Interactions between mouse ZP2 glycoprotein and proacrosin; a mechanism for secondary binding of sperm to the zona pellucida during fertilization

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SUMMARY

The mouse zona pellucida glycoprotein, mZP2, is thought to be the secondary receptor on eggs for retention of acrosome-reacted sperm during fertilization. Here, we present evidence that one of its complementary binding proteins on sperm is proacrosin/acrosin. mZP2 binds to proacrosin null sperm considerably less effectively than to wild-type sperm. Binding is mediated by a strong ionic interaction between polysulphate groups on mZP2 and basic residues on an internal proacrosin peptide. The stereochemistry of both sulphate groups and basic amino acids determines the specificity of binding. Structurally relevant sulphated polymers and suramin, a polysulphonated anticancer drug, compete with mZP2 for complementary binding sites on proacrosin/acrosin in solid-phase binding assays. The same competitors also displace attached sperm from the zona pellucida of eggs in an in vitro fertilization system. This combination of genetic, biochemical and functional data supports the hypothesis that mZP2-proacrosin interactions are important for retention of acrosome-reacted sperm on the egg surface during fertilization. Safe mimetics of suramin have potential as non-steroidal antifertility agents.

Key words: Fertilization, Proacrosin, Zona Pellucida Glycoprotein mZP2, Secondary binding, Mouse

INTRODUCTION

All mammalian eggs are surrounded by an extracellular matrix, the zona pellucida (ZP), which in the mouse paradigm is composed of three major sulphated glycoproteins designated mZP1, mZP2 and mZP3 (Wassarman, 1999). Current evidence indicates that O-linked oligosaccharides on mZP3 constitute the primary receptor that mediates binding of acrosome-intact sperm to the ZP surface, whereas mZP2 functions as the secondary receptor for retention of acrosome-reacted sperm (Florman and Wassarman, 1985; Bleil et al., 1988; Mortillo and Wassarman, 1991). mZP1 is thought to have a largely structural role. While this picture is fairly clear, the nature of the complementary binding or ligand molecules on sperm is highly contentious and a wide variety of candidates has been proposed, most of which are difficult to equate with the receptor properties of the ZP (Jones, 1990; McLeskey et al., 1998). Possible exceptions are β1,4-galactosyl transferase (β1,4-GalTase), which is thought to recognise N-acetylglucosamine residues on mZP3 oligosaccharides (Miller et al., 1992), and sp56, which contains Sushi domains characteristic of a superfamily of protein receptors (Bookbinder et al., 1995). Unexpectedly, however, β1,4-GalTase null (–/–) sperm bind more strongly to the ZP than wild-type (+/+) sperm (Lu and Shur, 1997), and recent evidence suggests that sp56 may be intra-acrosomal (Foster et al., 1997) and not on the surface membrane as originally reported (Cheng et al., 1994). Currently, therefore, there is no consensus of opinion on the nature of the primary mZP3-binding molecules on the mouse sperm surface.

Similar controversy surrounds the identity of the binding molecule on acrosome-reacted sperm for mZP2, although a leading candidate for this role is proacrosin and its active form, acrosin (Jones et al., 1996). Proacrosin is found within the acrosomal vesicle of all mammalian spermatozoa and, following exposure of the acrosomal matrix by the acrosome reaction, is correctly positioned to mediate binding between the newly exposed inner acrosomal membrane and the ZP. Previous work has shown that boar proacrosin/acrosin and homologous ZP glycoproteins bind strongly and specifically to each other (Brown and Jones, 1987; Jones, 1991; Urch and Patel, 1991; Jansen et al., 1995; Moreno and Barros, 2000). The interaction involves ionic bonds between polysulphate groups on ZP oligosaccharides and basic residues on the surface of procrosin/acrosin in a manner similar to that described between heparin and antithrombin III (Peterson et al., 1987; Arocas et al., 1999). Such a mechanism would be sufficiently tenacious to prevent sperm from detaching from the ZP surface and swimming away while at the same time not being so strong as to impede sperm penetrating through the ZP. To obtain genetic and biochemical evidence to support this hypothesis, we used sperm from proacrosin (–/–) mice and suramin (a polysulphonated drug) to probe proacrosin-mZP2 interactions. In addition, we used in vitro fertilization assays to investigate the functional relevance of the above concepts. Our results show that proacrosin/acrosin fulfils the three most

important criteria for a ZP-binding protein, namely, it is tissue-specific (i.e. on sperm), it is exposed in the correct location at the correct time (i.e. on the surface of the ZP following the acrosome reaction) and its mechanism of binding is compatible with the known biochemical properties of the receptor (i.e. sulphate groups on mZP2).

MATERIALS AND METHODS

Reagents

All chemicals, enzymes and general reagents were of the highest purity available commercially and were purchased from Sigma (London, UK) and BDH/Merck (Poole, UK). Sodium ^{125}I and [^{3}H]suramin were obtained from Amersham-LKB (Amersham, UK).

Production of proacrosin knockout mice

Proacrosin (–/–) mice (CD1 strain), in which the gene encoding for proacrosin was disrupted by targeted insertion of a PGK-Neo gene into exon 2, were developed at the University of Gottingen as described previously (Adham et al., 1997). A breeding colony of homozygous proacrosin (–/–) males and females was established at the Babraham Institute following UK Home Office guidelines.

Bacterial expression of proacrosin peptide

An internal peptide of mouse proacrosin, representing residues 38-288 was expressed as an N-terminal His-tag protein in *E.coli* strain DH5. The encoding cDNA fragment was derived by PCR from a full-length mouse proacrosin cDNA using the forward primer 5′-GCGGGATCCATTGTCAGTGGGCAGAGTGCG-3′ and the reverse primer 5′-GCGGAATTCATCAGCCGATCTTGGAAGCAATCCAG-3′. The amplified cDNA obtained was digested with restriction endonucleases *Bam*HI and *Eco*RI and inserted between the corresponding sites of pTricHis plasmid (Invitrogen BV, Leek, The Netherlands). *E. coli* (TOP10 strain) were transfected by electroporation and grown overnight to an OD550 value of 0.5 in 2× TY (tryptone-yeast) medium containing 200 μg/ml ampicillin. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 1 mM and the cells were left on a shaker at 37°C for 3 hours. Bacteria were then pelleted by centrifugation at 1200 ***g*** for 5 minutes, resuspended in MT-PBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4) and subjected to a further centrifugation step, after which the pellet was taken up into 4 ml MT-PBS. The bacterial cells were disrupted by sonication to release the inclusion bodies, which were pelleted by centrifugation at 10,000 ***g*** for 20 minutes at 4°C and subsequently extracted with 2% SDS/62.5 mM Tris-HCl, pH 7.2, for 3 minutes at 100°C. Supernatant containing solubilized proteins was collected by centrifugation at 10,000 ***g*** for 20 minutes and stored frozen at –20°C. Proteins were separated under reducing conditions by SDS-PAGE on 12% polyacrylamide gels and either stained with Coomassie Blue R-250 or transferred to PVDF membranes by Western blotting techniques. Blots were probed with either the anti-proacrosin rabbit antiserum (dilution 1:200) using standard procedures or with ^{125}I-mZP glycoproteins as described below.

Production of antibody to mouse proacrosin peptide

A synthetic peptide, representing residues 206-217 (CAGYPEGKIDTC) of mouse proacrosin, was conjugated to a purified protein derivative of tuberculin and injected subcutaneously into BCG-sensitised Dutch rabbits. Preimmune and immune antisera were collected by standard procedures, decomplemented at 56°C for 30 minutes and stored frozen at –70°C.

Purification of ^{125}I-labelled mZP2 and ^{125}I-labelled mZP3 glycoproteins

Purified ZPs were collected from ovaries of 21-day-old mice by homogenization (Bleil et al., 1988) and stored in 2 ml graduated siliconised tubes (Camlab, Cambridge, UK) in 0.4 ml 50% glycerol:50% 25 mM triethanolamine buffer, 150 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 8.5, at –20°C for up to 2 months before use. ZPs accrued from approx. 100 mice were diluted with 1 ml sterile 0.1 M sodium borate buffer, pH 8.5, and washed twice by centrifugation at 12,000 ***g*** for 10 minutes. The ZPs were then melted in 0.25 ml borate buffer for 30 minutes at 70°C and centrifuged again at 12,000 ***g*** for 10 minutes. Solubilized ZP glycoproteins were radiolabelled with 1 MBq (27 μCi) [^{125}I]NaI using Iodo-Gen (1,3,4,6-tetrachloro-3a, 6a-diphenylglycouril; Pierce & Warriner, Chester, UK) immobilised on glass tubes (Jones et al., 1991). The reaction was terminated after 30 minutes by the addition of 10 μl 2.5 M potassium iodide and free and protein-bound ^{125}I were separated on a G-25 Sephadex column previously conditioned with 0.1% bovine serum albumin (BSA) and equilibrated with sterile PBS. ^{125}I-labelled fractions (containing a mixture of mZP1, mZP2 and mZP3) were collected in 0.5 ml volumes of sterile PBS and stored at 4°C.

^{125}I-mZP2 and ^{125}I-mZP3 were purified by preparative SDS-PAGE on 10% polyacrylamide gels under non-reducing conditions. Strips of gel (approx. 0.5 cm wide) equivalent to approx. 120 kDa (mZP2) and 83 kDa (mZP3) were excised and proteins eluted into 10 mM Tris/acetate buffer, pH 8.2, using an ISCO model 1750 concentrator. Recovery was routinely 60-70%. Eluted proteins were dialysed extensively against PBS at 4°C and their specific activities calculated. Protein concentrations were measured by absorbance at OD_{280} (a value of 1.0 was regarded as equivalent to 1 mg/ml). Radioactivity was counted on a Packard Auto-Gamma counter.

^{125}I-mZP2 and ^{125}I-mZP3 are highly glycosylated and hence migrate as diffuse bands on SDS-PAGE (see Fig. 3E). Because there is some overlap between their positions on the gel, the purity of ^{125}I-mZP2 and ^{125}I-mZP3 preparations was assessed using dot blot assays with monoclonal antibodies IE3 to mZP2 and IE10 to mZP3 (IE3 and IE10 were generously provided by Dr Jurrien Dean, NIH, Bethesda, MD, USA). Bound antibody was detected using a biotinylated anti-rat IgG followed by peroxidase-conjugated avidin (Dako, High Wycombe, UK) and visualized by ECL (Amersham, UK).

Binding of ^{125}I-mZP2 and ^{125}I-mZP3 glycoproteins to expressed proacrosin peptide

Blots containing the reduced acrosin peptide were blocked with 5% BSA in PBS for 1 hour at 20°C, rinsed briefly in PBS and probed with 50-100×10^3 cpm/ml of ^{125}I-mZP glycoproteins in PBS for 1 hour. Blots were washed 3× for 10 minutes in 100 ml PBS, dried and exposed to pre-flashed X-ray film at –80°C. Autoradiographs were quantified by densitometry. In experiments to assess binding of ^{125}I-mZP glycoproteins in the presence of sulphated and non-sulphated compounds, blots containing the proacrosin peptide were cut into 1×3 cm strips and incubated with varying concentrations of competitor in 200 μl of PBS for 1 hour. Strips were rinsed briefly in PBS and probed with ^{125}I-mZP2 or ^{125}I-mZP3 as described above. Preliminary experiments established that binding of labelled probes was linear under the conditions of the assay. To investigate the optimum pH and salt concentration for binding, blots were incubated in the presence of varying concentrations of NaCl (0-500 mM) or at different pH values (3.2-8.0) using 50 mM Na_2HPO_4/citric acid buffer. For Scatchard plot analysis, increasing concentrations of probes were added to a constant amount of target protein in a final volume of 200 μl and bound radioactivity identified by autoradiography. For quantitation, labelled regions (0.5×1 cm) of the blots were excised and counted in an Auto-Gamma counter. Maximum binding is defined as the amount of probe retained in the absence of a competing agent. Non-specific binding is the amount of probe remaining on an equivalent area of blot that does not contain the target protein and specific binding as the amount of probe bound to target protein minus non-specific background (Jones, 1991). The number of molecules of bound probe were calculated

from the specific activity of labelling and Avogadro's constant (6.022×10^{23}).

Preparation of gametes

3-week-old female CD1 mice were superovulated with an i.p. injection of 7.5 i.u. follicle-stimulating hormone ('Folligon'; Intervet, Houghton, UK) followed 48 hours later by 7.5 i.u. human chorionic gonadotrophin ('Chorulon': Intervet). Mice were killed 14-16 hours post-ovulation and the oviducts dissected out into M199 medium (Gibco-BRL, Life Science Technologies, Paisley, Scotland). Cumulus masses containing eggs were recovered and transferred into 1 mg/ml hyaluronidase in M199 at 37°C for 2-5 minutes. When free of cumulus cells, eggs were immediately transferred through two washes of M199 using a mouth-operated siliconised micropipette and maintained in M199 at 37°C in 5% CO_2:95% air. Spermatozoa were obtained from mature CD1 male mice by removing cauda epididymides into 0.75 ml M199 and mincing the tissue 2-3 times with fine iridectomy scissors. Released spermatozoa were allowed to disperse for 30 minutes at 37°C in 5% CO_2: 95% air. A 75 μl sample of the dispersed sperm suspension was added to 500 μl M199 modified with 2 mg/ml BSA and 30 mg/ml pyruvate immediately before use (M199-M) to give a sperm concentration of $2.5\text{-}4\times10^6$/ml. Spermatozoa were incubated in 5% CO_2:95% air at 37°C for 1-2 hours to complete capacitation (assessed by monitoring motility). Sperm concentration was assessed using a haemocytometer.

Binding of ^{125}I-mZP2 and [^{3}H]suramin to spermatozoa from proacrosin (–/–) and (+/+) mice

Motile spermatozoa recovered from the cauda epididymidis of proacrosin (–/–) and (+/+) mice were capacitated as described above and the sperm concentration adjusted to 2×10^6/ml with M199 containing 1 mM p-aminobenzamidine (pAB). Samples (1 ml) were then subjected to 2 cycles of rapid freezing and warming to 37°C to permeabilize the sperm membranes. The pAB prevents loss of acrosomal contents. Samples were centrifuged at 800 ***g*** for 10 minutes and the resulting pellet resuspended in M199/pAB to a sperm concentration of 10^7/ml. A 10 μl portion was added to $0.5\text{-}1.0\times10^6$ cpm ^{125}I-mZP2 and this was diluted 20-fold with M199/pAB. The samples were incubated at 37°C in 5%CO_2:95% air for 1 hour. An equal volume of fixative (2% glutaraldehyde/2% formaldehyde in PBS) was added and after 30 minutes spermatozoa were pelleted by centrifugation at 800 ***g*** for 5 minutes. Sperm pellets were washed 5 times in 1 ml of 50 mM triethanolamine buffer, pH 8.0, and then resuspended in 0.5 ml of the same buffer. Sperm smears were prepared on clean glass slides, air-dried and stored in a desiccator until coated with Ilford K2 nucleur emulsion for autoradiography. Slides were exposed at 4°C for 2-14 days followed by development in Ilford Phenisol developer. For labelling with [^{3}H]suramin, washed permeabilized spermatozoa were prepared as above and incubated in suspension with 0.2 μl/ml [^{3}H]suramin (0.05 MBq or 0.8 μCi). In other experiments, spermatozoa were dispersed and washed in PBS/5 mM glucose/1 mM pAB, diluted to 1×10^6 spermatozoa/ml and 20 μl samples smeared and dried onto glass slides. These permeabilized cells were subsequently incubated with either 0.2 μl/ml [^{3}H]suramin or 10^6 cpm ^{125}I-mZP2 in PBS/1 mM pAB, pH 7.0, for 1 hour at room temperature, either alone or in the presence of 'cold' suramin (0.7 mM) or 'cold' ZP2 (0.05 mg/ml), respectively. The slides were then washed 5 times in PBS and dried in a desiccator before coating in Ilford K2 nucleur emulsion. Labelling was assessed by counting the number of silver grains over the heads of 100 spermatozoa viewed by phase-contrast microscopy. Background levels were assessed by counting the number of grains in 100 equivalent areas of the same slide using an eyepiece graticule.

Displacement of bound spermatozoa from the ZP ('pulse-chase' experiments)

The experimental protocol was similar to that described by Bleil et al. (Bleil et al., 1988). In each experiment, approx. 180 cumulus-free eggs were transferred into a 100 μl drop of M199-M medium in a 9 cm Petri dish and covered with sterile, PBS-saturated liquid paraffin maintained at 37°C. Capacitated spermatozoa were added to give a final concentration of 2.5×10^5/ml and the volume of the drop adjusted to 200 μl with M199-M. The Petri dish was returned to the incubator for 5 minutes. This represents the 'pulse' period. The eggs were then removed from the drop and washed through 3 drops (200 μl volumes) of M199-M to remove loosely adhering spermatozoa. For 0 min controls, approximately 20 eggs with bound spermatozoa were removed into 100 μl 2% glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4, and maintained at 37°C. Further batches of 20 eggs were transferred to 50 μl droplets of either M199-M alone (incubated controls) or M199-M containing the compound to be tested, and were incubated at 37°C in 5% CO_2:95% air for 5, 15, 25 or 35 minutes, at which times the eggs were removed and fixed in 2% glutaraldehyde as described above. This represents the 'chase' period. Compounds to be tested for their ability to displace bound spermatozoa from the ZP were dissolved in M199-M at the concentrations indicated in the Results section and pH was adjusted to 7.2 where necessary. Eggs were transferred to microscope slides, viewed with a ×40 objective lens using phase-contrast optics and the total number of spermatozoa adhering to each egg calculated by counting sperm heads at different focal planes.

RESULTS

Binding of ^{125}I-mZP2 and [^{3}H]suramin to spermatozoa from proacrosin (–/–) and (+/+) mice

In agreement with published work (Bleil and Wassarman, 1986; Bleil et al., 1988), we found that ^{125}I-mZP2 does not

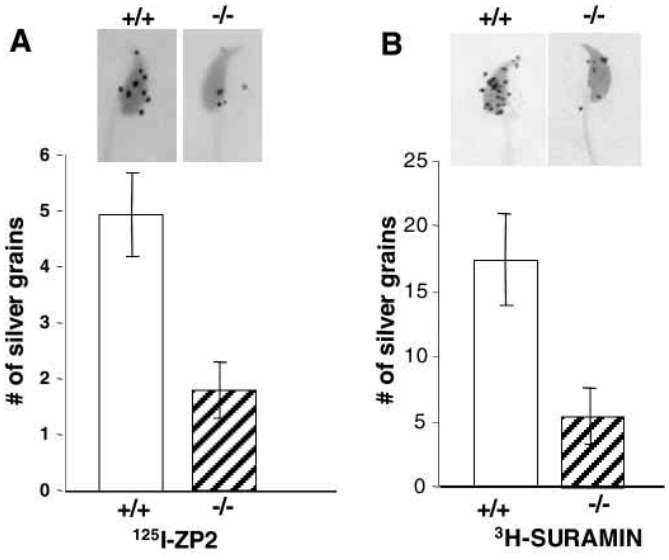


Fig. 1. ^{125}I-mZP2 and [^{3}H]suramin bind less efficiently to proacrosin (–/–) than (+/+) mouse sperm. Binding of (A) purified ^{125}I-mZP2 and (B) [^{3}H]suramin to proacrosin (+/+) and (–/–) sperm was detected by autoradiography. Micrographs (top) illustrate the typical distribution of silver grains over the sperm head. Data below show the mean number ± s.e.m. of silver grains over the head region of 100 sperm from each of nine proacrosin (–/–) and nine wild-type (+/+) mice in A, and three acrosin (–/–) and four wild-type (+/+) mice in B. Differences between (+/+) and (–/–) sperm were significant (P<0.01) in both A and B.

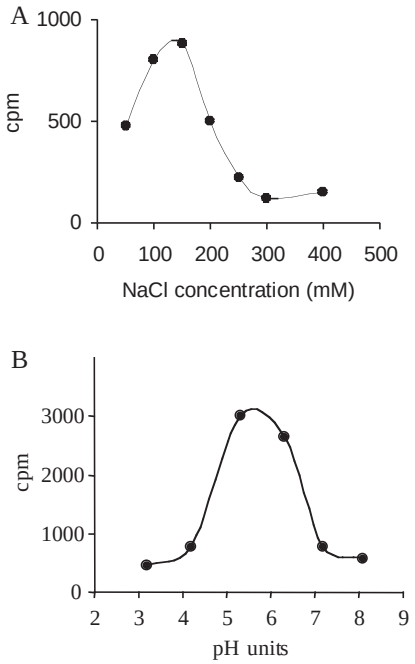


Fig. 2. Effects of ionic strength and pH on binding of ^{125}I-ZP glycoproteins to expressed proacrosin peptide. The relative uptake of unfractionated ^{125}I-ZP glycoproteins to expressed proacrosin peptide (expressed as c.p.m. bound, see Fig. 3) was measured in the presence of (A) 0-400 mM NaCl or (B) pH 3.2-8.0 at 100 mM NaCl.

bind to membrane-intact (+/+) spermatozoa. Typically, in fresh sperm suspensions, 15±2.5% became labelled after incubation with ^{125}I-mZP2 (this represents the background level of dead or spontaneously acrosome-reacted sperm), whereas after permeabilization 92±1.6% had significant numbers of silver grains overlying the head region. A similar result was obtained with [^{3}H]suramin, which has also been shown not to penetrate intact sperm plasma membranes (Jones et al., 1996). However, when permeabilized sperm from proacrosin (–/–) mice were probed with ^{125}I-mZP2 or [^{3}H]suramin, they had, respectively, 67% and 66% fewer silver grains overlying their heads than (+/+) sperm (Fig. 1A,B). Only background labelling was detected in the presence of 'cold' mZP2 or 'cold' suramin (results not shown). The difference in uptake of these probes between (+/+) and (–/–) sperm, therefore, must reflect the presence or absence of native proacrosin as it is normally complexed within the acrosomal matrix to various inhibitors and binding proteins.

Binding of ^{125}I-mZP2 and ^{125}I-mZP3 glycoproteins to expressed mouse proacrosin peptide

In preliminary experiments we assessed the purity of ^{125}I-mZP2 and ^{125}I-mZP3 probes using monoclonal antibodies IE3 (specific for mZP2) and IE10 (specific for mZP3) (East et al., 1985). Results from dot-blot assays containing equivalent amounts of purified mZP2 or mZP3 showed <0.1% crossreactivity with the appropriate antibody (results not shown). Optimisation of the conditions for binding of the labelled probes showed that uptake was highest in the presence of 100-150 mM NaCl and at pH values between 5.0 and 6.5 (Fig. 2). In future experiments, therefore, blots were probed in PBS adjusted to pH 6.25 with 25 mM Na_2HPO_4 and 10 mM sodium citrate to make conditions relevant to the situation on the ZP surface in vivo (see Discussion).

Since ^{125}I-mZP2 has been shown by autoradiographic analysis to bind more strongly than ^{125}I-mZP3 to acrosome-reacted mouse sperm (Bleil and Wassarman, 1986), it may be predicted that a similar difference will be observed with proacrosin/acrosin as the target protein. For this purpose, an internal peptide representing residues 38-288 was expressed in bacteria (Fig. 3A) (mouse sperm contain very small amounts of proacrosin, logistically precluding purification of the native protein) (Brown, 1983). This peptide encompasses the ZP-binding region defined in earlier studies on boar proacrosin (residues 47-272) (Jansen et al., 1995; Jansen et al., 1998). Additionally, various synthetic peptides representing the N terminus (residues 1-23) or the proline-rich C terminus (residues 323-399) show no binding activity. Western blots containing proteins extracted from inclusion bodies with SDS were either stained with Coomassie Blue (Fig. 3A, lanes 1,2) or probed with an unfractionated mixture of ^{125}I-mZP glycoproteins (Fig. 3B, lanes 3,4) or purified ^{125}I-mZP2 (Fig. 3C, lane 5) or purified ^{125}I-mZP3 (Fig. 3C, lane 6) and bound label was detected by autoradiography. Despite the presence of a large number of bacterial proteins on the blot, all three probes bound selectivity to a major component at approx. 32 kDa that was only present in IPTG-induced cultures. That the 32 kDa protein represented the internal proacrosin peptide was shown by its reactivity to the anti-acrosin antibody (Fig. 3D, lanes 7,8). If expressed proteins were separated by non-reducing SDS-PAGE, however, then binding to the 32 kDa protein was <tenfold of that shown. This suggests that during expression in bacteria there is incorrect folding of the proacrosin peptide, but following reduction, sufficient renaturation takes place on the blot to permit significant binding.

The expressed proacrosin peptide was then probed with ^{125}I-mZP2 and ^{125}I-mZP3 purified from the same original sample (Fig. 3E) to obtain a direct comparison of their relative affinity. As shown in Fig. 3F, for a constant amount of target protein ^{125}I-mZP2 shows between 1.5- to 2.0-fold higher binding than ^{125}I-ZP3. Saturation binding with ^{125}I-mZP2 followed by Scatchard plot analysis gave an estimated K_d of 0.97×10^{-6} M. Unfortunately, the low specific activity of labelling of ^{125}I-mZP3 precluded further analysis with this probe. Binding of ^{125}I-mZP3 to the proacrosin peptide is not unexpected since (1), some uptake of ^{125}I-mZP3 has been reported to acrosome-reacted sperm, albeit to a much lesser extent than for ^{125}I-mZP2 (Mortillo and Wassarman, 1991) and (2), mZP3 oligosaccharides contain a higher concentration of sulphate groups than mZP2. Chemical characterization of N-linked oligosaccharides from mZPs has revealed 2.8 mols sulphate/mol protein on mZP3 against 1.1 mols sulphate/mol protein on mZP2 (Noguchi and Nakano, 1993; Takasaki et al., 1999).

Mechanism of binding between ^{125}I-mZP2 and expressed proacrosin peptide

To investigate the mechanism of binding between ^{125}I-mZP2 and sperm proacrosin/acrosin, the expressed peptide was probed in the presence and absence of various competing agents. Binding was strongly inhibited in the presence of 1 mg/ml of fucoidan, dextran sulphate 500K, suramin and

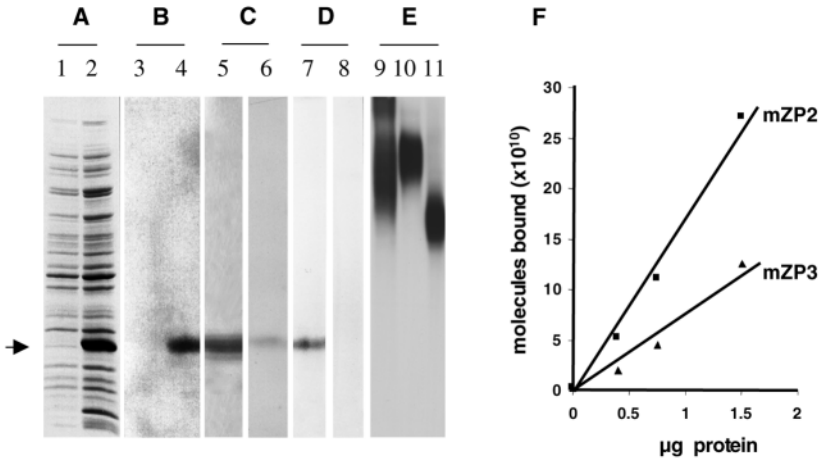


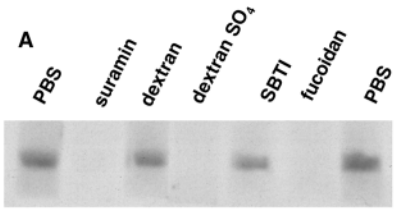
Fig. 3. Relative binding of ^{125}I-mZP2 and ^{125}I-mZP3 glycoproteins to bacterial expressed mouse proacrosin peptide. (A) Coomassie Blue stained proteins separated by reducing SDS-PAGE after extraction from inclusion bodies. Lane 1, pre-induction; lane 2, post-induction showing expression of a major 32 kDa peptide (arrow). (B) Binding of unfractionated ^{125}I-mZP glycoproteins to expressed 32 kDa peptide on western blots. Lane 3, pre-induced bacterial proteins probed with ^{125}I-mZP glycoproteins; lane 4, post-induction showing binding of ^{125}I-mZP glycoproteins to the 32 kDa peptide. (C) Binding of purified ^{125}I-mZP2 (lane 5) and purified ^{125}I-mZP3 (lane 6) to the 32 kDa peptide. (D) Immunological identification of the 32 kDa peptide as expressed residues 38-288 of proacrosin. Lane 7, immune serum. Lane 8, pre-immune serum. (E) Autoradiograph of purified ^{125}I-mZP glycoproteins separated by SDS-PAGE. Lane 9, unfractionated ^{125}I-mZP glycoproteins. Lane 10, purified ^{125}I-mZP2 equivalent to 120 kDa. Lane 11, purified ^{125}I-mZP3 equivalent to 83 kDa. (F) Relative binding of purified ^{125}I-mZP2 and ^{125}I-mZP3 to expressed proacrosin 32 kDa peptide. All experiments were repeated at least 3 times and results shown are representative of the data as a whole. See text for details.

suramin analogue NF062 but only weakly, or not at all, by 1 mg/ml dextran, chondroitin sulphates A and B, glucose-6-sulphate, suramin analogue NF036, galactan, soybean trypsin inhibitor (SBTI) or pAB (Fig. 4A,B). The absence of any inhibitory activity with SBTI or pAB confirms that ^{125}I-mZP2 binding does not involve the active site of the acrosin peptide. Instead, binding is dependent on the presence of a polysulphated structure, and in particular, the stereochemistry of the projecting sulphate groups, since not all sulphated polymers are inhibitory. Thus, chondroitin sulphates A, B and C are relatively poor competitors despite having a charge density similar to fucoidan and dextran sulphate (De Angelis and Glabe, 1987). We presume that the density of sulphate groups on the latter two compounds is such that some are always in a favourable orientation.

The strong competitive activity of suramin is worthy of further comment as it has a well-defined chemical structure (Fig. 5). Suramin is a symmetrical molecule with sulphonated naphthalene rings at its extremities that are responsible for its ability to bind to, and cross-link, proteins (Middaugh et al., 1992). The requirement for molecular symmetry is shown by analogue NF036 (Fig. 5), which is truncated at a central aromatic ring and has very poor competitive activity. Conversely, analogue NF062, despite having only one sulphonate group on each of the terminal naphthalene rings, has the same core structure as suramin, similar dimensions and is almost as effective as the parent compound.

The interaction between mZP2 and expressed proacrosin peptide, therefore, is similar to, but slightly different from, that reported in the pig (Jones, 1991; Urch and Patel, 1991). In the pig system galactan was a potent inhibitor of ZP-proacrosin binding whereas NF062 was very weak, suggesting there are differences in the stereochemistry of the reactants that may be related to the species specificity of fertilization.

To investigate if the mechanism of binding of ^{125}I-ZP2 to native proacrosin was similar to that described above for expressed proacrosin peptide, permeabilized spermatozoa from acrosin (+/+) mice were preincubated in the presence of 1 mg/ml of dextran, dextran sulphate, fucoidan, suramin and SBTI for 30 minutes, washed in PBS/pAB and probed with ^{125}I-ZP2 for 1 hour. Bound label was detected by



B

COMPETITOR (1mg/ml)	% BINDING
none	100.0
suramin	6.3±1.2
dextran	93.0±4.0
dextran SO_4 (500K)	5.7±2.1
SBTI	77.0±14.0
fucoidan	4.7±2.5
chondroitin SO_4 A	106.1±2.9
chondroitin SO_4 B	93.0±14.0
chondroitin SO_4C	84.0±6.9
glucose-6-SO_4	107.3±10.8
analogue NF036	96.6±8.9
analogue NF062	15.3±5.5
pAB	101.0±23.4
galactan	112.5±11.5

Fig. 4. Effects of sulphated versus non-sulphated competitors on binding of ^{125}I-mZP2 to expressed proacrosin peptide. (A) Typical autoradiograph of a western blot containing the expressed 32 kDa peptide probed with ^{125}I-mZP2 in the presence of a variety of competitors (all at 1 mg/ml). Suramin, dextran sulphate and fucoidan are all effective competitors whereas dextran and soybean trypson inhibitor (SBTI) are ineffective. (B) Relative efficacy of different competitors in preventing binding of ^{125}I-mZP2 to the 32 kDa peptide. Binding is expressed as a percentage relative to controls containing no competitor (taken as 100%). Results are means ± s.e.m. of 3-4 separate experiments.

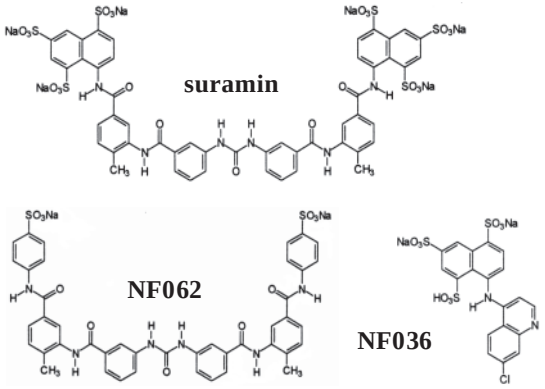


Fig. 5. Chemical structure of suramin and two of it analogues, NF062 and NF036. Note that suramin is a chemically symmetrical molecule containing three sulphonate groups on each of the terminal naphthalene rings. NF062 on the other hand carries only one terminal sulphonate group whilst NF036 is a truncated asymmetric molecule; see Jentsch et al. (Jentsch et al., 1987).

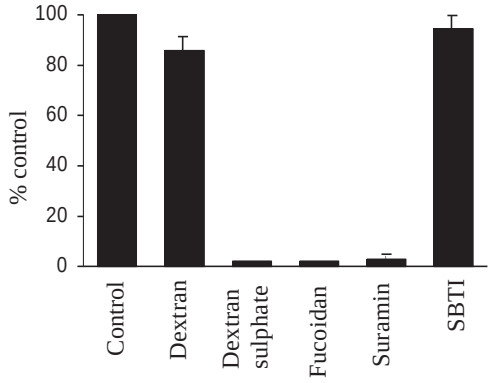


Fig. 6. Effects of sulphated and non-sulphated competitors on binding of ^{125}I-mZP2 to native mouse proacrosin in sperm heads as detected by autoradiography. Binding is expressed relative to controls containing no competitor (taken as 100%). Competitors were tested at 1 mg/ml.

autoradiography. Grain counts (Fig. 6) showed that dextran sulphate, fucoidan and suramin all inhibited ^{125}I-ZP2 binding by >95% whereas dextran and SBTI were ineffective competitors. This is consistent with the data in Fig. 4 and suggests that the expressed peptide and native proacrosin in sperm bind ^{125}I-ZP2 by similar mechanisms.

Efficacy of sulphated and non-sulphated compounds in displacing bound spermatozoa from the ZP

If proacrosin/acrosin behaves as a secondary ligand molecule at fertilization to interact with mZP2 and bind acrosome-reacted sperm to the zona surface, we would predict that those sulphated compounds that are potent competitors in blotting assays will also be effective at the cellular level. We hypothesise that if acrosome-intact sperm undergo primary binding to the ZP and then acrosome-react in the presence of an effective sulphated competitor, they should detach because the competitor has a higher affinity for proacrosin/acrosin than does mZP2. Low-affinity competitors should have little or no effect. To test this prediction, ovulated eggs free of cumulus cells were 'pulsed' with capacitated mouse sperm for 5 minutes to allow 10-30 sperm to attach to the zona surface, and then 'chased' into fresh medium ± competitors. The number of sperm remaining bound to the zona was counted over a 35 minute period. In the presence of 1 mg/ml fucoidan, dextran sulphate 500K, suramin or analogue NF062, >75% of sperm detached from the zona surface within the first 10 minutes and by 35 minutes the eggs were virtually devoid of sperm (Fig. 7A,B). Once again, suramin (at 710 μM) was the most potent competitor tested and showed a dose-response relationship down to 7.1 μM, at which level it became ineffective (results not shown). Compounds that were weak or non-competitive in the blotting assays (dextran 500K, glucose-6-sulphate, NF036, dextran sulphate 5K, galactan) did not displace sperm from the zona surface and retained numbers of sperm similar to controls (Fig. 7C). Between these extremes were chondroitin sulphates A, B and C that were partially effective and caused a reduction in bound sperm of between 40% and 55% (see Fig. 7C).

Suramin is known to inhibit the activity of some serine proteases including acrosin (Jones et al., 1996; Cadene et al., 1997) and there are long established reports that SBTI and pAB block fertilization in mice (Saling, 1981). To investigate whether protease activity is required for secondary binding, pulse-chase experiments were performed in the presence of SBTI (1 and 5 mg/ml), pAB (1 mg/ml) and bovine pancreatic trypsin inhibitor (BPTI) (1 mg/ml). Neither BPTI (results not shown) nor pAB (Fig. 7C) were able to displace attached sperm from the zona surface. SBTI had no effect at 1 mg/ml but at 5 mg/ml there was a reduction of approx. 50% (Fig. 7C), which is in agreement with observations by Bleil et al. (Bleil et al., 1988). The specificity of SBTI at this high concentration, however, is questionable. Thus, the ability of suramin to displace sperm from the zona surface seems unrelated to its anti-protease activity, which is consistent with the absence of such activity in other effective competitors such as dextran sulphate (500K) or fucoidan.

Two other possibilities may account for the displacement of sperm from the ZP surface by suramin, fucoidan, dextran sulphate and analogue NF062. First, these competitors may have induced premature acrosome reactions on attached sperm or adversely affected their motility. Attempts to determine the acrosomal status of the sperm displaced from the ZP have been unsuccessful because of the technical difficulties in recovering quantitatively the small numbers of sperm (10-20/egg) released into the medium. However, none of the compounds at the concentrations used in Fig. 7 induced acrosome reactions on capacitated sperm in vitro or affected their motility (results not shown). Second, the effective competitors may have induced exocytosis of cortical granules in the egg that subsequently caused detachment of bound sperm. To test this possibility, ZP-intact eggs were incubated in M199-M±1 mg/ml suramin or fucoidan or dextran sulphate or NF062 for 15 minutes at 37°C and the presence of cortical granule exudates on the surface of the oolema detected by staining with FITC-conjugated *Limulus polyphenus* lectin as described by Lee et al. (Lee et al., 1988). Control eggs were parthenogenetically activated with 7% ethanol. It was found that under the conditions used cortical granule release was not induced by any of the aforementioned competitors (results not shown). We have to conclude, therefore, that the most likely explanation for the results shown

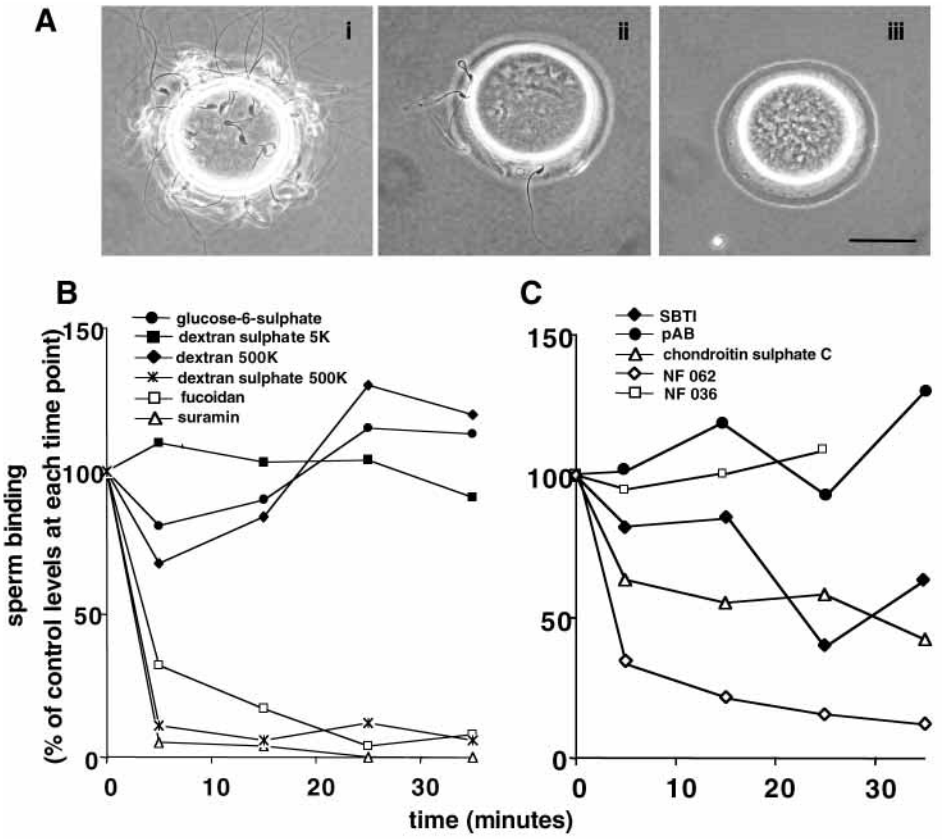


Fig. 7. Displacement of bound sperm from the ZP in the presence of suphated and non-sulphated competitors ('pulse-chase' experiments).
(A) Representative micrographs of (+/+) sperm bound to the ZP in (i) control eggs, (ii) eggs after 15 minutes incubation with 1 mg/ml fucoidan, (iii) eggs after 15 minutes incubation with 1 mg/ml suramin. Scale bar, 50 μm.
(B,C) Representative results showing the mean number of sperm bound to the ZP during the 35 minutes chase period in the presence of various competitors as indicated, expressed as a percentage of parallel controls at each time point. Zero minutes is taken as 100%. All compounds were tested at 1 mg/ml and experiments repeated at least 3 times.

in Fig. 7 is that the effective competitors displaced sperm from the ZP surface because of their ability to interfere with secondary binding mediated by exposed proacrosin/acrosin. It will be important in future work to determine the acrosomal status of sperm immediately before and after they bind to the ZP, the rate and proportion of sperm that subsequently undergo the acrosome reaction and the condition of the acrosome on sperm induced to detach by suramin etc. These are long-standing and contentious problems in the field of mouse fertilization but are now possible to study with specific markers for intra-acrosomal antigens, such as cyritestin and sp56, and specific inhibitors of the acrosome reaction, e.g. 3-quinuclidinyl benzilate.

DISCUSSION

The original observations by Bleil and Wassarman (Bleil and Wassarman, 1980) that purified mZP2 does not inhibit the binding of acrosome-intact sperm to mouse eggs in vitro, coupled with the direct demonstration that it only binds to acrosome-reacted mouse sperm (Bleil and Wassarman, 1986), strongly implies that mZP2 carries the secondary receptor for sperm. By inference, therefore, the complementary binding molecule on sperm must be present within the acrosomal matrix and/or bound to the inner acrosomal membrane. The present results, based on genetic, biochemical and functional assays, provides the most direct evidence to date that proacrosin/acrosin is a major mZP2-binding molecule.

It is known in several species that under controlled conditions in vitro acrosome-reacted sperm can bind to the zona surface and fertilize the egg (Yanagimachi, 1981). In the guinea pig in particular, it is predominantly acrosome-reacted sperm that bind to the ZP, a process that can be inhibited by sulphated fucoidan (Huang et al., 1981; Huang and Yanagimachi, 1984). Rabbit sperm recovered from the perivitilline space of fertilized eggs (and hence acrosome-reacted) also retain their capacity to bind to fresh zona-intact eggs (Kusan et al., 1984) and contain detectable amounts of acrosin on their inner acrosomal membrane (Valdivia et al., 1994). In the presence of a monoclonal antibody to homologous acrosin, hamster sperm were observed in vitro to attach to the ZP and then detach leaving 'tracks' over the surface, suggesting that binding was not sufficiently tenacious to retain the sperm head in one place (De Ioannes et al., 1990). These observations are consistent with an early view (Yanagimachi, 1981) that a 'sticky acrosomal matrix exposed by membrane vesiculation may bind the acrosome-reacting sperm to the zona as does bindin of sea urchin spermatozoa'. Sperm proacrosin/acrosin is noteworthy for its 'stickiness' during purification and has been shown to bind hydrophobically to cell membranes and liposomes (Parrish et al., 1978). Thus, it has the potential to function as a 'bridging' molecule between the inner acrosomal membrane and the zona matrix. Interestingly, the mechanism of mZP2-proacrosin binding is identical to that described for sea urchin bindin-vitelline envelope interactions, i.e. it is a strong ionic interaction between polysulphate groups on the egg receptor and basic residues on the sperm protein (De Angelis and Glabe, 1987). This appears to be a case of parallel evolution as there is no sequence similarity between acrosin and bindin. Like heparin-antithrombin III binding, specificity is determined

largely by the stereochemistry of the reacting groups (Peterson et al., 1987). Any 'leakiness' in the system is overcome by the simple expedient of having the proacrosin or bindin contained within the acrosomal vesicle and only released at their site of action. Both mZP2 and mZP3 contain sulphated oligosaccharides (Shimizu et al., 1983; Noguchi and Nakano, 1993; Takasaki et al., 1999), yet mZP3 does not bind as effectively as mZP2, emphasising once again the importance of the 3-dimensional alignment of sulphate groups relative to the docking site(s) on proacrosin. In view of the competitive effects of suramin on ^{125}I-mZP2-proacrosin binding, it may be speculated that a sulphated oligosaccharide structure is present on mZP2 that is stereochemically similar to suramin.

It is not possible at present to calculate the stoichiometry of binding between mZP2 and proacrosin in our assays. The content of proacrosin in a mouse sperm head is not known, although it has been calculated to be <tenfold that of ram, bull or boar sperm (Brown, 1983). Additionally, the finding that bacterial expressed proacrosin peptide has to be reduced with β-mercaptoethanol to obtain optimal binding of ^{125}I-mZP2 suggests incorrect folding of the protein. Since the proportion of expressed peptide molecules that undergo renaturation after blotting is also not known, the stoichiometry of binding cannot be determined.

Further support for the concept of stereospecificity comes from a recent crystallographic analysis of boar β-acrosin (Tranter et al., 2000), which has revealed that two clusters of basic residues (His 47, Arg50, Arg51 and Arg250, Lys252, Arg253), shown previously by site-directed mutagenesis to be involved in binding ZPs (Jansen et al., 1998), are located on the surface of the protein to either side of the active site. Binding of suramin and sulphated polymers to these clusters is compatible with their ability to inhibit enzyme activity by forming a 'bridge' across the active site. Although suramin binds to a wide range of proteins with varying affinities, it can discriminate between closely related proteins, suggesting that 'docking' sites are very precise. Within the family of serine proteases, for example, suramin inhibits the proteolytic activity of elastase strongly, trypsin very weakly and chymotrypsin not at all (Cadene et al., 1997). For elastase, the stoichiometry of binding is 4:1 with the suramin molecules forming bridges between specific clusters of basic residues exposed on the surface of the protein (Mely et al., 1997). We hypothesise that a similar situation applies to suramin-proacrosin interactions and that suramin is able to displace bound sperm from the zona surface because it has a higher affinity for proacrosin/acrosin than mZP2.

The question of whether or not proacrosin activates to β-acrosin immediately following the acrosome reaction on the surface of the ZP remains unresolved. The optimal pH for autoactivation in vitro is 8.2, a level of alkalinity unlikely to be found in vivo on the surface of the ZP. In our experience, when cumulus egg masses are placed in M199 medium containing Phenol Red indicator, there is a transient change in colour from red to yellow, suggesting a pH <7.0 within the cumulus mass (R. Jones, unpublished observations). In addition, proacrosin is normally complexed within the acrosome to inhibitors and binding proteins that, in theory, would retard autoactivation. Against this are observations that liposomes and ZP glycoproteins potentiate conversion of proacrosin to acrosin by a process of contact activation followed immediately by inhibition of enzyme activity (Parrish et al., 1978; Topfer-Peterson and Cechova, 1990; Lo Leggio et al., 1994). From the standpoint of ZP-binding, however, proacrosin and β-acrosin are equally effective (Jansen et al., 1995; Moreno and Barros, 2000), which is not entirely unexpected as they both contain the ZP-binding peptide represented by residues 38-288. It also implies that the small changes in tertiary structure during autoactivation, as revealed by the greater exposition of β-sheets and lower thermal stability observed by Fourier Transform Infrared spectroscopy (Fini et al., 1996), do not seriously impair the orientation of the polysulphate binding sites. Thus, in vivo, both proacrosin and acrosin could function as mZP2 binding proteins.

Although it has been shown that proacrosin (–/–) mice are fertile following natural mating, their sperm show delayed fertilization in vitro (Baba et al., 1994; Adham et al., 1997). More significantly, in an i.v.f. assay with equal numbers of (–/–) and (+/+) sperm present in the medium, all embryos derived from fertilized eggs are (+/+) (Adham et al., 1997). Thus, there is strong selective pressure in favour of sperm that contain proacrosin/acrosin as its presence increases their competitiveness to fertilize. This is consistent with its long evolutionary conservation (acrosin arose about 1 billion years ago as a gene duplication event from bacterial trypsin) (Klemm et al., 1991) and its strict tissue and organelle specificity. Our results do not exclude other roles for sperm proacrosin/acrosin as there is evidence from in vitro studies that it may be involved in the rate of dispersal of acrosomal contents following the acrosome reaction (Yamagata et al., 1998a). Other serine proteases are present in the mouse acrosome and are quantitatively more abundant than proacrosin (Yamagata et al., 1998b), emphasising once again the selectivity of the ^{125}I-mZP2 and [^{3}H]suramin probes in detecting a difference between acrosin (+/+) and (–/–) sperm. These proteases may have supporting roles during fertilization, thereby helping to explain why proacrosin (–/–) sperm are less competitive than (+/+) sperm in zona binding but are not completely incapable of doing so.

In conclusion, the data support the hypothesis that during fertilization in the mouse proacrosin/acrosin is the principal secondary binding protein on sperm for mZP2. The relatively weak affinity between mZP2 and proacrosin/acrosin would be sufficiently tenacious to retain acrosome-reacted sperm on the zona surface yet still allow penetration to be initiated. Strong competitors such as suramin disrupt proacrosin-mZP2 binding and cause detachment of sperm. Safe mimetics of suramin have obvious potential as non-steroidal antifertility agents for animal and human use if released in the vicinity of the egg, e.g. from long-acting IUDs.

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