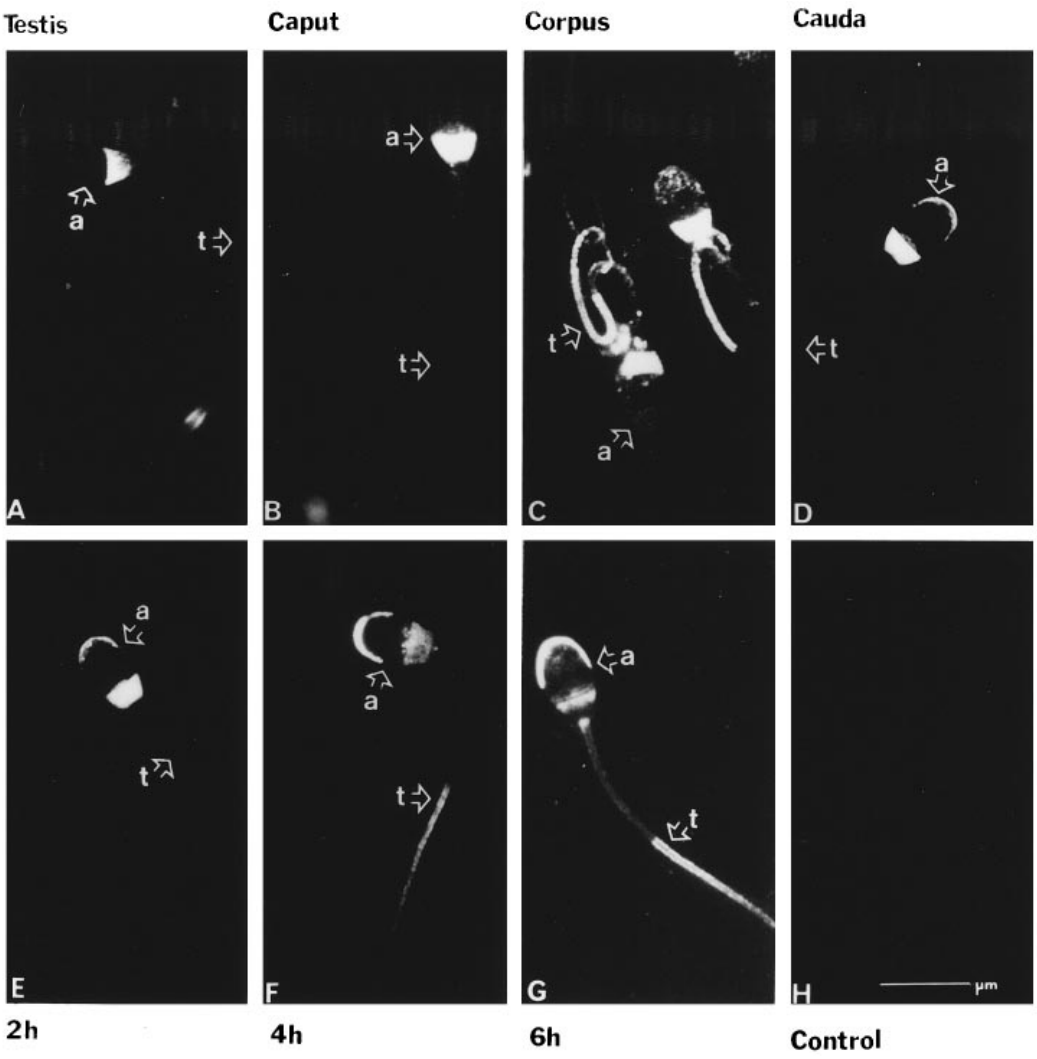

Figure 5 Distribution of proteins on sperm as recognized by anti-PiN during epididymal maturation and capacitation

Immunostaining of (**A**) testicular sperm, (**B**) caput sperm, (**C**) corpus sperm and (**D**) cauda sperm using anti-PiN as the first antibody and with the second antibody conjugated to fluorescein. Localization of the protein is shown at different time points during capacitation in cauda sperm: (**E**) 2 h, (**F**) 4 h, (**G**) 6 h and (**H**) control with cauda epididymal sperm where antiserum was adsorbed with excess antigen (1 μg/ml). a = acrosome, t = tail.

control sera (Table 4). Addition of this antiserum 3 h after initiation of capacitation also showed the same effect. Absorption of the antisera with Pi peptide (PiN) but not diptheria toxoid, reversed the fertilization rates to the control level, showing that PiN sites are specifically involved in fertilization (Table 4). When epididymal sperm were treated with anti-MuN during *in vitro* capacitation for the first 30 min, and the sperm were tested for their ability to fertilize the oocyte, no inhibition of fertilization was observed. However, when sperm were treated with anti-MuN 3 h after capacitation was initiated, the rate of fertilization of oocytes by these sperm was significantly reduced as compared with controls or sperm treated with anti-MuN at 0 h of capacitation.

Antisera interfere with the rate of acrosome reaction

The number of acrosome-reacted sperm increased with time of capacitation *in vitro*. However, when capacitation was carried out in the continuous presence of anti-MuN or anti-PiN the number of acrosome-reacted sperm measured at 3 or 6 h was significantly reduced, as measured by Naphthol Yellow staining (Table 5). The number of acrosome-reacted sperm measured at 2 h was significantly lower than that of control when sperm were capacitated in the presence of anti-PiN. The anti-MuN-treated group showed no difference in the number of acrosome-reacted sperm as compared with controls when measured at 2 h of incubation. This difference was significant after 3 h of capacitation in the presence of the antibody.

DISCUSSION

Evidence for the presence of GST on goat spermatozoa came from our earlier studies where immunoreactive GST was detected on these cells by anti-MuGST antiserum [1]. We also demonstrated that the same antiserum against Mu-GST could interfere with the capability of goat spermatozoa to fertilize goat oocytes *in vitro* [1]. Subsequently, GST was purified from goat-sperm extracts by GSH affinity chromatography. This affinity-

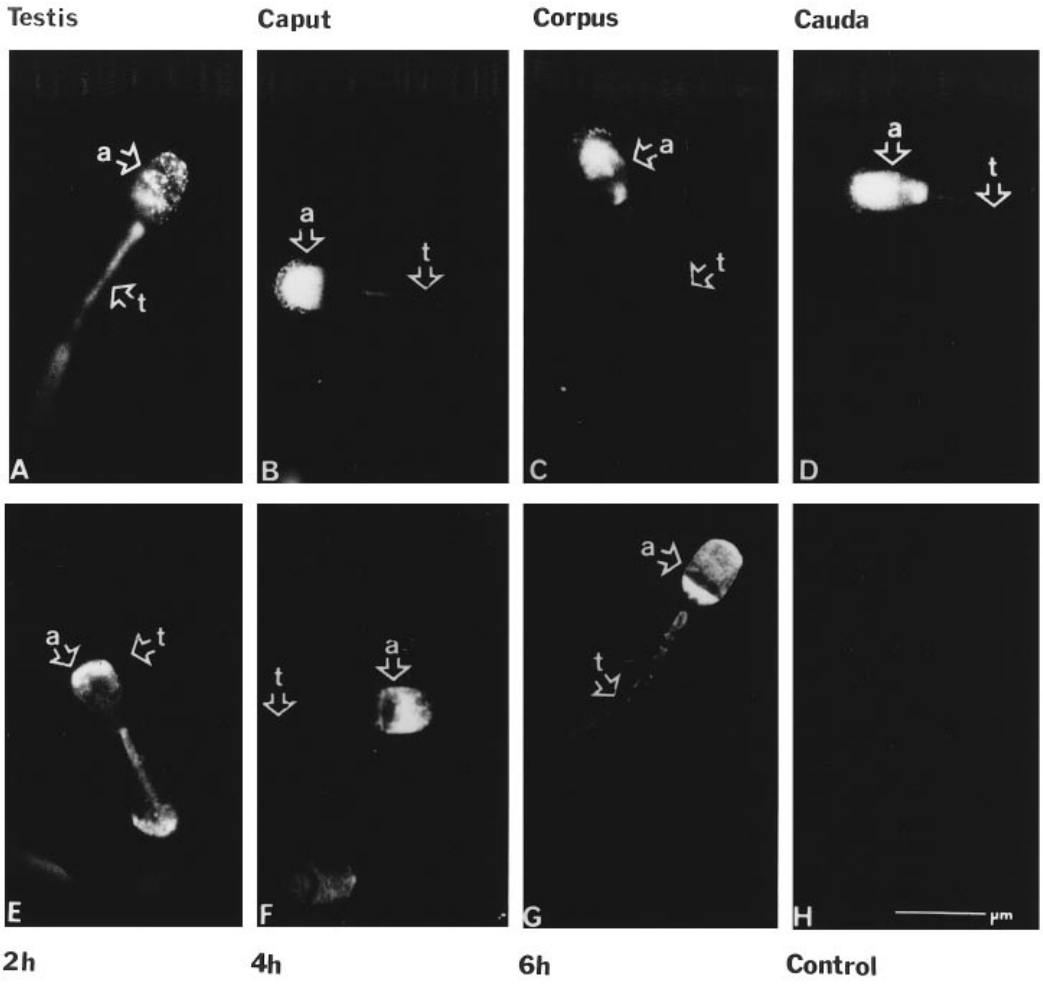

Figure 6 Distribution of proteins on sperm as recognized by anti-MuN during epididymal maturation and capacitation

Immunostaining of (**A**) testicular sperm, (**B**) caput sperm, (**C**) corpus sperm and (**D**) cauda sperm using anti-MuN as the first antibody and with the second antibody conjugated to fluorescein. Localization of the protein is shown at different time points during capacitation in cauda sperm: (**E**) 2 h, (**F**) 4 h, (**G**) 6 h and (**H**) control with cauda epididymal sperm where antiserum was adsorbed with excess antigen (1 μg/ml). a = acrosome, t = tail.

purified protein showed saturable enzyme activity with CDNB, a substrate for different GST classes, primarily Mu [10], and towards ethacrynic acid, an effective substrate for Pi-GST [34]. Definitive evidence for the presence of multiple GST isoforms was obtained both from SDS/PAGE, where GSP1 resolved into two bands, and from RP-HPLC, where GSP1 eluted as three distinct peaks. Peaks 1 and 2, which shared the same molecular mass and resolved in the same region on SDS/PAGE, could not be sequenced despite three attempts, apparently due to an N-terminus block. The third peak was sequenced as a polypeptide sharing 80 % similarity with human and rat Pi-GST [35]. Utilizing the property of restricted ability of the GST antibodies to recognize different classes of GST, the three eluted fractions were checked for immunoreactivity with specific antisera against Pi-GST and Mu-GST. Peaks 1 and 2 showed reactive sites for anti-MuN but not for anti-PiN. This established their identity as Mu-GSTs. Antibody reactivity on Western blots, N-terminal sequencing, enzyme activities with different substrates and RP-HPLC established that goat-sperm GST comprised isoforms of both Pi-GST and Mu-GST classes. SDS/PAGE of the three peaks showed that the molecular mass of the first two peaks corresponded to the reported molecular masses of Mu-GSTs [10] and the third peak was relatively smaller, resembling Pi-GST in size [36]. Sperm membrane fractions showed the same profile on RP-HPLC as with the NP-40 extracts, providing evidence for membrane association of the enzymes. Our studies with goat testicular and liver GST show that liver expresses only Mu-GST forms and testis expresses a Pi form in addition to Mu forms (B. Gopalakrishnan and C. Shaha, unpublished work). From the above observations it was established that goat sperm expressed both Pi and Mu-GSTs.

Having established the existence of two forms of GST on goat sperm it was necessary to substantiate the surface localization of GSTs, as molecules involved in the cell surface event of sperm–oocyte interactions should logically be on the surface. Evidence for this came from flow cytometric analysis and microscopic observation of sperm labelled with anti-MuN and anti-PiN antibodies at 4 °C, at which temperature no endocytosis or capacitation-induced changes can occur. In addition, GST activity was detectable on live spermatozoa under isotonic

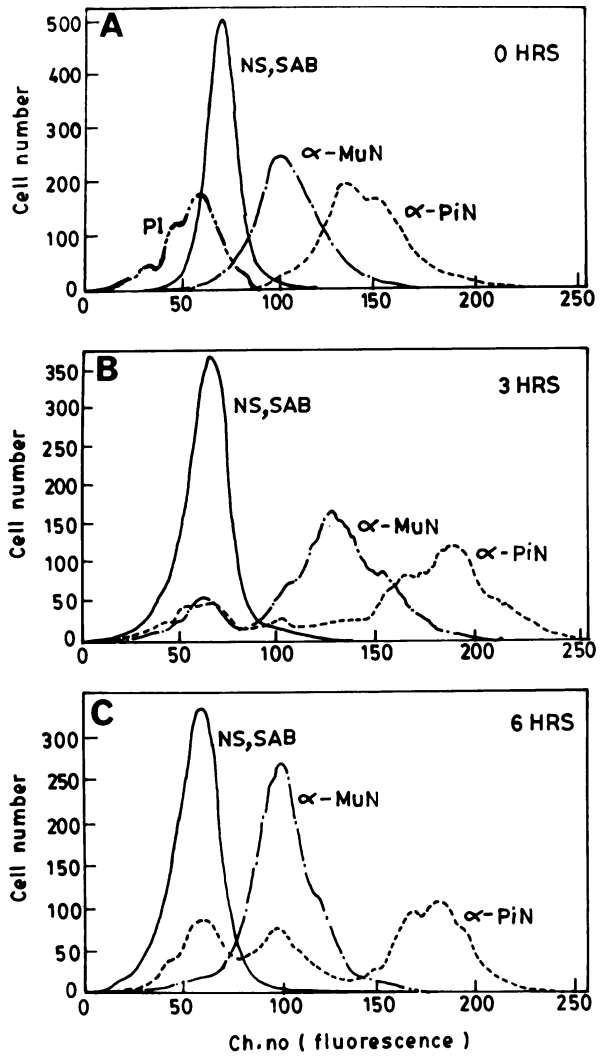

Figure 7 Flow cytometry of spermatozoa labelled with anti-MuN and anti-PiN during capacitation

Flow cytometry of sperm (**A**) at 0 h, (**B**) at 3 h, and (**C**) at 6 h of capacitation. Sperm population treated with normal rabbit serum (NS), second antibody (SAB), preimmune serum (PI), anti-PiN (α-PiN) and anti-MuN (α-MuN). The results of one experiment out of three is presented. Ch. no, channel number.

conditions and therefore confirmed surface localization of the molecules.

Reports of GST activity on rat sperm and human semen are available [11], and Pi-GST strongly expresses itself in neoplastic germ cells [37]. Recently, using DNA probes designed from tryptic peptide sequences from mouse spermatogenic cell fibrous sheath proteins, a GST designated mGSTM5 has been reported [9]. Both Mu and Pi class GSTs have been reported in rat [38,9] and human testis [39].

During epididymal transit, the sperm surface undergoes changes with additions or alteration of molecules, a phenomenon known to occur with a number of sperm proteins [40,41]. The decreased enzyme activity towards CDNB in cauda epididymal sperm, as compared with the caput region, demonstrate some form of alteration in the sperm GST molecule. The appearance of new Pi-GST sites on the anterior acrosome of cauda sperm, which was visualized by IIF, possibly indicate addition of Pi-GST sites during epididymal maturation or unmasking of fresh immunoreactive sites. In the available literature, PH-20 protein [42], 94, 72 and 59 kDa proteins of rat caput sperm [43], and a 28 kDa protein [44], are reported in a similar context.

Maturing spermatozoa experience physiological alterations collectively called capacitation, to become competent to fertilize the oocyte [40]. During *in vitro* capacitation both immunoreactive and enzymically active Pi-GST increase with time, indicating capacitation-induced exposure of fresh Pi-GST molecules or alteration of the existing molecules. Changes in the distribution patterns of certain antigens during sperm maturation are well documented [45]. Since sites recognized by anti-Pi-GST are not lost during acrosome reaction, it appears that Pi-GST is translocated to the inner acrosomal membrane after the event and is therefore localized at a very important cellular location relevant for sperm–oocyte interaction. A similar instance of translocation of proteins is observed with the PH-20 protein [46]. Fibronectin-like molecules on the human sperm surface [47], and a 37 kDa protein on rat sperm head [48] are among some proteins that undergo similar redistribution during capacitation. A transient increase in MuN reactive sites occurs at 3 h; however, reactivity to CDNB decreases in the course of capacitation. Whether this is an alteration of the molecule itself with modifications of the catalytic site, cannot be predicted at this stage of the investigations.

That both Pi-GST and Mu-GST present on sperm are important for the functional competence of sperm to fertilize the oocyte, is evident from the ability of antibodies against these proteins to inhibit fertilization when sperm are treated with antibodies before interaction with the oocyte. However, Mu-GST sites relevant for sperm function are only exposed after 3 h of capacitation, as antisera against Mu-GST are ineffective in preventing fertilization if sperm are treated for 30 min with antibodies to MuN at 0 h of capacitation, but fertilization is inhibited if Mu-GST sites are blocked at 3 h after the onset of capacitation. In contrast, Pi-GST sites, recognizable both at 0 and 3 h, are functionally relevant for fertilization, as blocking of these sites causes interference with fertilization. Whether these molecules are important for sperm–oocyte binding or fusion awaits further study.

Acrosome reaction is crucial for sperm–oocyte interaction and is an exocytotic event [49], which results in the release of enzymes required for fertilization. The rate of acrosome reaction of spermatozoa undergoing *in vitro* capacitation is reduced in the presence of anti-PiN and anti-MuN antisera. This indicates the direct involvement of GSTs in regulating the secretory event of acrosome reaction. Evidence for the loss of Mu-GST sites from sperm acrosomes after spontaneous acrosome reaction during capacitation *in vitro* or after Ca^{2+} ionophore-induced acrosome reaction, followed by appearance of sites in the posterior head and equatorial region, may indicate a vital relocalization process, whereby sites relevant to fertilization are exposed in the equatorial segment, a site which is important for sperm–oocyte adhesion [50]. The presence of Pi-GST sites on the acrosome before and after acrosome reaction may indicate participation of this molecule in various fertilization-related events. Appearance of these sites may also be due to unmasking of fresh sites. Preliminary investigations indicate the existence of binding sites for this protein on the zona pellucida of the oocyte (B. Gopalakrishnan and C. Shaha, unpublished work).

The most interesting aspect of this study is the ability of the antisera to inhibit fertilization but not the enzymic activity of GSP1. Incubation of the enzyme with the different antibodies for increasing periods of time did not affect the saturable GST

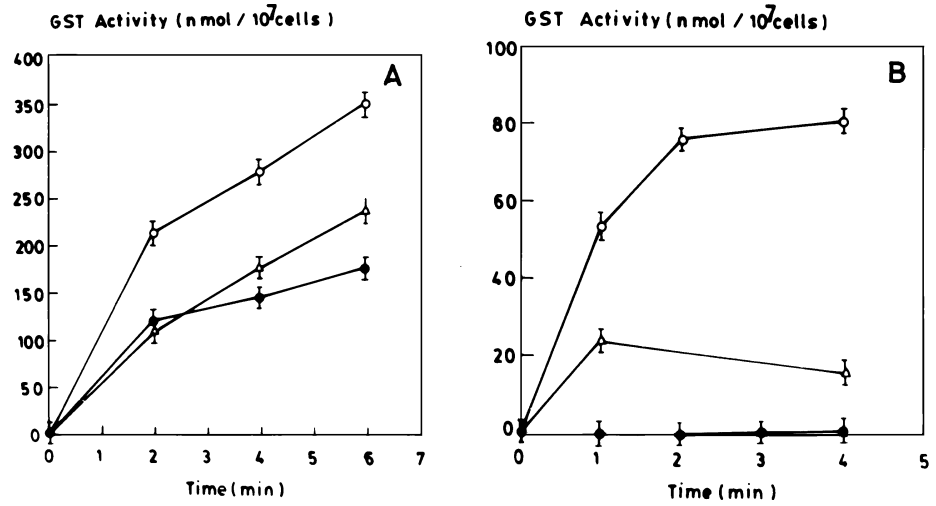

Figure 8 GST activity of epididymal and capacitated sperm

GST activity towards CDNB with (**A**) live sperm from caput (○), corpus (●) and cauda (△) epididymis, and (**B**) cauda sperm during *in vitro* capacitation at 0 (○), 2 (△) and 6 h (●).

Table 4 Effect of antisera on goat *in vitro* fertilization

In vitro fertilization of goat oocytes with epididymal and capacitated sperm treated with anti-PiN and anti-MuN, where antisera were preincubated with either the peptide antigen or diptheria toxoid to check specificity of effect. Results are expressed as means ± S.E.M. of three experiments. Epididymal sperm: control versus anti-PiN and control versus anti-PiN + DT, $P < 0.001$. Capacitated sperm: control versus anti-PiN and control versus anti-MuN, $P < 0.001$. Abbreviations: MII, oocytes in metaphase II stage; 1P, oocytes with one pronucleus; 2P, normal fertilized oocytes with two pronuclei; Poly, polyspermic oocytes with more than two pronuclei; F(%), total percentage of fertilized oocytes.

Groups	Total oocytes	MII	1P	2P	Poly	F (%)
Epididymal sperm						
Control	268	102	14	128	24	57 ± 2.4
Anti-PiN	164	102	10	50	2	32 ± 1.3
Anti-PiN + PiN	104	42	4	56	2	56 ± 1.5
Anti-PiN + DT	114	88	0	24	2	23 ± 2.0
Anti-MuN	138	40	2	96	0	69 ± 1.2
Capacitated sperm						
Control	84	36	0	48	0	57 ± 0.5
Anti-PiN	78	52	0	26	0	33 ± 0.7
Anti-MuN	92	66	6	18	2	22 ± 0.1

Table 5 Percentage of acrosome-reacted sperm in presence and absence of antisera

Epididymal sperm were incubated in capacitation media in the presence of anti-MuN and anti-PiN and the percentage of sperm which did not show staining with Naphthol Yellow (acrosome reacted) was compared with that of the control group of sperm incubated with the antisera. 2 h control versus anti-PiN, $P < 0.001$; 3 h control versus anti-MuN, $P < 0.001$; 3 h control versus anti-PiN, $P < 0.0001$; 6 h control versus anti-MuN, $P < 0.01$; 6 h control versus anti-PiN, $P < 0.0001$.

		Acrosome-reacted sperm (%)			
Groups	Time ...	0 h	2 h	3 h	6 h
Control		14 ± 1.1	31 ± 1.0	69 ± 0.3	76 ± 2.0
Anti-MuN		12 ± 0.5	26 ± 0.9	47 ± 2.5	64 ± 0.6
Anti-PiN		12 ± 1.1	12 ± 2.0	27 ± 0.3	32 ± 1.0

activity towards CDNB, which clearly demonstrated that the ability of the antibodies to interfere with fertilization is mediated by a mechanism which does not interfere with the catalytic activity of the molecule. Since enzyme activity was associated with protein-G-precipitable material, it was clear that the antigen bound to the antibody was still showing catalytic activity and hence the antibody was not binding to the catalytic site. Antibodies directed against a 12-residue synthetic peptide corresponding to fragment 2–13 of rat placental GST as the immunogen, was reported not to affect the enzymic activity of Pi-GST, but this was not correlated to any biological function [51]. This strongly suggests a multifunctional role of this protein on sperm, as it is unlikely that the detoxification function of the molecule will remain unutilized by sperm. PH-20 is one such protein, which has a hyaluronidase activity, required for penetration of the cumulus layer surrounding the oocyte, and has a different activity, required for the secondary binding of sperm to zona [52]. β-1,4-Galactosyltransferase is another enzyme which mediates sperm binding to oocyte [53].

In summary, we have shown that both Pi and Mu class GSTs are present on goat sperm and differ in their pattern of distribution on the cell during epididymal maturation and capacitation. GST sites are present on the sperm surface, and on acrosomal loss during acrosome reaction Mu-GST sites are lost from the anterior acrosome whereas Pi-GST sites are either translocated to the inner acrosomal membrane or fresh sites are exposed. *In vitro* fertilization inhibition data with antibodies present evidence that Mu-GST sites relevant for sperm function are exposed at 3 h of capacitation, whereas functional Pi-GST sites are present before and after initiation of capacitation. The mechanism of inhibition of fertility appears to be through the inhibition of acrosome reaction. The effects of the antisera on sperm function are mediated via mechanisms that do not affect the catalytic property of the molecule. Therefore, the results presented in this study indicate an important role for GSTs in sperm function, which is independent of the catalytic properties of the molecules.

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