Secretion of glutathione S-transferase isoforms in the seminiferous tubular fluid, tissue distribution and sex steroid binding by rat GSTM1

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The seminiferous tubular fluid (STF) provides the micro-environment necessary for spermatogenesis in the adluminal compartment of the seminiferous tubule (ST), primarily through secretions of the Sertoli cell. Earlier studies from this laboratory demonstrated the presence of glutathione S-transferase (GST) in STF collected from adult rat testis and in the spent media of ST cultures. This study describes the cellular source, isoform composition and possible function of GSTs in the STF. The major GST isoforms present in STF *in vivo* share extensive N-terminal similarity with rat GSTM1 (rGSTM1), rGSTM2, rGSTM3 and rGST-Alpha. Molecular masses of rGSTM2, rGSTM3 and rGST-Alpha from liver and testis sources were similar, unlike STF-GSTM1, which was larger by 325 Da than its liver counterpart. Peptide digest analysis profiles on reverse-phase HPLC between liver and STF isoforms were identical, and N-terminal sequences of selected peptides obtained by digestion of the various isoforms were closely similar. The above results confirmed close structural similarity between liver and STF-GST isoforms. Active synthesis and secretion of GSTs by the STs were

evident from recovery of radiolabelled GST from the spent media of ST cultures. Analysis of secreted GST isoforms showed that GST-Alpha was not secreted by the STs *in vitro*, whereas there was an induction of GST-Pi secretion. Detection of immunostainable GST-Mu in Sertoli cells *in vitro* and during different stages of the seminiferous epithelium *in vivo*, coupled with the recovery of radiolabelled GST from Sertoli cell-culture media, provided evidence for Sertoli cells as secretors of GST. In addition, STF of 'Sertoli cell only' animals showed no change in the profile of GST isoform secretion, thereby confirming Sertoli cells as prime GST secretors. Non-recovery of [³⁵S]methionine-labelled GSTs from germ cell culture supernatants, but their presence in germ cell lysates, confirm the ability of the germ cells to synthesize, but not to release, GSTs. Functionally, STF-GSTM1 appeared to serve as a steroid-binding protein by its ability to bind to testosterone and oestradiol, two important hormones in the ST that are essential for spermatogenesis, with binding constants of $< 9.8 \times 10^{-7}$ M for testosterone and 9×10^{-6} M for oestradiol respectively.

INTRODUCTION

The genesis of the work reported in this paper lies in earlier observations reported from this laboratory that immunoreactive and enzymically active glutathione S-transferase (GST) is present in the testicular seminiferous tubular fluid (STF) *in vivo* and is detectable in spent medium from seminiferous tubule (ST) cultures *in vitro* [1]. STF is primarily a secretory fluid contributed by the cells lining the seminiferous epithelium (SE) and contains diverse proteins with known and unknown functions [2]. GSTs are phase II drug-metabolizing enzymes [3], known to be either cytosolic [4] or microsomal [5], and our reports suggest a cell surface localization [6]. Observations of time-dependent accumulation of the GST protein in spent media of rat ST cultures suggested secretion of the protein into the STF [1]. Since GSTs could be identified in a secretory fluid, we decided to address several issues, including identification of cell types in the ST responsible for releasing GST isoforms into the STF and analysis of the possible functional roles of the secretory molecule in the testis.

In this study, we show that primary GST isoforms secreted *in vivo* by the STs share extensive N-terminal similarity with rat GSTM1 (rGSTM1), rGSTM2, rGST-Alpha and rGSTM3, similar molecular mass and a close similarity in peptide digest profiles with liver GST isoforms. The ability of ST cultures *in vitro* to actively synthesize and secrete different GST isoforms is also demonstrated. Immunostainable GST-Mu is present in the

cytoplasm of Sertoli cells *in vitro*, and during different stages of the SE cycle *in vivo*. In addition, the recovery of [³⁵S]methionine-labelled GST from Sertoli cell culture media is presented as evidence for Sertoli cells as prime secretors of GSTs. Germ cells are shown to be able to express GSTs, but unable to make a significant contribution to secretion in the STs. The secretion of GSTs by the STs is not hindered in 'Sertoli cell only' animals, confirming Sertoli cells as prime secretors. Functionally, GSTM1 binds to both testosterone and oestradiol, which makes it a very vital protein in the ST.

MATERIALS AND METHODS

Animals

Rats of Wistar strain of various ages were obtained from the animal facilities of the National Institute of Immunology, New Delhi, India. Rabbits of New Zealand White strain were procured from the same facility for raising antibodies.

Reagents

Anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase were procured from Jackson Laboratories (West Grove, PA, U.S.A.). The protein assay reagent bicinchoninic acid

Abbreviations used: GST, glutathione S-transferase; NP40, Nonidet P40; RP, reverse phase; SE, seminiferous epithelium; ST, seminiferous tubule; STF, seminiferous tubular fluid; TFA, trifluoroacetic acid.

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and trifluoroacetic acid were obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). The GST detection module was from Pharmacia Biotech (Uppsala, Sweden). Hyperfilm™ and molecular-mass markers were from Amersham Life Sciences (Bucks., U.K.) and endoproteinase Glu-C was from Boehringer Mannheim (Mannheim, Germany). The ABC staining kit was purchased from Vector Laboratories (Burlingame, CA, U.S.A.) and [³H] testosterone (102 Ci/mmol) and [³H] oestradiol (141 Ci/mmol) were from DuPont Chemicals (Boston, MA, U.S.A.). All other chemicals, if not specifically mentioned, were from Sigma Chemicals (St. Louis, MO, U.S.A.).

Cell and tissue culture

Collection of STF and preparation of ST for culture

STF collection was done as described earlier [1]. STs were isolated from 90-day-old male rat testes by treatment with 5 mg/20 ml collagenase/dispase at 37 °C for 20 min [7]. The tubules were washed extensively by sedimentation under gravity to remove the interstitial cells and trimmed into 2 mm segments, suspensions of which were incubated in serum-free F-12 Dulbecco's modified Eagle's medium in the presence of 25 µg/ml insulin, 10 µg/ml transferrin and 100 µg/ml gentamycin in an environment of 5% CO₂/95% air (v/v) at 33 °C for the required periods of time. The ST culture medium was collected, centrifuged and stored at -20 °C for further analysis. The basal testosterone levels in ST cultures, with or without contaminating Leydig cells, were found to be 3.0 ± 0.5 and 0.45 ± 0.02 ng/ml of spent culture medium respectively, showing there was negligible contamination by Leydig cells. The viability of the Sertoli germ cell system in the STs was checked by a thymidine incorporation assay as described previously [1].

Preparation of Sertoli cells from immature rats

Sertoli cells were prepared from 18-day-old rats for culture using collagenase/dispase, as described by Welsh and Weibe [8]. After 72 h of culture, cells were treated with 20 mM Tris (pH 7.2) to remove germ cells to obtain germ-cell-free Sertoli cells. Cells were cultured for a further 24 h, and the spent culture medium was collected and concentrated.

Preparation of total germ cells

Germ cells were prepared from 90-day-old rats by a mechanical procedure without any enzymic treatment, as described by Aravindan et al. [9] and cultured in medium supplemented with 10 mM sodium lactate, 2 mM L-glutamine, 0.7 mM sodium pyruvate, 10^{-7} M testosterone and 1% BSA in an environment of 5% CO₂/95% air at 33 °C for different time periods. The viability of germ cells was determined by adding 10 mg/ml of propidium iodide with 10^7 cells suspended in PBS (0.14 M NaCl/50 mM Na₂HPO₄/50 mM NaH₂PO₄, pH 7.4) and analysed on a Winbryte FACS machine [10].

Biochemical analyses

Preparation of cell extracts and tissue homogenates

Cells were extracted for 30 min in lysis buffer (0.14 M NaCl/2.7 mM KCl/1.5 mM KH₂PO₄/8.1 mM Na₂HPO₄, pH 7.4) containing 0.1% Nonidet P40 (NP40) and 0.17 units/ml aprotinin, 1 mM PMSF, 1 mM EDTA, 1 µg/ml pepstatin, 1 µg/ml *p*-hydroxymercuric benzoic acid [6] and 1 mM ethylene glycol-bis-(β-aminoethyl *N,N,N',N'*-tetra-acetic acid) as protease inhibitors on ice, centrifuged at 750 g for 30 min at 4 °C and stored at

-20 °C until use. Liver homogenates were prepared by homogenizing fresh liver tissue in lysis buffer without NP40, as described above, on ice in a Polytron homogenizer.

Purification of GST

Cell lysates, tissue homogenates, STF and culture supernatants were dialysed against PBS (pH 7.4) for 24 h with five changes of buffer. The lysates and homogenates were then passed through SM-2 beads to remove NP40, concentrated and loaded onto glutathione conjugated to Sepharose 4B. Elution of the protein was carried out as described previously by using 20 mM reduced glutathione [1].

SDS/PAGE

Electrophoresis was carried out using the buffer system of Laemmli [11] on 12% polyacrylamide gels.

Reverse-phase (RP)-HPLC of purified GST

RP-HPLC of purified GST from STF and spent ST culture media was carried out with a slightly modified version of the protocol of Hsieh et al [12]. Before RP-HPLC, samples were routinely confirmed for purity by SDS/PAGE. Purified GST from STF and liver cytosol were resolved on an RP Delta Pak C₁₈ column (3.9 mm × 30 cm) equilibrated with 60% solvent 1 (0.08% trifluoroacetic acid in water) and 40% solvent 2 (0.08% trifluoroacetic acid in 80% acetonitrile). A gradient of 40–70% was developed over a period of 70 min. The elution profile was monitored by UV absorption at 220 nm.

N-terminal amino acid sequence analysis

Purified proteins were transferred onto PVDF or Problot® membranes after SDS/PAGE and sequenced using Applied Biosystems 470A pulsed-liquid-phase protein sequencer as previously described [1].

Determination of molecular mass

Electrospray MS (ES/MS) was done on a VG Biotech Platform II (VG Biotech, Fison Instruments, Altrincham, U.K.) equipped with a quadrupole analyser and a pneumatic nebulizer-assisted LC/MS interface. Protein samples (10 µl) in 1:1 acetonitrile/water and 0.01% formic acid were infused through a Rheodyne injector into the carrier solvent (1:1 acetonitrile/water). The sample droplets were evaporated at -70 °C assisted by nitrogen as the drying gas. The data were analysed using the Mass Lynx data system. The spectra were obtained in multiple-channel analysis mode at a cone voltage of 40 V. The raw data were subtracted for background noise, smoothed and centroided to calculate the mass of the sample molecule.

Protein labelling with [³⁵S]methionine and [³⁵S]cysteine

Proteins, synthesised *de novo* by ST, germ cells or Sertoli cells, were labelled with Easytag™ express protein labelling mix (L-[³⁵S]methionine and L-[³⁵S]cysteine), specific activity 1175 Ci/mmol (NEN, Wilmington, DE, U.S.A.). ST, Sertoli cells or germ cells were first pre-cultured with Met(-) and Cys(-) medium containing one tenth of the required concentration of the two amino acids. After 4 h of incubation, cells were washed and resuspended in the same medium in the presence of 50 µCi of Easytag™ express protein labelling mix (L-[³⁵S]methionine and L-[³⁵S]cysteine) at 34 °C in 5% CO₂ in air for 24 h for Sertoli cells

Figure 1 RP-HPLC analysis of rat STF and liver GST

(A) Profile of rat STF-GST after RP-HPLC on a C_{18} RP column, showing the individual GST isoforms. For analysis, 60% solvent 1 and 40% solvent 2 were used to develop a gradient of 40–70% over a period of 70 min. (B) Rat liver GST profile after RP-HPLC. The peaks indicated by numbers are described in the text.

and ST and for a period of 5 h for germ cells. Displacement experiments were carried out by incubating Sertoli cells and ST with the normally required concentration of methionine and cysteine for 24 h in fresh medium after 24 h incubation with labelled methionine and cysteine. Germ cells were lysed in 1% sodium deoxycholate containing protease inhibitors. Spent culture media and cell lysates were dialysed and concentrated. GST was purified from dialysed media and cell extracts as described in the section 'Purification of GST' above. Samples were separated by SDS/PAGE under reducing conditions, dried and exposed for autoradiography to X-ray films. Autoradiographs were analysed by a Molecular Dynamics PhosphorImager (Sunnyvale, CA, U.S.A.) using Imagequant[™] software. Experiments were repeated with four different preparations.

Protein estimation

Protein was estimated either by using the bicinchoninic acid protein assay kit in a 96-well microplate using BSA as standard or the Bradford assay [13].

Proteolytic digestion of purified GST isoforms

GST isoforms, recovered from rat STF and liver separated by RP-HPLC, were subjected to proteolytic digestion using endoproteinase Glu-C [14]. Briefly, testis and liver GST isoforms were treated with endoproteinase Glu-C in phosphate buffer [20 mM Na_2HPO_4 /20 mM NaH_2PO_4 , pH 8.0] at 37 °C for 4 h. The substrate/enzyme ratio was maintained at 20:1. The digests were separated on an RP Delta Pak C_{18} column (3.9 mm $\times$ 30 cm) equilibrated with 60% solvent 1 and 40% solvent 2. A gradient of 5–70% was developed over a period of 130 min and monitored by UV absorption at 215 nm. Prominent peptides in the STF-GST isoform digests were sequenced and compared with reported liver sequences.

GST assay

The GST assay was carried out according to the method of Warholm et al [15], with some modifications as described previously [1].

Steroid-binding studies

The first set of binding studies was carried out according to the method of Homma and Listowsky [16], with modifications. STF preparations (20 mg/ml) were incubated with 6 nM [^3H]testosterone in PBS (pH 7.4) for 16 h at 4 °C and separated on Sephadex G-25 column (1 cm $\times$ 5 cm) in the same buffer. Fractions (1 ml) were collected and 50–100 μl fractions were assayed for GST activity and radioactivity was measured in a liquid-scintillation counter (Beckman, Palo Alto, CA, U.S.A.) with 500 μl of the fractions mixed with liquid-scintillation counting fluid. Some STF preparations were labelled with [^3H]testosterone in the presence of 60 nM, 600 nM and 6 μM unlabelled testosterone for specificity studies. Experiments were repeated with at least three different preparations. Similar studies were carried out with [^3H]oestradiol with displacement studies carried out with unlabelled oestradiol in similar doses as above. Binding studies were also performed with isolated GST isoforms from STF-GST in a similar manner. All experiments were carried out at least three times. Specific binding was calculated by subtracting the non-specific binding from total binding and K_d was calculated according to the method of Rosner [17].

Synthesis of peptides and production of antisera

Peptide synthesis and conjugation

Antibodies were raised against one 13-mer peptide designed from exon 4 (a region showing greatest homology within the GST class and between the two species of rat and human) of the GST-Mu

Table 1 Yields of amino acids at each step of Edman degradation during N-terminal sequencing of STF and liver GST (peaks 1 and 2)

Italics are used to indicate dissimilar amino acids between rat STF and liver sequences.

Cycle no.	STF peak 1 (GSTM1)		Liver peak 1 (GSTM1)		STF peak 2 (GSTM2)		Liver peak 2 (GSTM2)	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Proline	1220	Proline	19	Proline	663	Proline	275
2	Methionine	1070	Methionine	24	Methionine	2460	Methionine	238
3	Isoleucine	1516	Isoleucine	29	Threonine	860	Threonine	514
4	Leucine	1400	Leucine	36	Leucine	1925	Leucine	232
5	Glycine	484	Glycine	47	Glycine	1500	Glycine	270
6	Tyrosine	322	Tyrosine	36	Tyrosine	1060	Tyrosine	203
7	Tryptophan	865	Tryptophan	15	Tryptophan	1044	Tryptophan	159
8	Asparagine	905	Asparagine	23	Aspartic acid	1040	Aspartic acid	240
9	Valine	963	Valine	29	Isoleucine	1143	Isoleucine	206
10	<i>Tyrosine</i>	<i>322</i>	<i>Arginine</i>	<i>50</i>	Arginine	867	Arginine	120
11	Glycine	1323	Glycine	49	Glycine	1500	Glycine	259
12	Leucine	1400	Leucine	32	Leucine	1925	Leucine	209
13	Threonine	600	Xaa		Alanine	1417	Alanine	216
14	Histidine	214	Histidine	20	<i>Alanine</i>	<i>584</i>	<i>Histidine</i>	<i>85</i>
15	Proline	733	Proline	15	Alanine	1400	Alanine	194
16	Isoleucine	725			Isoleucine	743	Isoleucine	185
17	Tyrosine	252			Arginine	600	Arginine	185
18	Leucine	923			Leucine	1575	Leucine	198
19	Leucine	846			Phenylalanine	750	Phenylalanine	158
20	Leucine	1000			Xaa		Leucine	200
21					<i>Proline</i>	<i>525</i>	<i>Tryptophan</i>	<i>253</i>
22					Tyrosine	670	Xaa	
23					Threonine	500	Xaa	
24					Aspartic acid	630	Aspartic acid	158
25					Threonine	300	Xaa	

Table 2 Yields of amino acids at each step of Edman degradation during N-terminal sequencing of STF and liver GST (peaks 4 and 5)

Italics are used to indicate dissimilar amino acids between rat STF and liver sequences.

Cycle no.	STF peak 3 (GST-Alpha)		Liver peak 3 (GST-Alpha)		STF peak 4 (GST-M3)	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Proline	943	Proline	127	Proline	530
2	Glycine	1540	Glycine	200	Methionine	611
3	Lysine	424	Lysine	127	Threonine	383
4	Proline	890	Proline	111	Leucine	360
5	Valine	1033	Valine	117	Glycine	528
6	Leucine	1587	Leucine	127	Tyrosine	600
7	Histidine	430	Histidine	400	Tryptophan	444
8	Tyrosine	862	Tyrosine	94	Aspartic acid	458
9	Phenylalanine	923	Phenylalanine	118	Isoleucine	330
10	Aspartic acid	933	Aspartic acid	122	Arginine	375
11	Glycine	880	Glycine	152	Glycine	471
12	<i>Tyrosine</i>	<i>200</i>	<i>Arginine</i>	<i>80</i>	Leucine	350
13	Glycine	930	Glycine	134	Alanine	364
14	Arginine	225	Arginine	22	Histidine	236
15	Methionine	923	Methionine	91	Alanine	364
16	Glutamic acid	693	Glutamic acid	100	Isoleucine	280
17	Proline	300	Proline	62	Arginine	300
18	Isoleucine	260	Isoleucine	44	Leucine	320
19	Arginine	63	Arginine	15	Leucine	370
20	Tryptophan	63	Tryptophan	22	Leucine	370
21					Glutamic acid	225
22					Tyrosine	325
23					Threonine	166
24					Aspartic acid	275
25					Serine	40
26					Serine	40
27					Tyrosine	150

Figure 2 Molecular mass of GST isoforms in STF and liver

(A) Comparative molecular masses of GST isoforms from STF and liver. (B) Profile of STF-GSTM2 on analysis by ES/MS.

with the sequence Lys-Gln-Leu-Pro-Lys-Phe-Gln-Asp-Gly-Asp-Leu-Thr-Leu-Tyr coding from amino acids 51 to 64 of the GST-Mu reported sequence and designated MuC. The peptide was conjugated to diphtheria toxoid and antibodies were raised by conventional protocols [18].

Immunocytochemical techniques

Immunocytochemistry was carried out as described by Shaha et al. [19] with slight modifications. Sections (5 µm) of adult rat testes fixed with Bouin's fluid were deparaffinized and pre-incubated with normal goat serum (1:100) for 1 h, followed by a first antibody (anti-MuC, 1:100 dilution) for 1 h at room temperature. After three washes with PBS (pH 7.4), incubation with a second antibody (anti-rabbit IgG HRP, 1:100) was carried out for 1 h at room temperature. Control staining was carried out with either preimmune serum alone, or with immune serum adsorbed with excess antigen (anti-MuC, 1 µg/ml), immune serum adsorbed with excess diphtheria toxoid (1 µg/ml) or with an unrelated hyperimmune serum. After three washes, immunoreactive products were visualized by use of the ABC staining kit. Sections were counterstained with 0.1% haematoxylin. For immunofluorescence with Sertoli cells in culture, cells were grown in chambered slides (Carlson Scientific, Peotone, IL, U.S.A.) and stained as described above, except that FITC-conjugated second antibody was used. Immunofluorescence was observed under a Nikon Optiphot fluorescence microscope. Incubation with the antibodies was performed in the

same way as described above. Immunocytochemistry experiments were conducted with three different sets of testes fixed with Bouin's fluid.

Germ-cell-free animal model

Male offspring devoid of germ cells were produced by a single injection of busulphan in arachis oil (10 mg/kg body weight) into pregnant rats on day 13 of pregnancy [20]. The first day of presence of spermatozoa in the vaginal smear was taken as day 0. The male pups ('Sertoli cell only' animals) were allowed to grow for 90 and 110 days, after which the animals were killed and tubular fluid was collected. Testes were frozen immediately after decapsulation in liquid nitrogen for preparing frozen sections of 6 µm thickness in a cryostat (Bright, Huntingdon, U.K.). Testes from male pups of the same age born to vehicle-treated female rats were taken as controls.

RESULTS

GST isoforms in rat STF and liver

The elution pattern of STF and liver GST isoforms on RP-HPLC were closely similar. STF-GST resolved into four primary peaks and one minor peak and liver GST into five primary peaks and one minor one (Figures 1A and 1B). The elution times of peaks 1, 2, 3 and 4 of STF were comparable with the elution times of liver peaks 1, 2, 3 and 4 respectively. The major GST isoforms detected were rGSTM1 (peak 1), GSTM2 (peak 2),

Figure 3 Endoprotease Glu-C digest profiles of rat STF-GST isoforms

Peptide digest profiles of: (A) GSTM1; (B) GSTM2; (C) GSTM3; and (D) GST-Alpha. Numbered peaks indicate the peptides used for sequencing. For analysis, 60% solvent 1 and 40% solvent 2 were used to develop a gradient of 5–70% over a period of 130 min.

GST-Alpha (peak 3) and GSTM3 (peak 4) in STF, as confirmed by N-terminal sequencing (Tables 1 and 2). The similarity of the STF peaks with reported sequences of rGSTM1, GSTM2, GST-Alpha and GSTM3 were 93, 94, 90 and 100% respectively. Liver showed the presence of GSTM1 (peak 1), GSTM2 (peak 2) and GST-Alpha (peak 3), expression of GSTM3 (peak 4) being very low. The similarity of the three liver peaks with reported sequences of GSTM1, GSTM2 and GST-Alpha were 100, 100 and 95% respectively. In both tissues GSTM2 was the most predominant component. Two prominent peaks present in the liver cytosol, designated as peaks 5 and 6, were N-terminal blocked. Minor peaks were not sequenced due to limitations in the quantity of material available for sequencing. These results established the identity of the STF-GST peaks.

Molecular mass

The molecular masses of corresponding peaks from liver and STF were similar in the case of GSTM2, GSTM3 and GST-Alpha. GSTM1 from STF was larger by 325 Da than liver GSTM1 (Figure 2A). The profile of mass analysis of STF-GSTM2, the most predominant component in STF, is shown in Figure 2(B) as a representative sample.

Peptide maps of endoproteinase Glu-C digests of GST isoforms

Peptide maps generated by endoproteinase Glu-C digests of STF-GSTM1, STF-GSTM2, STF-GSTM3 and STF-GST-Alpha are shown in Figures 3(A) to 3(D) respectively. The N-terminal sequences of the selected peptides (marked by numbers in Figure 3) obtained, and their similarity with reported GST isoform sequences, are detailed in Table 3. The elution profiles of the STF-GST isoform digests on RP-HPLC were similar to the digests of similar peaks from the liver (results not shown).

Table 3 Amino acid sequences of endoprotease Glu-C digests of STF-GST isoforms and their comparison with reported rat GST sequences

rs, reported sequences; see [59].

Fragments	Sequence	Similarity (%)
STF peak 1 (peptide 1) rGSTM1 (rs)	KRYAMSDAPDYDRSQ KRYAMGDAPDYDRSQ 31 45	93
STF peak 1 (peptide 2) rGSTM1 (rs)	RSQWLNE RSQWLNE 43 49	100
STF peak 2 (peptide 1) rGSTM2 (rs)	KMKYMGDAPDYDG KKYSMGDAPDYDR 31 43	70
STF peak 3 rGST-alpha (rs)	KVLHYPYIPPGEKE IVLHYPYIPPGEKE 105 118	93

Synthesis and secretion of GST isoforms by ST cultures and Sertoli cells

Radiolabelled GST was recovered by GSH affinity chromatography from spent ST culture media incubated with [35 S]methionine and [35 S]cysteine. The proteins were separated by SDS/PAGE at 24 kDa as a single thick band (Figure 4A, lane a) and possessed GST activity [$29.6 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg of tissue}^{-1})$]. This labelling could be chased out with cold methionine (Figure 4A, lane b). Volume quantification was used to confirm the observations on gel (Figure 4B). This reflected actual secretion of the GST into the media. Sertoli cell culture media when purified on a GSH affinity column showed the presence of labelled GST

Figure 4 Incorporation of [^{35}S]methionine and [^{35}S]cysteine by ST and Sertoli cell cultures

(A) Lane a, autoradiograph of [^{35}S]methionine- and [^{35}S]cysteine-labelled GST released by ST in culture media in 24 h. Labelled GST was purified on a GSH-Sepharose 4B affinity column and run on SDS/PAGE, followed by exposure to X-ray films for 7 days. Lane b, GST released by ST culture media, labelled with [^{35}S]methionine and [^{35}S]cysteine in the presence of unlabelled methionine for 24 h. Lane c shows molecular mass markers. (B) Volume quantification by PhosphorImager showing the relative densities of lanes a and b of (A). (C) Lane a, autoradiograph of [^{35}S]methionine- and [^{35}S]cysteine-labelled GST purified on a GSH-Sepharose 4B affinity column, released by Sertoli cells in culture media. Lane b, GST released by Sertoli cells in culture labelled with [^{35}S]methionine and [^{35}S]cysteine in the presence of unlabelled methionine. (D) Volume quantification by PhosphorImager showing the relative densities of lanes a and b of (C). (E) Immunolocalization of GST-Mu using GST-Mu-specific antisera in cultured Sertoli cells from rats of 18 days of age. (F) Significant reduction in staining intensity when antibody was adsorbed with excess antigen.

on 12% SDS/PAGE (Figure 4C, lane a). This labelling could be displaced in presence of cold methionine (Figure 4C, lane b). Volume quantification confirmed the observations on gel (Figure 4D). Sertoli cells in culture showed the presence of immuno-reactive GST in their cytoplasm (Figure 4E), which could be

Figure 5 GST isoforms in STF secretion *in vitro*

Profile of GST isoforms detected in the spent culture media of STs at 16 h of culture after RP-HPLC on a C_{18} RP column. For analysis, 60% solvent 1 and 40% solvent 2 was used to develop a gradient of 40–70% over a period of 70 min. Peaks 1 and 2 are rGSTM1 and rGSTM2, peak 3 is rGST-Pi and peak 4 is rGSTM3.

Figure 6 GSTs in germ cells

(A) Autoradiograph of [^{35}S]methionine- and [^{35}S]cysteine-labelled GST present in germ cell lysates (lane a) and released by germ cells in culture media (lane b). Labelled GST was purified on GSH-Sepharose 4B affinity column and run on SDS/PAGE, followed by exposure to X-ray films for 10 days. Lane c shows molecular-mass markers. (B) Volume quantification by PhosphorImager showing the relative densities of lanes a and b of (A).

displaced if the anti-MuC antibody was adsorbed with excess peptide (Figure 4F). The analysis of GSTs purified by GSH affinity chromatography from spent ST culture media and comparison with RP-HPLC analysis profiles of STF-GSTs showed that GST-Alpha was not secreted *in vitro*; however, the

Figure 7 Immunocytochemical localization of GSTs in rat testis

(A) Section of rat testis stained with anti-GSTMu antibody (anti-MuC) showing ST at stage 5 of the SE cycle. Sertoli cells with immunoreactive brown deposits are visible extending from the base towards the lumen. Haematoxylin-stained heads of step 17 elongated spermatids are visible closely associated with the Sertoli cell cytoplasm. Pachytene spermatocytes and step 5 round spermatids are visible unstained. (B) Section showing ST at stage 7 of the SE cycle with spermatids about to be spermeated. Note the intense immunoreactivity associated with the late step 19 spermatid cytoplasm that at this stage is located near the head. Round spermatids and pachytene spermatocytes are not stained. (C) Stage 8 ST showing spermatozoa released into the lumen. Immunoreactivity is visible at the base and tip of the Sertoli cells. (D) Stage 12 of the SE cycle showing staining of Sertoli cells. Step 12 spermatids are visibly associated with the Sertoli cell body. (E) Section at stage 7 of cycle stained with anti-MuC antibody absorbed with excess antigen showing significant displacement of staining. For (A)–(E) magnification is $200\times$. (F) Higher-magnification ($1000\times$) photomicrograph showing step 5 spermatids associated with Sertoli cells. (G) High-magnification ($1000\times$) photomicrograph showing a Sertoli cell extending from the base of the ST towards the lumen, with no spermatid head closely apposed to the cytoplasm, as the spermatids have just been released. L = lumen, n = Sertoli cell nucleus, i = interstitium, el = elongated spermatids, sc = Sertoli cells, p = pachytene spermatocytes, st = spermatids, pl = preleptotene spermatocytes, r = residual body, rb = residual body still attached to the elongated spermatid.

secretion of GST-Pi was induced in culture (Figure 5). The identity of GST-Pi was established by N-terminal sequencing. Therefore, barring a change in the pattern of GST-Alpha and -Pi secretion observed *in vitro*, no other change was detected in the isoform composition of the ST fluid collected *in vivo*.

Synthesis of GSTs by germ cells

[35 S]Methionine and [35 S]cysteine incorporation studies showed significant accumulation of radiolabelled GST in the cell lysates run on SDS/PAGE, but there was no significant amount detectable in the spent culture media (Figure 6A, lane b). The faint bands seen in Figure 6, lane b, may indicate release of some

GST by germ cells. After 5 h of experimenting, the viability of the germ cells was 95%, as measured by propidium iodide staining using FACS (results not shown).

Immunocytochemical studies

Antisera against GST-MuC detected immunoreactive GST-Mu in the Sertoli cells of sections fixed with Bouin's fluid. In all stages of the SE, the Sertoli cells showed the distribution of immunoreactive material from the base of the SE towards the lumen in finger-like projections, which is typical of Sertoli cell shape, shown in stages 4, 7, 8 and 12 of the cycle of the SE in Figures 7(A)–7(D). The specificity of staining was checked by

Figure 8 Germ-cell-free animals

(A) Size of testicles of males from control mothers not treated with busulphan (control) and testicles from males whose mothers were treated with busulphan on day 13 of pregnancy (bu-tr). (B) Section through the testis of a male from a busulphan-treated mother showing drastic reduction in the size of the STs and empty lumen. (C) Control testis showing normal size of tubules and populated ST photographed at the same magnification as (B) (200 $\times$). (D) a, RP-HPLC of STF from control animals; b, RP-HPLC of STF from germ-cell-free animals.

saturating the antibody-binding sites with excess antigens which showed significant reduction in immunoreactive deposits (Figure 7E). Counter-staining of testes showed sperm heads deeply embedded in crypts within the Sertoli cells surrounded by immunoreactive material (Figure 7F). Sertoli cells just after spermiation, without any embedded sperm head, showed clear staining (Figure 7G). Interstitial cells showed some reactivity Figures 7(A) and 7(B). No staining of germ cells was visible with this antiserum.

Table 4 Comparison of STF-GST activities in normal control and germ-cell-free rat testes

Results are given as means $\pm$ S.E.M.; $n = 3$. * $P < 0.001$; ** $P < 0.0001$.

Age of rats (days)	Enzyme activity [nmol $\cdot$ min $^{-1}$ $\cdot$ (mg of protein) $^{-1}$]	
	Control	Busulphan-treated
90	92.4 $\pm$ 6.5	137.84 $\pm$ 7.3*
110	144.3 $\pm$ 10.3	268.93 $\pm$ 2.6**

Figure 9 Steroid binding by GST molecules

(A) Elution of [3 H]testosterone incubated with STF (20 mg/ml) on Sephadex G-25 as two separate peaks of radioactivity. The first peak co-eluted with GST, as shown by elution of GST activity with the first peak. $\circ$, [3 H]Testosterone; $\bullet$, GST activity. The inset shows the displacement experiments with excess unlabelled testosterone. a = [3 H]testosterone, b = [3 H]testosterone + 60 nM unlabelled testosterone, c = [3 H]testosterone + 600 nM testosterone, d = [3 H]testosterone + 6 μ M testosterone. (B) Elution of [3 H]oestradiol incubated with STF (20 mg/ml) from a Sephadex G-25 column showing two peaks of radioactivity. The first peak co-eluted with GST activity. $\circ$, [3 H]Oestradiol; $\bullet$, [3 H]oestradiol bound in the presence of unlabelled oestradiol. The inset shows the GST activity of both groups, tube numbers 3–6.

Germ-cell-free animal model

There was a significant reduction in the size of the testis of males born of busulphan-treated mothers in comparison with males of the same age from untreated mothers (Figure 8A). There was a complete lack of germ cells within the ST of males from treated

Figure 10 Steroid binding by rGSTM1 and rGSTM2

(A) Elution of [^3H]testosterone + GSTM1 and GSTM2 on a Sephadex G-25 column. The first larger peak represents the steroid bound to rGSTM1 ($\square$) while the smaller peak shows binding of the same isoform in the presence of unlabelled steroid ($\triangle$). Note that the binding of GSTM2 to the labelled steroid occurs where no first peak is detected; only the free steroid peak is seen ($\circ$). (B) Elution of [^3H]oestradiol + GSTM1 and M2 on a Sephadex G-25 column. The first larger peak represents the steroid bound to rGSTM1 ($\square$), whereas the smaller peak shows binding of the same isoform in the presence of unlabelled steroid ($\triangle$). Note the binding of GSTM2 to the labelled steroid occurs where no first peak is detected ($\circ$).

mothers as compared with controls (Figures 8B and 8C). There was no change in the GST isoform profile of the STF of germ-cell-free males in comparison with control males when analysed by RP-HPLC (Figure 8D, a and b respectively). There was an apparent increase in enzyme activity (Table 4) in the animals lacking germ cells when expressed per mg of testis; this should not be considered an actual increase as the treated testis had a much lower protein content due to reduced weight. There was no change in the GST isoform profile of the STF of germ-cell-free animals (Figure 8D).

Steroid-binding studies

Two major radioactivity peaks eluted from Sephadex G-25 separation of a mixture of total STF and [^3H]testosterone. Radioactivity was associated with the GST activity peak, followed by another peak representing the free (i.e. unbound) counts (Figure 9A). The binding of radioactive testosterone could be effectively competed out with excess unlabelled testosterone in a dose-dependent manner (inset). Similar results were obtained with the binding of labelled oestradiol to STF (Figure 9B). Among the isoforms separated on RP-HPLC, only GSTM1 bound to labelled oestradiol and testosterone (Figures 10A and 10B). The K_d values for GST binding to testosterone and oestradiol were $< 9.8 \times 10^{-7}$ M and 9×10^{-6} M respectively.

DISCUSSION

GSTs are important detoxification enzymes. They also serve as intracellular-binding proteins for transport of hormones and other molecules [4]. By virtue of the extensive variety of demonstrated and proposed functions, and the emerging large variation in isoforms, GSTs are an important supergene family. Several studies have focused on the identification of GST isoforms in whole rat and human testis or testicular cells [21–29], while

some investigations have attempted to selectively localize the GST isoforms in specific testicular compartments [30–33]. Identification of GST isoforms in whole testis homogenates is of little value, as testis is a heterogeneous tissue and different cells may express different isoforms. The ST is the most important part of the testis, as it houses the germ cells that are required for propagation of species. The ST is lined by the SE, composed of germ and Sertoli cells, and is divided into basal and adluminal compartments by the blood/testis barrier [34]. This barrier prohibits movement of molecules from the interstitium to the adluminal compartment. Because of this segregation, the later stages of germ cells are totally dependent upon the micro-environment provided by secretions of the Sertoli cells [35,36]. Therefore, the fluid within the ST, that surrounds the germ cells, has significant influence on germ cell survival and integrity, making the proteins secreted into the STF very important from a germ cell growth and survival point of view. This study is the first report, described in four parts, of GST isoforms present in the STF. First is the demonstration of GST isoforms in STF and their identity. Second is the evidence that GST isoforms are actively synthesized and secreted *in vitro* and third is the identification of Sertoli cells as the prime secretors. The fourth part provides data demonstrating the ability of STF GSTM1 to bind to two physiologically relevant steroids, testosterone and the oestradiol.

Earlier reports from our laboratory described the presence of significant amounts of GST in rat STF *in vivo*. The identification of the different isoforms in the STF in the current study shows that the STF-GST isoforms are structurally similar to the liver isoforms. The slightly larger molecular mass of STF-GSTM1 observed may be due to glutathionylation [37]. Many Sertoli cell secretory proteins closely resemble their somatic cell counterparts [38]; therefore, the observed comparable structural similarity in GST isoforms from the STF and liver is not surprising. It is possible, because of special functional requirements in the testis,

that a significant amount of GST enzymes are routed through the secretory pathway in the testicular cells, perhaps in a similar manner as liver GSTs are secreted in the bile [39]. These results are in agreement with a study that shows that the total cells of the ST express GSTM1, GSTM2 and GSTM3 [33]. However, this study [33] did not investigate isolated cells or secretory GSTs. The attenuated expression of GSTM3 in the liver and the detection of GSTM2 as the predominant isoform in the STF is in agreement with previously published reports [4,12,32] with whole testis homogenates. The similarity of peptide digest profiles of individual isoforms from the liver and the STF confirm the structural similarity between the molecules from two different organs. The sequences of the peptides obtained from the peptide digests of the STF-GST isoforms show minor substitution of amino acids in STF isoforms as compared with liver sequences, which may have occurred in response to different functional requirements in the testis. Therefore, taken together, the comparable RP-HPLC profile of liver and STF-GST isoforms, the N-terminal amino acid sequences, molecular mass data and peptide digest profiles, clearly confirm the structural similarity of GST isoforms in the liver and the ST.

Results from this laboratory have earlier described a time-dependent release of GST by ST cultures *in vitro* [1]. Our current studies confirm active synthesis and secretion of GSTs by the ST cultures, which is evident from the recovery of radiolabelled GST from ST culture supernatants. Secretion of mammalian GSTs is not widely known, although reports of release of GST into human plasma by platelets and malignant tumour cells [40] and by liver into bile [39] exist. The ability of the cells of the SE to secrete GST isoforms *in vitro* is confirmed by analysis of spent culture media. The enhanced expression of GST-Pi and the absence of GST-Alpha is similar to the situation observed by Abramovitz et al. in primary hepatocyte cultures [41]. One can speculate that the variable regulation of isoform secretion observed in the ST cultures compared with *in vivo* situations may be due to the absence of interstitial tissue *in vitro*. Since it is known that complex cell-cell interactions exist between the interstitial cells and the cells of the ST, it is tempting to speculate that regulatory molecules from the interstitial cells control GST secretion; however, further studies have to be carried out before a conclusive statement can be made.

Taking into account that movement of molecules from the interstitium to the adluminal compartment of the ST is restricted, the two cell types lining the SE were investigated as sources of STF-GST. Detection of significant amounts of radiolabelled GST in Sertoli-cell conditioned media, the presence of multiple GST isoforms in the STF and the presence of immunostainable GST-Mu located in the SE in a pattern consistent with Sertoli cell staining [42], points towards the Sertoli cells as possible GST secretors. In addition, the observation of an unchanged pattern of STF-GST secretion in 'Sertoli cell only' animals clearly established Sertoli cells as the prime secretors of STF-GSTs. The inability to detect GST-Alpha in the Sertoli cells immunocytochemically, although they were recovered by RP-HPLC from STF, may be due to limitations in the quantity of proteins required for detection by immunocytochemistry. It is well established that Sertoli cell proteins secreted into the STF help in germ-cell development [35], and recent reports show that proteins secreted into the ST lumen can also have a 'lumicrine' effect on the function of the downstream epididymal epithelium [43]; therefore localization of GST in this compartment may suggest functional importance of the protein in germ cell development and transport.

Although Sertoli cells are acknowledged as the major contributors of secretory products in the STF [35], germ cells are

also known to release proteins *in vitro* [44]. However, significant amounts of radiolabelled GST in germ cell lysates, in contrast to the amount present in the germ cell culture supernatants, testify that these cells synthesize GST but do not contribute to a significant release. It is possible that germ cells acquire proteins from Sertoli cells, a phenomenon reported for some molecules [45]. However, data on incorporation of radiolabelled amino acids and the presence of GST messenger RNA in germ cells (A. V. S. K. Rao and C. Shaha, unpublished work) confirm the ability of these cells to synthesize GST, although that does not discount the possibility of some Sertoli cell proteins being acquired by the germ cells. GST-Mu in mouse germ cells [46] and GST-Pi in human germ cell tumours have been described [47]. Although the function of GSTs in germ cells is yet to be determined, one can speculate as to the potential role of GSTs in detoxification reactions, as the close proximity of these cells to the Sertoli cells make them vulnerable to injurious products from phagocytic nurse cells. Results from this laboratory with mature goat sperm implicate GSTs as multifunctional proteins according protection to sperm from reactive oxygen species [48]. Therefore investigations in that direction with germ cells may offer clues to the function of GSTs in combating free radicals in these cells.

Large amounts of GST in the ST is an indication that this molecule is functionally important in this compartment. Since STF-GSTs are enzymically active, it is perhaps obvious that they will take part in detoxification reactions. However, clues from a series of studies coming from Listowsky and co-workers on the ability of liver GSTs to bind to bilirubin, glucocorticoids, progestins, oestradiol and thyroid hormones [16,49–52], prompted us to investigate the potential role of this protein of STF source in steroid binding. The ability of oestradiol and testosterone to bind to STF-GSTs appears to implicate GSTs as prominent sex-steroid-binding proteins in the ST. This observation is particularly meaningful, because there is a great requirement of steroid binding and carrier proteins in the Sertoli cells and the STF for transport of molecules to different cellular sites and cells respectively. Both testosterone [53] and oestradiol [54] are important for spermatogenesis, and the concentration of testosterone in the STF is 20 times higher than in the peripheral circulation [55]. Oestradiol is known to regulate spermatogenesis, and several generations of germ cells possess oestradiol receptors [56]. The fact that GSTM1 could bind to the sex-steroids generates the possibility that different GST isoforms may be performing different functions and makes GSTM1 a very important component of the ST. Two examples of binding proteins in the same compartment are the androgen binding protein, which is known to carry androgens, and transferrin, which transports iron [38]; however, these molecules are not known to perform any other function. The binding of STF-GST to the steroids appears to be specific, as radiolabelled steroids are displaced from the protein by excess of unlabelled forms and sub-saturating amounts fail to displace labelled steroid. The K_d values obtained with both testosterone and oestradiol reflect high-affinity binding compared with binding of liver GSTs to progesterone and androsterone as reported earlier [57]. Recently, binding of rat liver GSTM1 and GSTM2 to α -tocopherylquinone has been reported, where both the subunits are indicated as transport proteins [58]. Irreversible covalent labelling may not accurately reflect cellular processes where the relative dissociation rates of steroids from steroid-binding proteins are important to make the steroid available for cellular processes.

In summary, we have established that GSTs are actively synthesized and secreted by the ST of Sertoli cells, where they appear to be present in all stages of the SE cycle. The ability of germ cells to synthesize and not to secrete this protein is also

recognized. Most importantly, the functional involvement of GSTM1 in sex-steroid binding function, in addition to the catalytic function of GSTs, makes them vital multifunctional proteins in the ST compartment.

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