Zygote **7** (May), pp 105–111. © 1999 Cambridge University Press Printed in the United Kingdom

Functional interactions between sulphated polysaccharides and proacrosin: implications in sperm binding and digestion of zona pellucida

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Date submitted: 29.6.98. Date accepted: 10.9.98

Summary

Acrosin is a serine protease located within mammalian acrosome as inactive proacrosin. Sulphated polymers bind to proacrosin and acrosin, to a domain different from the active site. Upon binding, these polymers induce proacrosin activation and some of them, such as fucoidan, inhibit sperm binding to the zona pellucida. In this work we have studied the interaction of solubilised zona pellucida glycoproteins (ZPGs), heparin and ARIS (Acrosome Reaction Inducing Substance of Starfish) with boar and human acrosin. We have found that ARIS, solubilised ZPGs and fucoidan, but not heparin, inhibit the binding of the monoclonal antibody against human acrosin C5F10 to boar or human proacrosin. These results suggest that fucoidan, solubilised ZPGs and ARIS bind to a related domain on the proacrosin surface. Moreover, ARIS was able to induce human proacrosin activation. On the other hand, neither ARIS nor heparin from porcine intestinal mucosa or bovine lung induced hamster sperm acrosome reaction or sperm motility. Recent data showed that acrosin is involved in dispersal of the acrosomal matrix after acrosome reaction. Thus, the control of the ZPG glycan chains over proacrosin activation may regulate both sperm penetration rate and limited proteolysis of zona pellucida proteins.

Keywords: Acrosin, ARIS, Heparin, Secondary binding, Zona pellucida

Introduction

Proacrosin is the zymogen form of the serine protease acrosin (EC 3.4.21.10) that is found within the acrosome of mammalian spermatozoa (see review by Barros *et al.*, 1996). It has been purified and cloned from several species including human, cattle, rabbit, boar and rat (Baba *et al.*, 1989*b*; Keime *et al.*, 1990; Klemm *et al.*, 1991; Richardson & O'Rand, 1996). Upon the acrosome reaction or *in vitro* at pH 8.0, proacrosin is activated and after autocatalytic cleavage at amino acid 23, a two-chain active acrosin molecule is formed (α-acrosin) with a light and a heavy chain. Further processing at the C-terminus produces several different molecular weight forms (Baba *et al.*, 1989*a*,*b*). In several species, proacrosin and acrosin have been shown to bind to the glycan chains of homologous solubilised zona pellucida glycoproteins (ZPGs; K_d=10^{-8} M) (Jones, 1991; Urch & Patel, 1991; Williams & Jones, 1990,1993; Hardy & Garbers, 1994). However, this binding is not restricted by species specificity (Williams & Jones, 1990, 1993; Hardy *et al.*, 1994). Moreover, proacrosin/acrosin also binds to other polysulphated chains such as fucoidan, heparin and dextran sulphate, but not to short length or desulphated chains (Jones, 1991; Urch & Patel, 1991).

It has been shown that chondroitin sulphates A, B and C, as well as fucoidan, solubilised ZPGs and heparin, potentiate the conversion of proacrosin to either α- or β-acrosin, but subsequently inhibit its amidase activity (Parrish *et al.*, 1980; Töpfer-Petersen & Cechova, 1990; Eberspaecher *et al.*, 1991; Lo Leggio *et al.*, 1994). Thus, these results suggest that stimulation of proacrosin conversion into acrosin by polysulphates is a general phenomenon that is not species- or organ-specific and may function during the *in vivo* conversion

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https://doi.org/10.1017/S0967199499000453 Published online by Cambridge University Press

of proacrosin into acrosin. In fact, glycosaminoglycans isolated from tubal fluid or follicular fluid induce in vitro human proacrosin activation (Wincek *et al.*, 1979; Drahorad *et al.*, 1988, 1991).

The polysulphated binding domain (PSBD) of the proacrosin/acrosin system is still not well determined. This domain, however, is different from the active site, it is located on the enzyme surface, and is rich in basic residues. Site-directed mutagenesis in rabbit proacrosin has located this domain within a restricted loop containing residues 50 and 51 (Ricardson & O'Rand, 1996). On the other hand, in boar, it seems that it is more broadly distributed (Jansen *et al.*, 1995). We have shown that the monoclonal antibody (Mab) C5F10, against human acrosin, binds to the PSBD (Moreno *et al.*, 1998). This Mab is able to inhibit the activation of proacrosin induced by fucoidan and the binding of human sperm to homologous zona pellucida. Thus, the PSBD has a key role in proacrosin activation and sperm penetration.

In spite of the fact that fucoidan and heparin do not participate during *in vivo* gamete interaction, they have been the most used polysulphated polymer models to study proacrosin–zona pellucida interactions. Therefore, we wanted to study the interaction of the PSBD with sulphated polysaccharides of known function during fertilisation. We chose two naturally occurring sulphated saccharides: solubilised ZPGs and ARIS (Acrosome Reaction Inducing Substance of Starfish). ARIS is a saccharide polymer consisting of pentasaccharide repeating units containing fucose-sulphated groups (Koyota *et al.*, 1997). ARIS alone induces the acrosome reaction, but only at high calcium levels or in high pH seawater (for review see Hoshi *et al.*, 1988).

Since ARIS shares structural similarities with mammalian ZPGs glycan chains, we decided to study whether pig solubilised ZPG and ARIS may bind to the PSBD and/or activate proacrosin.

Materials and methods

Reagents

Unless otherwise stated, all the reagents were purchased from Sigma (St Louis, MO) or Merck (Darmstadt, Germany). Heparin from bovine lung was purchased from Calbiochem (La Jolla, CA).

Supply of spermatozoa

Human semen samples were obtained from an infertility clinic following the ethical guidelines of the Pontifical Catholic University of Chile. Boar semen samples were obtained from a local farm. Hamster spermatozoa were obtained from the caudae epididymides of adult animals and incubated in TALP-10K medium (Yanagimachi, 1982; Barros *et al.*, 1984) supplemented with 10 mg/ml bovine serum albumin (BSA). To achieve capacitation, spermatozoa were incubated in drops of 100 µl under mineral oil for 3 h at a concentration of 1.5×10^6/ml in a 5% CO_2 atmosphere.

Extraction to obtain human and boar proacrosin

Human proacrosin, used as an acid sperm extract, was obtained according to the method described by Leyton *et al.* (1986). Briefly, semen samples were diluted 10-fold in 150 mM NaCl and incubated for 1 h at 37 °C. The sample was centrifuged at 200 *g* in a clinical centrifuge at room temperature. The pellet was resuspended in 0.1% Triton X-100 and centrifuged again. Acrosin was obtained from this pellet in acid solution containing 1 mM HCl, 10% glycerol, 0.02% sodium azide and 50 mM benzamidine at pH 3.0 and left at 4 °C overnight. This suspension was centrifuged using a Sorvall model RC2-B centrifuge at 27000 *g* for 30 min and the supernatant stored at –20 °C. Immediately prior to the experiment, a 200 µl aliquot of frozen human acid extract was dialysed overnight against 2 l of 1 mM HCl at pH 3.0.

Boar proacrosin was used as an acid extract and obtained as described previously (Parrish & Polakoski, 1977). Boar semen ejaculate was adjusted with 1 M benzamidine to a final concentration of 50 mM and filtered through Miracloth. Twenty millilitre aliquots were carefully placed in 25 ml of 1 M sucrose, 50 mM benzamidine, 0.02% sodium azide, and were centrifuged for 30 min at 5000 rpm in a model HNS clinical IEC centrifuge at room temperature. Boar acrosin was then extracted and stored under the same conditions described for human acrosin.

Acrosin activity

Acrosin activity was measured spectrophotometrically at 25 °C by following the hydrolysis of *N*-benzoyl-L-arginine-ethyl-ester (BAEE) and by the addition of 50 or 100 µl of the enzyme solution, at 253 nm. The assays were performed in 3 ml volumes, utilising a substrate mixture of 50 mM Tris, 50 mM $CaCl_2$ and 500 µM BAEE at pH 8.0. A molar absorption difference of 1150 $M^{-1}cm^{-1}$ was used to convert changes in optical density to micromoles of BAEE hydrolysed (Whitaker & Bender, 1965). One international unit of activity was defined as that amount of acrosin hydrolysing 1 (mol BAEE/min at 25 °C.

Enzyme-linked immunoassay (ELISA)

Nunc-inmuno plates (Nunc) of 96 wells were incubated overnight at 4 °C with 50 µl of PBS, pH 7.0, containing 40 µg/ml of boar acid extract. The free reactive sites

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were blocked by incubating with PBS–BSA for 3 h at room temperature. Fifty microlitres of Mab solution (C5F10 or E5E10) in PBS–BSA were added to the well and incubated for 2 h. The wells were then washed with PBS-Tween and incubated with an anti-mouse IgG conjugated to alkaline phosphatase in PBS–BSA, which was added to the wells and incubated for 1 h at room temperature. The immune complex was developed with the alkaline phosphatase reaction prepared with 1 mg/ml of *p*-nitrophenyl phosphate (PNPP) in 50 mM $NaHCO_3$, 50 mM Na_2CO_3, 1 mM $MgCl_2$. The reaction was stopped with 3 M NaOH and the absorbance was measured at 405 nm (Harlow & Lane, 1988).

Interaction of polysulphate polymers with boar proacrosin

Boar acid sperm extract in ELISA plates (2 µg per well) was pre-incubated with different concentrations of fucoidan (0–50 mg/ml), heparin (0–50 mg/ml), soybean trypsin inhibitor (SBTI, 0–50 µg/ml) or heat-denatured solubilised ZPGs (0.005, 0.05, 0.5 and 5 µg per well). Extracts were then incubated with Mab C5F10 and the binding to boar proacrosin was detected as described previously. The Mab binding in each case was evaluated as a percentage of control (0), which was considered as 100%.

Human acid sperm extract in ELISA plates (2 µg per well) was pre-incubated with different concentrations of ARIS (0.1, 0.2, 0.5 mg/ml). Extracts were then incubated with Mab C5F10 and the binding to human proacrosin was detected as described previously. The Mab binding in each case was evaluated as a percentage of control (0), which was considered as 100%.

Effect of ARIS on human proacrosin activation

Human proacrosin (30 µg) was added to a solution of 1 M Tris-HCl at pH 8.0 (100 (l) containing 0, 10 or 100 µg/ml of ARIS. The samples were incubated for different times (5, 10, 20 min) at room temperature. They were then adjusted to pH 2.8 with 1 M HCl and an aliquot was assayed at pH 8.0 with BAEE as described previously.

Effect of ARIS on hamster sperm acrosome reaction

ARIS, heparin from porcine intestinal mucosa or heparin from bovine lung (100 µg/ml) was added at the start of sperm capacitation. At the indicated times (0, 3, 6, 8 h) one aliquot of 10 µl was taken for acrosome reaction evaluation by phase contrast microscopy (Barros *et al.*, 1992). At each time, 100 or more living spermatozoa were counted.

Results

Interaction of polysulphate polymers with boar proacrosin

In ELISA plates pre-incubated with fucoidan, the binding of Mab C5F10 to boar proacrosin/acrosin was inhibited (Fig. 1*A*). When boar proacrosin/acrosin was pre-incubated with heparin or with SBTI the binding of the antibody was not affected (Fig. 1*A*, *B*).

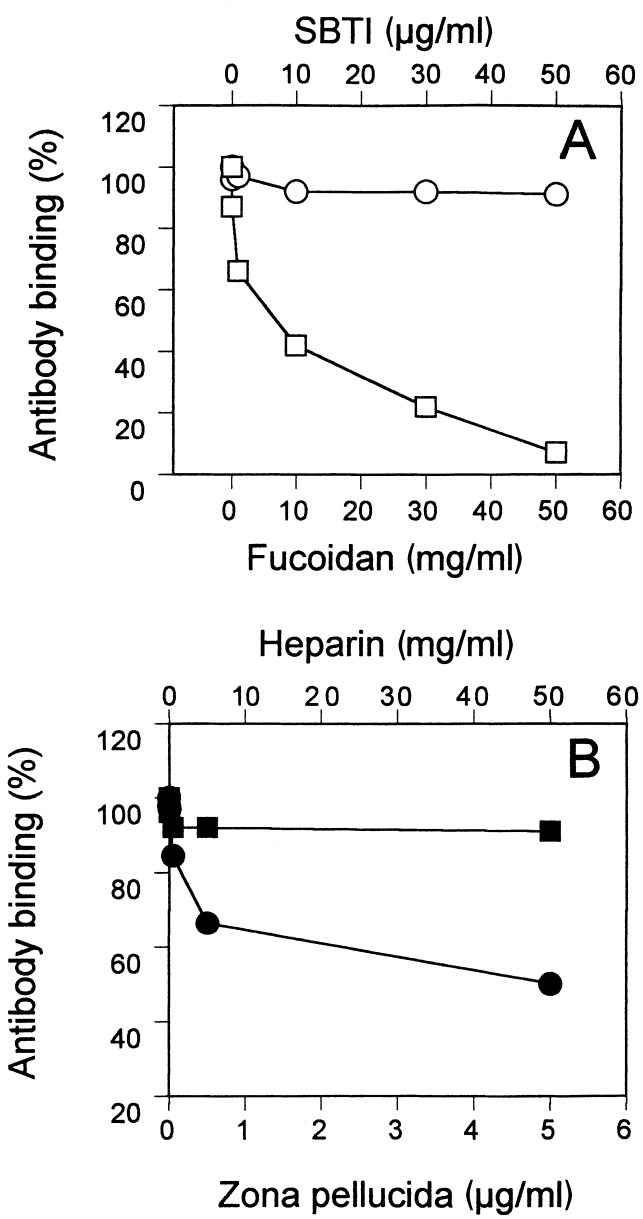


Figure 1 Interaction of polysulphated polymers with boar proacrosin/acrosin. The binding of the monoclonal antibody C5F10 was inhibited in the presence of fucoidan (*A*, open squares) or solubilised zona pellucida glycoproteins (*B*, filled circles). Neither SBTI (*A*, open circles) nor heparin (*B*, filled squares) inhibited the binding of Mab C5F10 to boar proacrosin/acrosin.

https://doi.org/10.1017/S0967199499000453 Published online by Cambridge University Press

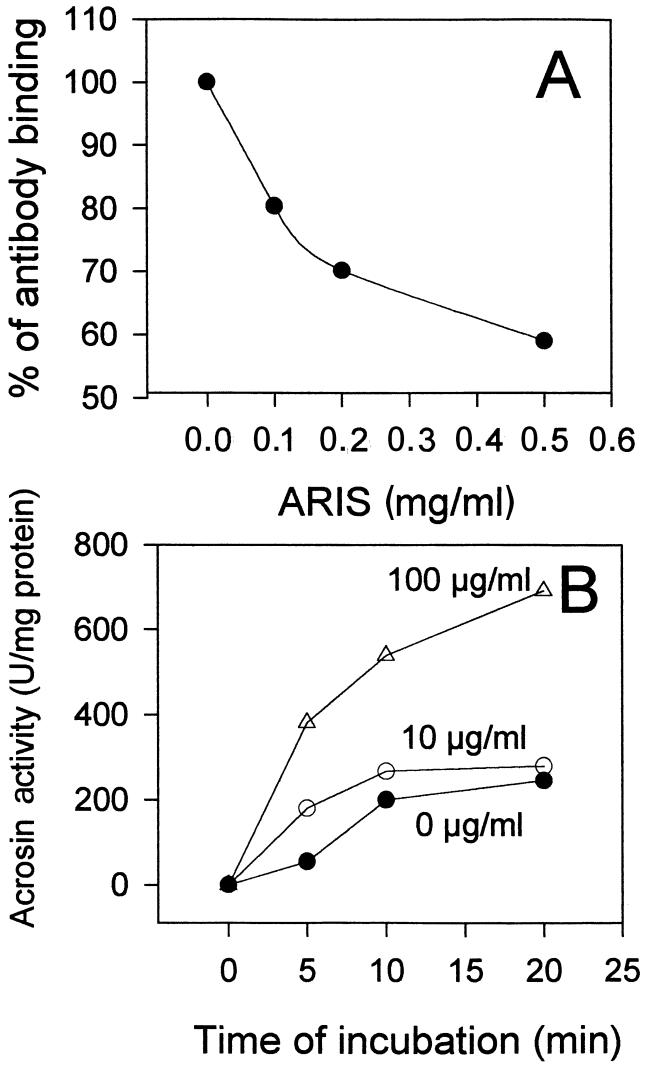


Figure 2 Interaction of ARIS with the human proacrosin/acrosin system. The binding of the monoclonal antibody C5F10 to human permeabilised spermatozoa (*A*) was inhibited in the presence of ARIS. Human proacrosin can be activated *in vitro* (*B*, 0 µg/ml). In the presence of ARIS (10 or 100 µg/ml) there is a change in the kinetics of human proacrosin activation (*B*).

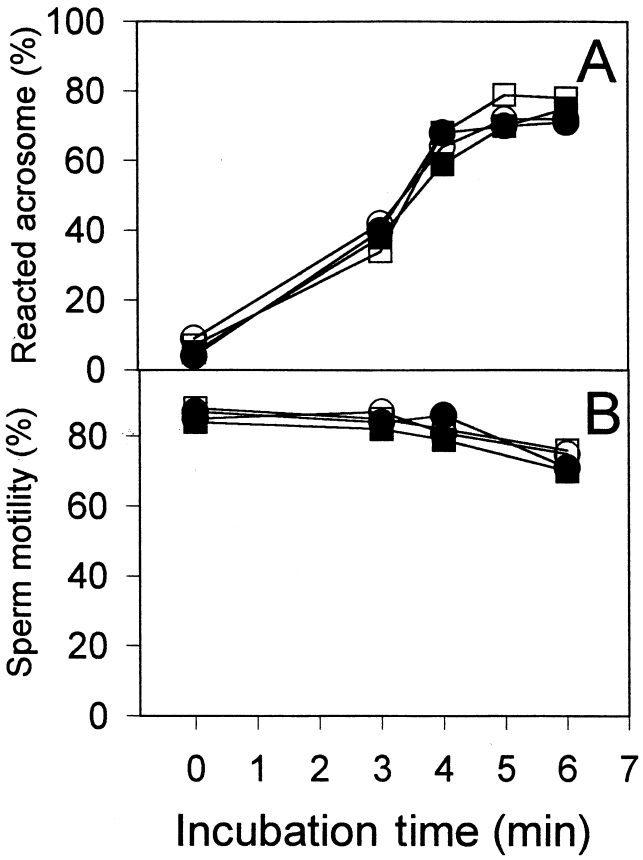


Figure 3 Effect of ARIS upon hamster sperm acrosome reactions. Hamster spermatozoa were incubated in TALP-10K medium, supplemented with BSA (10 mg/ml) at 37 °C. At this time we added 100 µg/ml of either ARIS (filled squares), heparin from porcine intestinal mucosa (open squares) or heparin from bovine lung (open circles). The other group was incubated without any polysulphate in the medium, as a control (filled circles). At the indicated times, aliquots of the incubation medium were removed and the percentage of living acrosome-reacted sperms (*A*) or motile spermatozoa (*B*) was recorded using a phase-contrast microscope ($n = 3$).

When boar proacrosin/acrosin was pre-incubated with heat-denatured solubilised ZPGs, the binding of Mab C5F10 was inhibited in a dose-dependent manner (Fig. 1*B*). The same happened when boar proacrosin/acrosin was pre-incubated with ARIS (Fig. 2*A*), attaining up to 40% inhibition at 0.5 mg/ml.

Effect of ARIS on human proacrosin activation

The time course reaction of human proacrosin acid extract in 1 M Tris-HCl at pH 8.0 and at room temperature was not linear with time, the appearance of acrosin activity following a sigmoidal curve (Fig. 2*B*). In the presence of 10 µg/ml of ARIS the activation curve changed to hyperbolic. In the presence of 100 µg/ml of ARIS the activation of human proacrosin was completely hyperbolic. Moreover, it appears that ARIS induces proacrosin activation and stimulates enzyme activity (Fig. 2*B*).

Effect of polysulphates on the acrosome reaction

The time course of hamster acrosome kinetic reactions (Fig. 3*A*) follows a sigmoidal curve, as has been shown previously (Barros *et al.*, 1984). The presence of 100 µg/ml of ARIS, or heparin from either porcine intestinal mucosa, or bovine lung, had no effect on the hamster sperm acrosome reaction. We also tested whether these polysulphates have any effect on sperm motility. Neither ARIS nor heparin had any effect on sperm motility and viability (Fig. 3*B*).

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Discussion

In a previous report (Moreno *et al.*, 1998) we showed that Mab C5F10 is directed against the PSBD of human proacrosin. In this report we have extended our studies to show that it is also directed to the PSBD on boar proacrosin. Moreover, we have shown that solubilised ZPGs and ARIS inhibit the binding of this Mab to proacrosin.

In starfish, three components of the egg jelly, namely a highly sulphated glycoprotein of an extremely large molecular size (ARIS), a steroid saponin named Co-ARIS and a sperm-activating peptide (asterosap), are responsible for triggering the acrosome reaction (Amano *et al.*, 1992; Nishigaki *et al.*, 1996). The structure of the main saccharide of ARIS is composed of a 10–11 repeating unit composed of Xyl1→3Gal1→(SO_3^-)3,4Fuc1→(SO_3^-)3,4Fuc1→4Fuc (Koyota *et al.*, 1997). Removal of one sulphate from the first fucose destroys the biological activity of this fragment (Koyota *et al.*, 1997). Heparin and other glycosaminoglycans are able to induce the acrosome reaction in bovine and rabbit sperm (Lenz *et al.*, 1982, 1983; Lee *et al.*, 1986). This biological activity of heparin and glycosaminoglycans, like that occurring with ARIS, is also related to the degree of sulphation of the polymer (Lenz *et al.*, 1983; Lee *et al.*, 1986). In this work we have shown that neither ARIS nor heparin from porcine intestinal mucosa or bovine lung induces acrosome reaction or affects sperm motility in the hamster. In our system, *in vitro* hamster acrosome reaction rates were very high (almost 80% at 4 h of incubation), and probably in that condition heparin or ARIS does not show any effect. On the other hand, we believe that acrosome reaction and fertilisation rates induced by heparin, as it does in cattle and rabbit, are not a widely distributed effect on mammalian sperm. In fact, human spermatozoa have heparin binding sites on head and tail (Delgado *et al.*, 1982; Lasalle & Testart, 1992). Although there is good correlation between heparin binding sites and zona-free hamster egg penetration, heparin treatment does not induce acrosome reaction (Lalich *et al.*, 1989; Lasalle & Testart, 1992).

The role of the PSBD on proacrosin/acrosin in mammalian gamete interaction has been inferred from the inhibitory effect during fertilisation exerted by fucose-sulphate-containing polysaccharides such as fucoidan, solubilised homologous ZPGs or isolated glycan chains from zona pellucida glycoproteins (Huang & Yanagimachi, 1984; Berger *et al.*, 1989; Yurewicz *et al.*, 1991; Windt *et al.*, 1991). In addition, a Mab directed against the PSBD on human acrosin inhibited binding of human spermatozoa to homologous zona pellucida, suggesting a role for this domain during sperm penetration (Moreno *et al.*, 1998). Proacrosin can be activated by different negatively charged polymers such as fucoidan (Lo Leggio *et al.*, 1994), negatively charged liposomes (Straus & Polakoski, 1982), DNA (Eberspaecher *et al.*, 1991), heparin (Urch & Hedrick, 1988; Lo Leggio *et al.*, 1994; Töpfer-Petersen and Cechova, 1990), solubilised ZPGs (Töpfer-Petersen & Cechova, 1990) and ARIS (this report).

What is the biological meaning of this non-specific induced proacrosin activation by polysulphates? During sperm penetration, mammalian spermatozoa must digest both zona pellucida and acrosomal matrix in order to release the hydrolytic enzymes and cross the zona pellucida. Within mammalian acrosomes there are proteases other than acrosin (Schleuning *et al.*, 1976; Berruti, 1980; Kaufmann *et al.*, 1987; Akama *et al.*, 1994; Kohno *et al.*, 1998) and other structural proteins with zona pellucida or proacrosin binding activity (Mori *et al.*, 1993, 1995; Cheng *et al.*, 1994; Baba *et al.*, 1994*a*; Richardson *et al.*, 1994; Foster *et al.*, 1997). Thus, during sperm penetration, not only acrosin may be involved in secondary binding to the zona. Proacrosin/acrosin may be part of a multimeric complex involved in the interaction of acrosome-reacted mammalian spermatozoa with the glycan chains of zona pellucida glycoproteins. In this context, the PSBD may function as an allosteric regulator of proacrosin activation induced by ZPGs during *in vivo* penetration. Activated proacrosin α- or β-acrosin) may be involved in acrosomal matrix or zona pellucida digestion or both. In fact, trypsin inhibitors do not inhibit the acrosome reaction, but do affect acrosomal dispersion in guinea pig (Huang *et al.*, 1985). Moreover, a mouse lacking acrosin shows an altered rate of protein dispersal from the acrosome (Yamagata *et al.*, 1998). It is worth noting that it could be possible that more than one hydrolytic enzyme is involved in acrosomal matrix or zona pellucida digestion. Therefore, the non-specific interaction of the PSBD with polysulphates might allow different glycan chains of ZPGs to interact with proacrosin and regulate its activation. This role of acrosin during mammalian fertilisation could explain the fertilising capacity of mouse spermatozoa lacking a proacrosin/acrosin system (Baba *et al.*, 1994*b*; Adham *et al.*, 1997). In these spermatozoa the hydrolytic enzymes responsible for zona pellucida digestion are still active but either the multimeric complex involved in the secondary binding or the acrosomal matrix dispersion is not working properly.

Acknowledgements

This work was supported by a research grant from the Chilean Research Fund (FONDECYT) 1971234 to C.B. and TWAS 96-089 and PLACIRH PLI-291/97 grant to R.D.M. R.D.M. was a recipient of a post-doctoral fellowship from the Catholic University of Chile, grant

https://doi.org/10.1017/S0967199499000453 Published online by Cambridge University Press

RF 94025 #15. We thank Mr Pedro Cortés for the photographic work.

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