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The interaction of boar sperm proacrosin with its natural substrate, the zona pellucida, and with polysulfated polysaccharides

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Summary

Boar sperm acrosin is an acrosomal protease with trypsin-like specificity, and it functions in fertilization by assisting sperm passage through the zona pellucida by limited hydrolysis of this extracellular matrix. In addition to a proteolytic active site domain, acrosin binds the zona pellucida at a separate binding domain that is lost during proacrosin autolysis. In this study, we quantitate the binding of proacrosin to the physiological substrate for acrosin, the zona pellucida, and to a non-substrate, the polysulfated polysaccharide fucoidan. Binding was analogous to sea urchin sperm bindin that binds egg jelly fucan and the vitelline envelope of sea urchin eggs. Proacrosin was found to bind to fucoidan and to the zona pellucida with binding affinities similar to bindin interaction with egg jelly fucan. These interactions were competitively inhibited by similar relative molecular mass polysulfated polymers. Since bindin and proacrosin have distinctly different amino acid sequences, their interaction with acidic sulfate esters demonstrates an example of convergent evolution wherein different macromolecules localized in analogous sperm compartments have the same biological function. From cDNA sequence analysis of proacrosin, this binding may be mediated through a consensus sequence for binding sulfated glycoconjugates. Proacrosin binding to the zona pellucida may serve as both a recognition or primary sperm receptor, as well as maintaining the sperm on the zona pellucida once the acrosome reaction has occurred.

Key words: cell adhesion molecules, fertilization, acrosin, fucoidan.

Introduction

Sperm–egg interaction is a species-specific cell recognition and binding event and a necessary prerequisite for fertilization. This interaction involves protein–carbohydrate interactions and is inhibited by carbohydrates in a wide variety of species (Ahuji, 1982; Shalgi *et al.* 1986). The extracellular matrix of the mammalian egg, the zona pellucida (ZP), is composed of sulfated glycoproteins (Bliel and Wassarman, 1980; Shimizu *et al.* 1983; Hedrick and Wardrip, 1987), and it contains the ligands that interact with sperm receptors. In the mouse, an O-linked oligosaccharide from the unfertilized egg ZP3 component of the ZP interacts with mouse sperm (Florman and Wassarman, 1985), whereas both the 55 M_r glycoprotein families (designated α and β) and the 90 M_r glycoprotein family of porcine ZP contain ligands for porcine sperm (Berger *et al.* 1989). Several mammalian sperm receptors have been described with either a specific recognition function or a less specific or secondary binding function. Examples of these include macromolecules that bind protease inhibitors, sperm autoantigens with lectin-like activity, and galactosyl transferase (Poirier *et al.* 1986; Benau and Storey, 1987; O'Rand *et al.* 1988; Hathaway and Shur, 1988). In sea urchin sperm, an acrosomal protein called bindin binds specifically and tightly to the egg jelly fucan and to the vitelline envelope. Bindin was first characterized as a lectin, but it has recently been redescribed as a binder of polysulfated macromolecules (DeAngelis and Glabe, 1990).

The sperm enzyme acrosin assists sperm penetration of the ZP by specific limited hydrolysis of ZP glycoproteins (reviews: Urch, 1991; Hedrick *et al.* 1988). An additional function for acrosin in fertilization has been described as ZP binding, possibly at a domain other than the proteolytic active site (Brown and Jones, 1987). Support for this latter function comes from western blot analyses, where sperm extract macromolecules that bound ZP had similar mobilities to macromolecules with epitopes recognized by proacrosin antibodies (Urch *et al.* personal communication; Jones and Brown, 1987; Töpfer-Petersen and Henschen, 1987). This approach has recently implicated proacrosin/acrosin as part of the several ZP binding molecules in the guinea pig, where the acrosome-

reacted sperm makes initial contact with the ZP. This study characterized the interaction of those sperm extracts with fucoidan, through the use of polysulfated polysaccharide inhibition, as a sea urchin bindin-like phenomenon (Jones and Williams, 1990). In the pig, western blots of purified proacrosin bound ^{125}I-ZP with the same M_r as the fucose-binding protein of boar sperm (Jones, 1989), and the sperm receptor for ZP that bound HRP-fucose has the same amino acid N-terminal sequence as proacrosin (Töpfer-Petersen and Henschen, 1988). These observations taken together suggest that proacrosin is a primary recognition receptor for porcine ZP on the surface of boar sperm. This interpretation conflicts with the recent description of a boar sperm receptor with the same M_r as proacrosin, but which was antigenically dissimilar to it (Peterson and Hunt, 1989). However, placed on the sperm, the interaction of two acrosin domains with the ZP allows for an elegant mechanism of binding and hydrolysis that would assist sperm penetration of the ZP (Urch, 1991; Hedrick *et al.* 1988).

This novel binding function of a proteolytic proenzyme is thought to be modulated through a fucose-binding domain in proacrosin, distinct from the proteolytically active site, with this functional binding domain exposed on the sperm surface to interact with the ZP (Töpfer-Petersen and Henschen, 1988). There are other examples of enzymes binding non-substrates with high affinities, some of which also inhibit enzymatic activity. One of the most studied systems is the binding and inhibition of glutathione transferase by the non-substrate bile acids (Hayes and Mantle, 1986; Boyer and Vessey, 1987; Young and Briedis, 1990), although the physiological importance of this interaction has not yet been determined. In the case of proacrosin-binding polysulfated polysaccharides, these polymers inhibit sperm–ZP binding in the pig (Peterson *et al.* 1984), as well as inhibiting the amidase and protease activities of active boar acrosins (Urch and Hedrick, 1988). Therefore, proacrosin binding to, and modulation of acrosin activity by, the ZP would have significant physiological importance in fertilization.

In this investigation, we qualitatively and quantitatively describe the binding of purified proacrosin to polysulfated polysaccharides and to the ZP by using analogous binding experiments to those described for sea urchin bindin (DeAngelis and Glabe, 1987). This binding represents a novel mechanism for recognition between mammalian gamete molecules and for the utilization of a proteolytic enzyme for more than a single function. A similar investigation, with similar findings, has been reported in a study by Jones (1991).

Materials and methods

Materials

Fucoidan from *Fucus vesiculosus* (Sigma, St Louis, MO) was treated with pronase to remove contaminating protein in the following fashion: 50 mg of fucoidan was treated with 0.5 mg of pronase (Sigma) at 37°C for 24 h on a shaker, with a few drops of toluene added as a bacteriostatic agent. A second 0.5 mg pronase was then added for an additional 12 h. The solution was lyophilized and dissolved in water. *Xenopus laevis* jelly coat (the kind gift of Dr Jerry Hedrick, University of California, Davis) was also treated in a similar manner.

Fucoidan was partially acid hydrolyzed and desulfated after the methods of DeAngelis and Glabe (1987). Briefly, mild acid hydrolysis was performed in 0.1 M hydrochloric acid at 100°C for 2 min. After hydrolysis, the samples were plunged into a dry ice/ethanol bath and lyophilized prior to gel filtration on Sephadex G-100 in 1 mM 3-[*N*-morpholino] propanesulfonic acid (Mops), pH 8.0. For desulfation, samples were mixed, after Dowex 50 column equilibration in 0.1 M pyridine, with dimethyl sulfoxide/pyridine (50:1) and kept in an air-free environment for 9 h. The pyridine was removed with N_2 and then the samples were dialyzed extensively with water and lyophilized.

Dextran sulphate (M_rs 5000 and 500 000), mannose-6-phosphate, chondroitin sulphate A and C, poly(vinyl alcohol), and poly(vinyl sulfate) were obtained from Sigma (St Louis MO). Dextran (500 000 M_r) was obtained from Pharmacia (Piscataway, NJ). Poly(vinyl phosphate), poly(vinyl styrene sulfonic acid), polyacrylic acid, and the ammonium salt of polyacrylic acid were obtained from Polysciences (Warrington, PA). The poly(vinyl sulfate) was dissolved in water, separated from undissolved materials by centrifugation and filtration, and lyophilized. Metachromatic shift assays using toluidine blue were performed as outlined in DeAngelis and Glabe (1987). Only polyanions demonstrating metachromasia were utilized. The other chemicals were used as supplied.

Sea urchin egg jelly fucan was the kind gift of Dr Charles Glabe, University of California, Irvine. Yeast polyphosphate mannan was the kind gift of Dr Steven Rosen, University of California, San Francisco. Both polymers were used as supplied.

All other reagents and chemicals used in this study were of the highest quality commercially available.

Zona pellucida isolation

Porcine ovaries were obtained from slaughter houses, and ZP and ZP families were prepared after the method of Hedrick and Wardrip (1986). Heat-solubilized ZP were prepared by heating ZP for 30 min at 70°C in 5 mM ammonium bicarbonate. SDS-dissociated ZP were prepared by heating ZP in 0.5% SDS at a 100°C for 2 min.

Radiolabelling of fucoidan and zona pellucida

Pronase-treated fucoidan was activated with cyanogen bromide after the method of Glabe *et al.* (1982). The sample was then desalted on Sephadex G-50, and fractions containing carbohydrate were pooled, lyophilized and resuspended in 0.2 M sodium borate, pH 8.0. The activated fucoidan was added to 5.0 mg of tyrosine and allowed to react overnight at 4°C. The resulting complex was re-chromatographed on Sephadex G-50, and fractions containing carbohydrate were pooled and concentrated. The resulting tyrosine–fucoidan complex was radioiodinated using Iodogen as a catalyst, and desalted prior to use with Sephadex G-50 in 1 mM Mops, pH 8.0. The ZP, either heat solubilized or SDS dissociated, was radiolabelled in a similar manner using Iodogen, and similarly desalted prior to use.

Proacrosin purification and immobilization

Boar proacrosin was purified following the method of Parrish and Polakoski (1978) with only minor modifications. Contaminating protein at M_r 28 000 (possibly a proacrosin-binding protein) was removed using gel filtration in the presence of 8 M

urea or 6 M guanidine. Proacrosin was immobilized onto agarose beads using Actigel-ALD (Sterogene Biochemicals, San Gabriel, CA) in 0.1 M sodium acetate at pH 3.0. After blocking the unreacted aldehyde groups with ethanolamine, the proacrosin-Actigel was washed and kept in 1 mM Mops, 50 mM benzamidine HCl, pH 8.0.

Proacrosin activation and blotting

Proacrosin was activated by adding 0.1 vol of 1.1 M Tris buffer, pH 8.0, following the appearance of active acrosins enzymatically (Urch and Hedrick, 1988). The reaction was stopped by the addition of HCl to pH 3.0. The resultant autoactivation time points were separated on SDS–PAGE and western blotted onto nitrocellulose overnight at 25 volts. The nitrocellulose sheets were blocked in 5 % skimmed milk in Tris-buffered saline, and 50 000 cts min^{-1} of ^{125}I-fucoidan or ^{125}I-ZP (preadsorbed with skimmed milk-blocked nitrocellulose sheets) was added. The sheets were incubated overnight, rinsed in 0.05 % NP-40 in Tris-buffered saline for 30 min (4× changes), air dried and autoradiography of the bound label performed. The autoradiograms were photographed and reflective densitometry with the Visage 60 BioAnalyzer was performed.

Filter binding assay

The filter assay is a modification of the procedure of DeAngelis and Glabe for sea urchin bindin interaction with fucoidan (1987). Agarose-immobilized proacrosin (3.5 μg) was added to ^{125}I-tyrosine-fucoidan (0.5–6.0 μg) in a total volume of 1.0 ml with 1 mM Mops, pH 8.0. The initial concentration of fucoidan was determined by radioactivity. After incubation for 15 min, the reaction mixture was filtered through glass fiber filters (Whatman GF/C) with 2×4.0 ml washes of 1 mM Mops, pH 8.0. The filters were counted in a gamma counter for 2 min to determine the amount of fucoidan bound to the proacrosin beads. For the binding of ZP, 1.65 μg of proacrosin bound to Actigel-ALD was added to ^{125}I-ZP (4.5–55 μg) and incubated for 150 min at the same pH and ionic strength as above.

Scatchard plot analysis of the saturation curves was performed by plotting bound/free *versus* bound amounts of the radiolabelled components. For the determination of relative binding affinities, saturating levels of fucoidan or ZP were added to the assay along with varying amounts of the test chemical. The results are graphed as the percentage of maximal binding, or the amount of binding obtained at saturating levels of fucoidan or ZP.

Results

Agarose-insolubilized beads of proacrosin could be saturated by radiolabelled ZP and fucoidan, and binding affinities were determined from Scatchard analysis of these saturation curves. Assuming M_rs of 2×10^6 for heat-solubilized ZP and 1×10^5 for fucoidan, binding affinities of 2×10^{-8} M for ZP and 6×10^{-8} M for fucoidan were obtained. The fucoidan binding affinity was similar to that reported for bindin [$K_d=5.5\times10^{-8}$ M (Glabe *et al.* 1982)].

The ability of sulfated and nonsulfated polysaccharides and polymers to compete with fucoidan- or ZP-proacrosin binding was measured with the filter assay. The binding of both radiolabelled materials to proacrosin was more sensitive to ionic strength than the

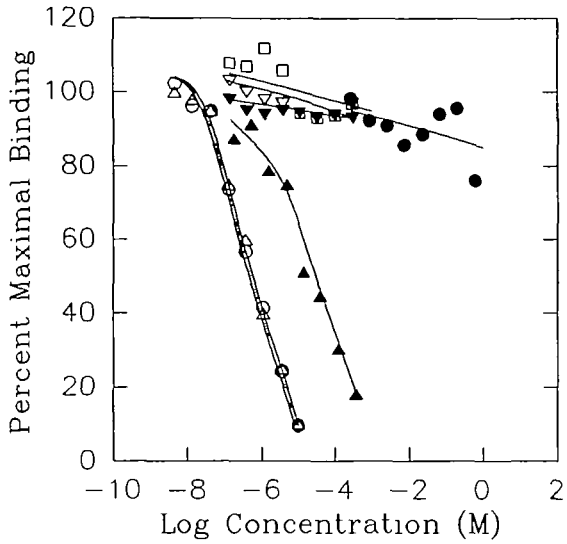

Fig. 1. The effect of added carbohydrates on the fucoidan-proacrosin binding assay measured as the percentage of maximal binding at saturating levels of ^{125}I-fucoidan. The carbohydrates are: unlabeled fucoidan (○); dextran sulphate (M_r 5000) (▲); dextran sulphate (M_r 500 000) (△); chondroitin sulphate A or C (▽); hyaluronic acid (▼); dextran (M_r 500 000) (□); fucose (●).

binding of fucan or fucoidan to sea urchin bindin; also proacrosin binding was totally inhibited at the ionic strength maximum for bindin interaction [0.1–0.6 M (DeAngelis and Glabe, 1988)]. However, there were several similarities between proacrosin and bindin. Dextran sulphate (both M_rs) and 'cold' fucoidan competed effectively with ^{125}I-fucoidan for proacrosin, whereas, neither the nonsulfated carbohydrates hyaluronic acid, dextran and fucose, nor the sulfated carbohydrates, chondroitin sulphates A or C inhibit the interaction (Fig. 1). The lack of competition with fucose is contradictory to the interpretation of proacrosin as a fucose-lectin (Töpfer-Petersen and Henschen, 1988). Inorganic sulfate at constant ionic strength was also without effect. Similar inhibition of ^{125}I-ZP–proacrosin interaction was obtained with the sulfated polysaccharides (data not shown) and the unlabeled components competed effectively in both the homologous and heterologous assays. When ZP was added to the fucoidan–proacrosin assay, the degree of inhibition was greater than with the same molar equivalent of fucoidan, assuming a M_r for ZP of 2×10^6. However, in an attempt to determine which ZP component is the ligand for proacrosin, the SDS–PAGE isolated and electroeluted components did not compete effectively for intact ZP or fucoidan. This was not a direct effect of SDS, since SDS-dissociated ZP was as effective a competitor as heat-solubilized ZP. When intact ZP was western blotted, both proacrosin and biotinylated DIP-acrosin bound to the acidic end of the 55 M_r family (data not shown). This glycoprotein family is not hydrolyzed by acrosin in intact ZP (Urch, 1991; Hedrick *et al.* 1988). These data suggest that proacrosin/acrosin binding to the non-substrate portion of the ZP is similar

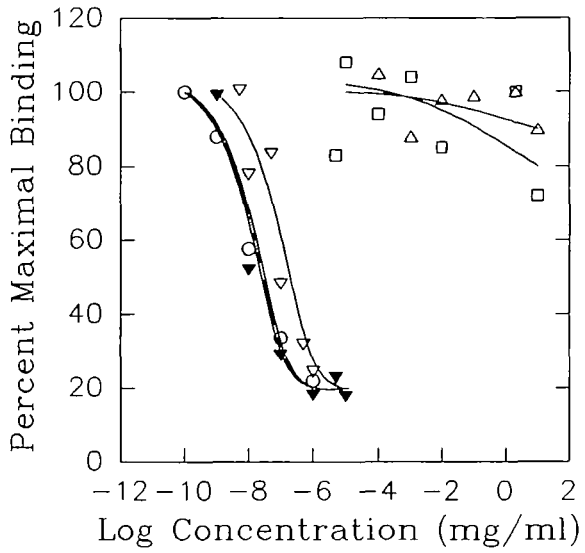

Fig. 2. The effect of polyvinyl polymers on the ZP–proacrosin binding assay measured as the percentage of maximal binding at saturating levels of ^{125}I-ZP. The polymers are: poly(vinyl sulfate) (○); poly(vinyl styrene sulfonic acid) (▼); polyacrylic acid (▽); poly(vinyl phosphate) (□); poly(vinyl alcohol) (△).

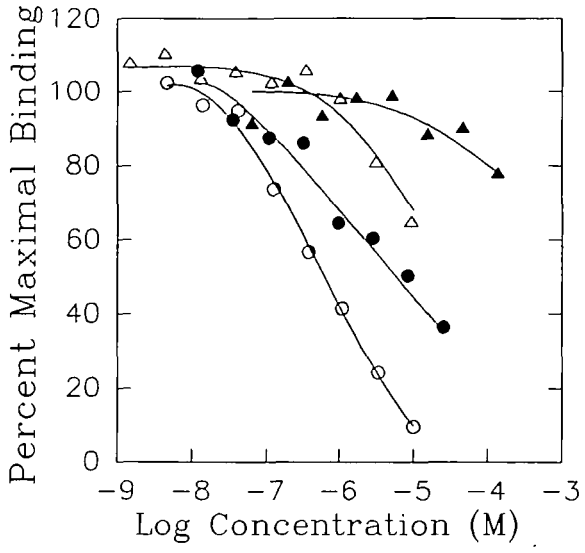

Fig. 3. The effect of partially acid hydrolyzed fucoidan on the fucoidan–proacrosin binding assay measured as the percentage of maximal binding at saturating levels of ^{125}I-fucoidan. The molecular sizes of the fucoidan pieces from gel filtration analysis are: intact fucoidan (M_r >100000) (○); M_r approx. 30000 fucoidan (●); M_r approx. 10000 fucoidan (△); M_r approx. 4000 fucoidan (▲).

to its binding to fucoidan. Sea urchin jelly fucan inhibited the interaction of both radiolabelled materials with the same affinity as fucoidan, a result similar to bindin. Interestingly, *Xenopus laevis* egg jelly inhibited fucoidan–, but not ZP–, proacrosin interaction.

The role of the polymeric back bone in proacrosin binding to the ZP was investigated by the addition of polyanions to the ZP binding assay (Fig. 2). Poly(vinyl sulfate) was able to compete with ^{125}I-ZP for proacrosin, while both poly(vinyl phosphate) and poly(vinyl alcohol) did not inhibit binding, and at high concentrations they increased apparent binding 2- to 3-fold. Poly(styrene sulfonic acid) inhibited ^{125}I-ZP–proacrosin binding, indicating polymeric sulfonic acid groups are important in addition to the sulfate esters. Polyacrylic acid, but not the ammonium salt of polyacrylic acid, inhibited binding, even though they both exhibited dye metachromasia consistent with the carboxylic acid residues being on the polymer. This effect may be due to a generalized ionic strength phenomenon; however, neither polymeric uronic acid groups [hyaluronic acid above (Fig. 1)], nor mannose-6-phosphate or polyphosphated mannan from yeast competed effectively in either assay (data not shown). The same inhibition results were obtained with fucoidan binding to proacrosin. The polymeric backbone does not seem to be of importance in the binding assays with either ZP or fucoidan, a finding similar to that of sea urchin bindin.

Since the competition for fucoidan appeared to be dependent on the M_r of the competing agent [the lower M_r dextran sulfate was tenfold less effective a competitor (Fig. 1)], fucoidan was acid hydrolyzed and the resultant products separated on gel filtration. The smaller M_r fractions were not as effective as intact fucoidan (Fig. 3). Similar data were obtained with pronase-digested ZP. The larger glycopeptides were found to interact with a proacrosin affinity column, whereas smaller glycopeptides did not (data not shown). The partial removal of sulfate ester groups from fucoidan by solvolytic desulfation resulted in 10-fold less inhibition of proacrosin–fucoidan binding (Fig. 4). All these data are quite similar to those described for sea urchin bindin, and indicate that sulfate (or sulfonic acid) esters on a macromolecular backbone are the important ligand features for binding.

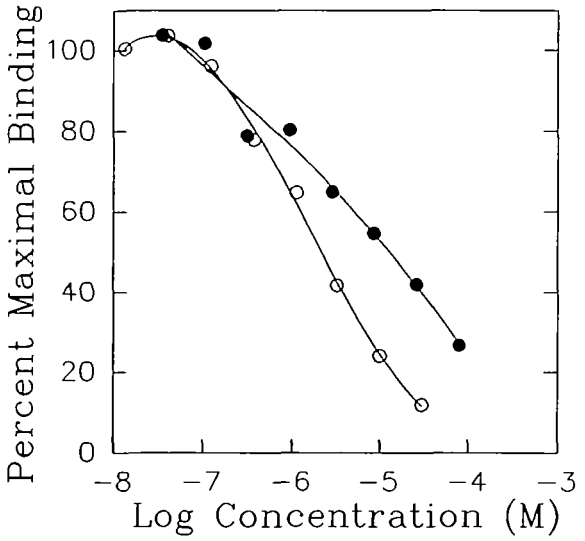

Fig. 4. The effect of partial desulfation of fucoidan on the fucoidan–proacrosin binding assay measured as the percentage of maximal binding at saturating levels of ^{125}I-fucoidan. Intact fucoidan (○); partially desulfated (●).

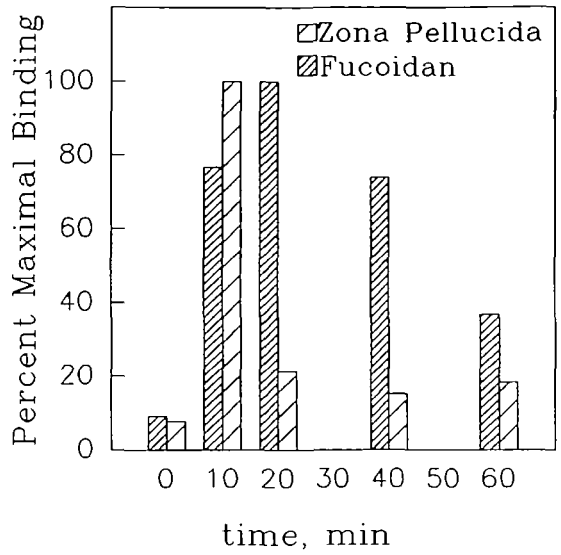

Fig. 5. Percentage of maximal binding of ^{125}I-fucoidan and ^{125}I-ZP to m_α-acrosin (M_r 49 000) on nitrocellulose blots as a function of proacrosin autolysis time.

Proacrosin was autoactivated to active acrosins by incubation at pH 8.0. The resultant autolysis products were blotted onto nitrocellulose after SDS–PAGE, and these western blots were probed with both ^{125}I-ZP and ^{125}I-fucoidan. Both forms of proacrosin (M_r 55 000 and 53 000) and the first active acrosin m_α-acrosin (M_r 49 000) bind the radiolabelled components avidly. With continued C-terminal processing of m_α-acrosin, and the appearance of m_β-acrosin (M_r 38 000), there is a loss of fucoidan binding. However, the ability to bind ZP is lost sooner than fucoidan binding (Fig. 5). m_β-acrosin binds fucoidan and ZP, but not as avidly as the m_α-acrosin. This would indicate either that the binding domain for the two components is not the same size or that a specific recognition sequence for ZP is lost before the recognition sequence for fucoidan. These data are consistent with those for polysulfated polysaccharide inhibition of amidase and protease activities of acrosin, since inhibition is lost with autolysis of acrosin (Urch and Hedrick, 1988). In addition to the m_β-acrosin, there are low relative molecular mass components that bind to the labeled materials as avidly as m_α-acrosin, indicating that acrosin autolysis releases a competent binding domain, which is also consistent with previous studies (Töpfer-Petersen and Henschen, 1988).

Discussion

Cell–cell recognition and adhesion involve specific interactions between proteins and/or carbohydrates on the cell surface. Several of these macromolecular factors have been isolated and described. Cell adhesion molecules (CAMS) have been isolated and characterized from differentiating neural crest cell surface [N-CAM (Brackenbury *et al.* 1981)], from liver cells [L-CAM (Sorkin *et al.* 1988)] and from the adherens junction [A-CAM (Geiger *et al.* 1987)]. Protein–carbohydrate interactions are important for recognition and attachment of cell homing factors [e.g. leucocyte (Stoolman *et al.* 1984)], stem cell proliferation factors (Vohmeyer *et al.* 1988) and for the species-specific aggregation of sponge cells (Coombe *et al.* 1987). The molecules involved in recognition and binding are typically multidomain molecules that are either members of the IgG superfamily or lectins (Brackenbury, 1988) Yednock *et al.* 1987). In addition to these interactions, carbohydrate–carbohydrate cell surface interactions have been described recently (Eggens *et al.* 1989).

Fertilization requires the successful recognition and binding of gametes. In a variety of species, gamete binding is inhibited by carbohydrates (Ahuji, 1982; Shalgi *et al.* 1986), although other mechanisms may also be involved, such as the fibronectin amino acid adhesion sequence arg-gly-asp-ser which inhibits binding of mouse gametes (Bunch and Saling, 1988). In the pig, polysulfated polysaccharides inhibit sperm–egg interaction (Peterson *et al.* 1984), and these same polysaccharides inhibit the amidase and protease activities of boar acrosin (Urch and Hedrick, 1988). With sea urchin gametes, the necessary elements for attachment are polysulfated egg jelly fucan and sperm bindin (DeAngelis and Glabe, 1988). This charge–charge interaction retains species-specific agglutination properties through the novel use of a sulfate docking site on bindin, modulated with species-specific recognition factors (DeAngelis and Glabe, 1990). In this present study, we describe a recognition mechanism between mammalian gamete molecules, based on a charge–charge interaction of ZP nonsubstrate components for a separate and distinct binding domain on the proteolytic enzyme acrosin. This mechanism may play a role in maintaining the penetrating sperm on the ZP, and allow for cycles of hydrolysis and binding that facilitate sperm penetration of the ZP (Urch, 1991; Hedrick *et al.* 1988).

Using analogous experimentation to sea urchin bindin and its interaction to fucoidan and egg jelly fucan (Glabe *et al.* 1982), proacrosin was found to bind to its natural substrate, the ZP, and to fucoidan with high avidity similar to the sea urchin bindin–fucoidan or bindin–egg jelly fucan interaction (K_ds$>10^{-8}$ M). Like bindin, proacrosin–fucoidan binding was competitively inhibited with macromolecular polysulfated polysaccharides and polymers, whereas nonsulfated carbohydrates had no effect on binding (Figs 1, 3, and 4). Because there is very little structural similarity in amino acid sequence between bindin (Gao *et al.* 1986) and boar proacrosin (Baba *et al.* 1989*a*; Adham *et al.* 1989), these data represent an example of convergent evolution: sperm molecules stored in analogous granules behaving in an analogous fashion by binding to similar polysulfated polysaccharides with similar affinities. These data also represent an example of similar binding function with very different primary structures, consistent with a growing body of information that suggests primary sequence information does not alone predict specific secondary structural considerations in several

classes of molecules (Bowie *et al.* 1990; Bowie and Sauer, 1989; O'Neill and DeGrado, 1990).

Even though they have functions, there were several differences between sea urchin bindin and proacrosin in binding to fucoidan, that were not unexpected. Bindin functions in the milieu of sea water and the binding to fucoidan and egg fucan is maximum at 0.1–0.6 M ionic strength. Proacrosin binding to both ZP and fucoidan is inhibited by ionic strengths above 0.066 M. DeAngelis and Glabe argued for specificity of bindin interaction by comparing fucoidan binding to polymers of basic amino acids with fucoidan binding to bindin (DeAngelis and Glabe, 1988). They reported that polylysine- and polyhistidine-^{125}I-fucoidan binding was more sensitive to ionic strength than bindin-^{125}I-fucoidan binding. Also all three polymers tested [polyacrylic acid, poly(vinyl sulfate) and poly(vinyl phosphate)] inhibited ^{125}I-fucoidan binding to polylysine, polyarginine and polyhistidine (DeAngelis and Glabe, 1988), whereas bindin–fucoidan interaction was only inhibited by polyvinyl sulfate. Proacrosin binding to either ZP or fucoidan is more sensitive to ionic strength than the interaction of fucoidan with any of the polyamino acids reported. However, there is retention of binding specificity, since poly(vinyl sulfate), but not poly(vinyl phosphate), inhibits ZP- and fucoidan-binding assays.

Proacrosin–fucoidan interaction was competed for effectively by polyacrylic acid, whereas bindin–fucoidan interaction was not. The ammonium salt of polyacrylic acid did not compete, and it is not clear why the salt was not effective, since both acid and salt exhibited dye metachromasia. Fucoidan contains other charged carbohydrates, e.g. 7–25% uronic acids, depending on the species of *Fucus* that it was extracted from (Medcalf and Larsen, 1977). These acid groups may be involved in proacrosin binding, from the result that fucoidan desulfation decreases, but does not eliminate competition (Fig. 4). Since macromolecular size is very important for effective competition (Fig. 3), macromolecular ZP has sufficient charge density of acidic groups (both sulfate and carboxylic acidic groups) for effective proacrosin binding.

High concentrations of nonsulfated carbohydrates did not compete with fucoidan (or ZP) for proacrosin binding (Fig. 1). Neoglycoprotein probes of fucose attached to either horse-radish peroxidase or to bovine serum albumin have been used to label intact sperm (Töpfer-Petersen *et al.* 1988). They have also been used to identify bands on western blot analyses of sperm extracts with the same M_rs as proacrosin, active acrosin and breakdown products of acrosin (Jones, 1989). The data presented in this study suggest that the molecular size of the binding molecule is important. It may be that macromolecular fucose (either in the form of fucoidan or fucose neoglycoprotein) may be recognized by proacrosin, whereas the monosaccharide is not. Previous inhibition data also indicated that fucose was not an inhibitor of acrosin amidase or protease activity (Urch and Hedrick, 1988).

The autolytic processing of proacrosin to active acrosins takes place at both the N and C termini of the molecule (Zelezna *et al.* 1989; Baba *et al.* 1989*b*). The C-terminal end of the molecule is not as homologous among acrosins as the rest of the sequence. These data led Adham *et al.* (1989) to suggest that the C terminus is the species-specific region of proacrosin. In support of this hypothesis, our study demonstrated the loss of ZP binding by m_α-acrosin through autolysis before the loss of binding to the non-specific fucoidan (Fig. 5). This is intriguing since the ZP ligand for proacrosin/acrosin appears to be the acidic end of the 55 M_r families: ZP families that are not hydrolyzed by acrosin (Urch, 1991; Hedrick *et al.* 1988). Since the interaction of proacrosin with ZP is so closely mimicked by polysulfated polysaccharides, these polymers should prove invaluable for determining and understanding the ligand structure of the ZP. Also since the ZP ligand is a non-substrate for the protease domain of acrosin, the 55 M_r families should act in a similar fashion to fucoidan and inhibit the protease and amidase activities of acrosin. Their function physiologically then would be not only as ligands, but also as inhibitors of acrosin to limit the random and total proteolysis of the ZP. Non-substrate inhibition of acrosin would be similar to the action of other non-substrates, e.g. bile acid inhibition of glutathione transferases (Hayes and Mantle, 1986; Boyer and Vessey, 1987; Young and Briedis, 1990). The data presented in Fig. 5 suggest that the differences seen with the proacrosin binding domains for ZP and fucoidan may be due to specific recognition sequences for both. The importance of the C-terminal end of proacrosin in recognition and binding can now be properly tested with the assay described in this study, by investigating the binding affinities of different proacrosins for the same ZP, or the same proacrosin for different ZPs.

Recently, Holt *et al.* (1989) described the amino acid sequence cys-ser/thr-val-ser/thr-cys-gly-X-gly-X-X-X-arg/lys-X-arg/lys as a consensus sequence for the binding of sulfated glycoconjugates (Table 1). Acrosin has at least one, and perhaps as many as four, sequences that are similar to this reported sequence. These might function as potential binding sites for the sulfated glycoconjugates, fucoidan and ZP. One of these sequences spans the active site serine and may account for the observed competitive inhibition with polysulfated polysaccharides (Urch and Hedrick, 1988). Surprisingly, these sequences are all located in the interior or N-terminal end of the molecule, and not on the C-terminal end. Even though active acrosins still retain this internal sequence, there is less avid binding with time, consistent with the loss of important recognition sequences through the C-terminal processing of proacrosin to active acrosins. Also the replacement of arginine by lysine in the most conserved acrosin binding sequence would result in binding more sensitive to ionic strength, based on the data collected by DeAngelis and Glabe (1988). Recently, Jones (1990) determined that trypsinogen and chymotrypsinogen bound polysulfated polysaccharides. However, the binding constants determined were considerably lower than proacrosin, indicating that the unique binding

Table 1. *Consensus sequence for binding sulfated glycoconjugates**

cys	[ser / or / thr]	val	[ser / or / thr]	cys	gly	X	gly	X	X	X	[arg / or / lys]	X	[arg / or / lys]

Acrosin and related pig protease sequences, sequence 137-148

Acrosin	**cys**	trp	**val**	**thr**	-	**gly**	trp	**gly**	tyr	leu	-	**lys**	glu	**lys**†
Trypsin	**cys**	leu	ile	**ser**	-	**gly**	trp	**gly**	asn	thr	-	**lys**	ser	ser‡
Chymotyr	**cys**	ala	thr	**thr**	-	**gly**	trp	**gly**	lys	thr	-	**lys**	tyr	asn‡

Boar acrosin, other possible binding sequences

									cys					
196-209	**cys**	gln	gly	asp	ser	**gly**	-	**gly**	pro	leu	met	**arg**	asp	**arg**
169-180	**cys**	asn	ser	**thr**	arg	trp	asn	**gly**	-	-	-	**arg**	ile	**arg**
287-302	phe	gln	arg	pro	pro	**gly**	pro	ser	gln	gln	ser	**arg**	pro	**arg**
										gly	pro			

−, missing; X, any amino acid.

*Holt, G. D. *et al.* (1989) *J. biol. Chem.* **264**, 12 138–12 140.

†Baba, T. *et al.* (1989a) *J. biol. Chem.* **264**, 11 920–11 927.

‡Dayhoff, M. O., (1976) Atlas of Protein Sequence and Structure, Vol. 5, National Biomedical Research Foundation, Washington, D.C.

character of proacrosin results from changes in the sequence of these related molecules. When potential binding sequences in acrosin are compared to trypsin and chymotrypsin (Table 1), both these proteases lack the basic amino acid pair at the end of the consensus sequence. We assert therefore that acrosin was selected through evolution by modification to this basic pair of amino acids. This hypothesis is testable utilizing site-directed mutagenesis, where a basic pair of amino acids could be inserted into porcine trypsin. We would predict that this modified trypsin would then bind the non-substrate polysulfated polysaccharides in a manner similar to acrosin. Also similar site-directed mutagenesis of proacrosin to remove that basic pair should produce enzymatically active, but binding deficient, acrosins. Possible recognition sequences in the C-terminal portion of acrosin for binding ZP and fucoidan could also be established through such a study.

The striking similarities of cellular location and biological function of sea urchin bindin and proacrosin through convergent evolution, give the following quotation made by Lajos Piko (1969) an interesting new perspective: 'It is tempting to speculate that the outer acid layer of the zona may be analogous to the gelatinous coat of the sea urchin egg....' It would appear that evolution has utilized a similar motif of charge–charge interaction between gamete molecules in both the sea urchin and mammals.

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References

ADHAM, I. M., KLEMM, U., MAIER, W.-M., HOYER-FENDER, S., TSAOUSIDOU, S. AND ENGEL, W. (1989). Molecular cloning of preproacrosin and analysis of its expression pattern in spermatogenesis. *Eur. J. Biochem.* **182**, 563–568.

AHUJI, K. K. (1982). Fertilization studies in the hamster. The role of cell surface carbohydrates. *Expl Cell Res.* **140**, 353–362.

BABA, T., KASHIWABARA, S., WATANABE, K., ITOH, H., MICHIKAWA, Y., KIMURA, K., TAKADA, M., FUKAMIZU, A. AND ARAI, Y. (1989*a*). Activation and maturation mechanisms of boar acrosin zymogen based on the deduced primary structure. *J. biol. Chem.* **264**, 11 920–11 927.

BABA, T., MICHIKAWA, Y., KAWAKURA, K. AND ARAI, Y. (1989*b*). Activation of boar proacrosin is effected by processing at both the N-terminal and C-terminal portions of the zymogen molecule. *FEBS Lett.* **244**, 132–136.

BENAU, D. A. AND STOREY, B. T. (1987). Characterization of the mouse sperm plasma membrane zona-binding site sensitive to trypsin inhibitors. *Biol. Reprod.* **36**, 282–292.

BERGER, T., DAVIS, A., WARDRIP, N. J. AND HEDRICK, J. L. (1989). Sperm binding to the zona pellucida and inhibition by solubilized components of the zona pellucida. *J. Repro. Fert.* **86**, 559–565.

BLIEL, J. D. AND WASSARMAN, P. M. (1980). Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse zonae pellucidae possessing receptor activity for sperm. *Cell* **20**, 973–882.

BOWIE, J. U., REIDHAAR-OLSON, J. F., LIM, W. A. AND SAUER, R. T. (1990). Deciphering the message in protein sequences: Tolerance to amino acid substitutions. *Science* **247**, 1306–1310.

BOWIE, J. U. AND SAUER, R. T. (1989). Identifying determinants of folding and activity for a protein of unknown structure. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2152–2156.

BOYER, T. D. AND VESSEY, D. A. (1987). Inhibition of human cationic glutathione *S*-transferase by nonsubstrate ligands. *Hepatology* **7**, 843–848.

BRACKENBURY, R. (1988). Expression of neural cell adhesion molecules in normal and pathologic tissues. *Anal. N.Y. Acad. Sci.* **540**, 39–46.

BRACKENBURY, R., RUTISHAUSER, U. AND EDELMAN, G. M. (1981). Distinct calcium-independent and calcium-dependent adhesion systems of chicken embryo cells. *Proc. natn. Acad. Sci. U.S.A.* **78**, 387–391.

BROWN, C. R. AND JONES, R. (1987). Binding of zona pellucida proteins to a sperm polypeptide of M_r 53,000 and identification of zona moieties involved. *Development* **99**, 333–339.

BUNCH, D. O. AND SALING, P. (1988). The recognition sequence, RGDS, disrupts mouse sperm-zona pellucida interaction. *J. Cell Biol.* **107**, 175a.

COOMBE, D. R., JAKOBSEN, K. B. AND PARISH, C. R. (1987). A role for sulfated polysaccharide recognition in sponge cell aggregation. *Expl Cell Res.* **170**, 381–401.

DEANGELIS, P. L. AND GLABE, C. G. (1987). Polysaccharide structural features are critical for the binding of sulfated fucans to bindin, the adhesive protein from sea urchin sperm. *J. biol. Chem.* **262**, 13946–13952.

DEANGELIS, P. L. AND GLABE, C. G. (1988). Role of basic amino acids in the interaction of bindin with sulfated fucans. *Biochemistry* **27**, 8189–8194.

DEANGELIS, P. L. AND GLABE, C. G. (1990). Specific recognition of sulfate esters by bindin, a sperm adhesion protein from sea urchins. *Biochim. biophys. Acta* **1037**, 100–105.

EGGENS, I., FENDERSON, B. A., TOYOKUMI, T. AND HAKOMORI, S. (1989). A role of carbohydrate–carbohydrate interaction in the process of cell recognition during embryogenesis and organogenesis: A preliminary note. *Biochem. biophys. Res. Commun.* **158**, 913–920.

FLORMAN, H. M. AND WASSARMAN, P. M. (1985). O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* **41**, 313–329.

GAO, B., KLEIN, L. E., BRITTEN, R. J. AND DAVIDSON, E. H. (1986). Sequence of mRNA coding for bindin, a species-specific sea urchin protein required for fertilization. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8634–8638.

GEIGER, B., VOLK, T., VOLBERG, T. AND BENDORI, R. (1987). Molecular interactions in adherens-type interactions. *J. Cell Sci. Suppl.* **8**, 251–272.

GLABE, C. G., GRABEL, L. B., VACQUIER, V. D. AND ROSEN, S. D. (1982). Carbohydrate specificity of sea urchin bindin: A cell surface lectin mediated sperm egg adhesion. *J. Cell Biol.* **94**, 124–128.

HATHAWAY, A. J. AND SHUR, B. D. (1988). Novel cell surface receptors during mammalian fertilization and development. *Bioessays* **9**, 153–158.

HAYES, J. D. AND MANTLE, T. J. (1986). Inhibition of hepatic and extrahepatic glutathione *S*-transferase by primary and secondary bile acids. *Biochem. J.* **233**, 407–415.

HEDRICK, J. L., URCH, U. A. AND HARDY, D. M. (1988). Structure-function properties of the sperm enzyme acrosin. In *Biocatalysis in Agricultural Biotechnology* (ed. J. Whitaker and P. Sonnet) pp. 215–229. Washington, DC: American Chemical Society Books.

HEDRICK, J. L. AND WARDRIP, N. J. (1986). Isolation of the zona pellucida and purification of its glycoprotein families from pig oocytes. *Anal. Biochem.* **157**, 63–70.

HEDRICK, J. L. AND WARDRIP, N. J. (1987). On the macromolecular composition of the zona pellucida from porcine oocytes. *Devl Biol.* **123**, 257–262.

HOLT, G. D., KRIVAN, H. C., GASIC, G. J. AND GINSBURG, V. (1989). Antistasin, and inhibitor of coagulation and metastasis, binds to sulfatide (Gal(3-SO_4)β1-1 cer) and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J. biol. Chem.* **264**, 12138–12140.

JONES, R. (1989). Identification of carbohydrate-binding proteins in mammalian spermatozoa (human, bull, boar, ram, stallion and hamster) using [^{125}I]fucoidan and [^{125}I]neoglycoprotein probes. *Human Reprod.* **4**, 550–557.

JONES, R. (1990). Unusual fucoidan binding properties of chymotrypsinogen and trypsinogen. *Bioch. Biophys. Acta* **1037**, 227–232.

JONES, R. (1991). Interaction of the zona pellucida glycoproteins, sulfated carbohydrates and synthetic polymers with proacrosin, the putative egg-binding protein from mammalian sperm. *Development* **111**, 1155–1163.

JONES, R. AND BROWN, C. (1987). Identification of a zona-binding protein from boar spermatozoa as proacrosin. *Expl Cell Res.* **171**, 503–550.

JONES, R. AND WILLIAMS, R. M. (1990). Identification of zona- and fucoidan-binding proteins in guinea-pig spermatozoa and mechanisms of recognition. *Development* **109**, 41–50.

MEDCALF, D. G. AND LARSEN, B. (1977). Fucose-containing polysaccharides in the brown algae *Ascophyllum nodosum* and *Fucus vesiculosus*. *Carbohydr. Res.* **59**, 531–537.

O'NEILL, K. T. AND DEGRADO, W. F. (1990). How calmodulin binds its targets: Sequence independent recognition of amphiphilic a helices. *TIBS* **15**, 59–64.

O'RAND, M. G., WIDGREN, E. E. AND FISHER, S. J. (1988). Characterization of the rabbit sperm membrane autoantigen, RSA, as a lectin-like zona binding protein. *Devl Biol.* **129**, 231–240.

PARRISH, R. F. AND POLAKOSKI, K. L. (1978). Boar m_α-acrosin. Purification and characterization of the initial active enzyme resulting from the conversion of boar proacrosin to acrosin. *J. biol. Chem.* **253**, 8424–8432.

PETERSON, R. N. AND HUNT, W. P. (1989). Identification, isolation, and properties of a plasma membrane protein involved in the adhesion of boar sperm to the porcine zona pellucida. *Gamete Res.* **23**, 103–118.

PETERSON, R. N., RUSSELL, L. D. AND HUNT, W. P. (1984). Evidence for specific binding of uncapacitated boar sperm to porcine zonae pellucidae. *J. exp. Zool.* **231**, 137–147.

PIKO, L. (1969). Gamete structure and sperm entry in mammals. In *Fertilization, Comparative Morphology, Biochemistry and Immunology* Vol II (ed. C. B. Metz and A. Monroy) p. 359. New York: Academic Press.

POIRIER, G. R., ROBINSON, R., RICHARDSON, R., HINDS, K. AND CLAYTON, D. (1986). Evidence for a binding site on the sperm plasma membrane which recognizes the murine zona pellucida. *Gamete Res.* **14**, 235–243.

SHALGI, R., MATITYAHU, A. AND NEBEL, L. (1986). The role of carbohydrates in sperm-egg interaction in rats. *Biol. Reprod.* **34**, 446–452.

SHIMIZU, S., TSUJI, M. AND DEAN, J. (1983). *In vitro* biosynthesis of three sulfated glycoproteins of murine zonae pellucidae by oocytes grown in follicle culture. *J. biol. Chem.* **258**, 5858–5863.

SORKIN, B. C., HEMPERLY, J. J., EDELMAN, G. M. AND CUNNINGHAM, B. A. (1988). Phosphomannosyl receptors may participate in the adhesion interaction between lymphocytes and high endothelial venules. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7617–7621.

STOOLMAN, L. M., TENFORDE, T. S. AND ROSEN, S. D. (1984). *J. Cell Biol.* **99**, 1535–1540.

TÖPFER-PETERSEN, E., FRIESS, A. E. AND SCHILL, W.-B. (1988). The acrosome reaction in boar spermatozoa. *Human Reprod.* **3**, 319–326.

TÖPFER-PETERSEN, E. AND HENSCHEN, A. (1987). Acrosin shows zona and fucose binding, novel properties for a serine protease. *FEBS Lett.* **226**, 18–42.

TÖPFER-PETERSEN, E. AND HENSCHEN, A. (1988). Zona pellucida binding and fucose-binding of boar sperm acrosin is not correlated with proteolytic activity. *Biol. Chem. Höppe-Seyler* **369**, 69–76.

URCH, U. A. (1991). Biochemistry and function of acrosin. In *The Biology and Chemistry of Mammalian Fertilization* (ed. P. M. Wassarman) pp. 233–248. Chicago: CRC Press.

URCH, U. A. AND HEDRICK, J. L. (1988). The inhibition of boar acrosin amidase activity by sulfated polysaccharides. *Biol. Chem. Höppe-Seyler* **369**, 727–732.

VOHMEYER, K., BRANDT, W., NAGE, G. A. AND GABIUS, H.-J. (1988). Evidence for the involvement of protein-carbohydrate interaction in hematopoietic, multipotential colony-stimulating factor-dependent stem cell proliferation. *Life Sci.* **43**, 1591–1598.

YEDNOCK, T. A., STOOLMAN, L. M. AND ROSEN, S. D. (1987). Receptors involved in lymphocyte homing: Relationship between a carbohydrate binding receptor and the MEL-14 antigen. *J. Cell Biol.* **104**, 725–731.

YOUNG, P. R. AND BRIEDIS, A. V. (1990). Binding of inhibitors to the major glutathione *S*-transferase from bovine brain. Competitive binding between bilirubin and glutathione. *Biochim. biophys. Acta* **1038**, 114–118.

ZELEZNA, B., CECHOVA, D. AND HENSCHEN, A. (1989). Isolation of the boar sperm acrosin peptide released during the conversion of α-form into β-form. *Biol. Chem. Höppe-Seyler* **370**, 325–329.

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