Development 111, 1155-1163 (1991)
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Interaction of zona pellucida glycoproteins, sulphated carbohydrates and synthetic polymers with proacrosin, the putative egg-binding protein from mammalian spermatozoa

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Summary

Fertilization in mammals is a unique cell–cell recognition event that involves specific receptors on the surface of each gamete. Previous work has shown that proacrosin, a protein found within the acrosome of mammalian spermatozoa, binds non-enzymatically to zona pellucida glycoproteins (ZPGPs) that surround the egg and that this binding can be inhibited by sulphated polysaccharides such as fucoidan. The mechanism of this interaction has been investigated using ^{125}I-ZPGPs and ^{125}I-fucoidan as probes. Results show that it involves poly(sulphate) groups on zona glycoproteins that bind with high affinity (K_d=1.2 to 5.0×10^{-8} M) to complementary 'docking' sites on proacrosin. The spatial orientation of these sulphates, together with the tertiary structure of the target protein, determines the selectivity of polymer binding. Thus, dextran sulphate and poly(vinyl sulphate) are strong inhibitors of the above probes whereas dextran, chondroitin sulphates A and C and poly(vinyl phosphate) are ineffective. Proacrosin, therefore, has properties analogous to those described for 'bindin', the egg adhesion protein found within the acrosomal vesicle of sea urchin spermatozoa.

Key words: fertilization, sulphate binding, fucoidan, proacrosin, zona pellucida.

Introduction

Proacrosin is the zymogen form of the serine proteinase acrosin (EC 3.4.21.10) that is found exclusively within the acrosome of mammalian spermatozoa (reviewed by Hedrick *et al.* 1988). It has been purified and characterized extensively from several species and recently the genes for human and porcine proacrosin have been identified and sequenced (Adham *et al.* 1989; Baba *et al.* 1989*a*,*b*). The protein is synthesized postmeiotically in round spermatids in the testis (Arboleda and Gerton, 1987; Adham *et al.* 1989) and is subsequently processed during late spermatogenesis and epididymal maturation to produce the mature molecule characteristic of fully formed spermatozoa (Hardy *et al.* 1987; Arboleda and Gerton, 1988). It is found mostly within the acrosomal matrix where it is complexed to a binding protein and a naturally occurring inhibitor (Hedrick *et al.* 1988). Unusually for a serine proteinase, it autoactivates by modification of both N-terminal and C-terminal ends. At the N terminus there is site-specific cleavage between amino acids 23 and 24 to produce a 2-chain molecule consisting of a 'light' chain (relative molecular mass 4.2×10^3) cross-linked by disulphide bridges to a 'heavy' chain (Fock-Nüzel *et al.* 1984) while at the C terminus there is removal of a proline-rich domain, 72 to 75 amino acids long (Baba *et al.* 1989*a*).

From a functional standpoint, the enzyme has long been presumed to facilitate penetration of spermatozoa through the zona pellucida, the extracellular matrix that surrounds mammalian eggs. Whilst this possibility cannot be discounted, doubts about it have been raised on several occasions (Bedford and Cross, 1978), especially in light of recent findings on the ability of proacrosin/acrosin to bind non-enzymatically to zona glycoproteins (Brown and Jones, 1987; Jones and Brown, 1987; Töpfer-Petersen and Henshen, 1987; Jones *et al.* 1988). It has been suggested that proacrosin actually mediates secondary or tight binding of spermatozoa to the zona pellucida following the acrosome reaction and that the process is essentially a protein–carbohydrate interaction since it can be blocked by certain kinds of sulphated polysaccharides (Jones *et al.* 1988; Jones and Williams, 1990). Such an hypothesis correlates with earlier observations that the inhibitory effects of sulphated polymers on fertilization *in vitro* are directed more against spermatozoa than eggs (Huang *et al.* 1982; Boldt *et al.* 1989).

In this communication, we have investigated the structural properties of sulphated polymers and homologous zona glycoproteins that are critical for me-

diating their recognition and binding to proacrosin from boar spermatozoa. Results indicate that it is the density and stereochemical alignment of poly(sulphate) groups along the polymer chain that are the important parameters for interaction with basic residues on the surface of proacrosin. This work provides direct biochemical evidence that mammalian proacrosin has properties analogous to bindin, the egg adhesion protein that is found within the acrosomal granule of sea urchin spermatozoa (Vacquier, 1986). Similar conclusions have been reached in a parallel study to this by Urch and Patel (1991).

Materials and methods

Chemicals

All routine chemicals were of the highest purity available commercially and were purchased from Sigma, British Drug Houses or Pharmacia. Poly(vinyl phosphate) and poly(styrene sulphonate) (sodium salt) were supplied by Polysciences, galactan (from larch) by Kodak Laboratories and xylan (from *Triticum spelta*) and poly(vinyl sulphate), (potassium salt) by Sigma.

Preparation and purification of ^{125}I-fucoidan and ^{125}I-zona pellucida glycoprotein probes

Crude fucoidan (Sigma) from *Fucus vesiculosis* was purified by β-elimination and extensive pronase digestion (DeAngelis and Glabe, 1987). It had an average relative molecular mass (M_r) of 100×10^3 as determined by gel filtration on Sephadex G-75 and did not contain detectable amounts of protein. A compositional analysis after acid hydrolysis revealed 7.4% fucose, 8% xylose, 5% galactose, 1% mannose, 1% arabinose and 7% uronic acid (R. M. Williams and R. Jones, personal communication). These values are close to those reported originally by Medcalf and Larsen (1977) for fucans from this source. The purified fucoidan was conjugated with fluoresceinamine and iodinated with ^{125}I-Na and 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril ('Iodogen' Pierce) as described by Glabe *et al.* (1983). Fucoidan was estimated colorimetrically with orcinol/H_2SO_4 reagent (Vasseur, 1948) using purified material as standard.

Whole zona pellucidae were isolated from pig ovaries (Hedrick and Wardrip, 1987) and iodinated with ^{125}I-Bolton and Hunter reagent as supplied by Amersham Inter. (Jones *et al.* 1988). Zonae were solubilized by heating at 70°C for 30 min in 5 mM ammonium bicarbonate pH 9.0 and supernatants collected after centrifugation at 10000 *g* for 10 min. Total protein was measured by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Electrophoresis of samples by non-reducing SDS–PAGE/autoradiography revealed a labelled glycoprotein at M_r approx. 90×10^3 and a diffuse band with an average M_r approx. 55×10^3 (Fig. 1). Hedrick and Wardrip (1987) have shown that the M_r 55×10^3 component consists of 2 immunologically distinct families of glycoproteins referred to as M_r 55α and M_r 55β. The terminology of Hedrick and Wardrip (1987) will be used in this paper.

The M_r 90×10^3 and M_r $55\alpha+55\beta\times10^3$ zona glycoproteins were purified by separation of whole material by non-reducing SDS–PAGE and electroelution from gel slices using an ISCO model 1750 concentrator. SDS was removed from the proteins by acetone precipitation and renaturation as described by Hager and Burgess (1980). When the iodinated glycoproteins were re-analysed on SDS–PAGE/autoradiography, single bands were observed at M_r 90×10^3 and approx. M_r 55×10^3. The M_r 55α and M_r 55β components were not separated from each other as this involves endo-β-galactosidase digestion (Yurewicz *et al.* 1987), a step we wished to avoid because of the possibility that it might perturb reactive groups on part of the carbohydrate moiety.

Iodinated total zona pellucida glycoproteins (^{125}I-ZPGPs) were digested with protease (1 i.u. ml^{-1}) from *Streptomyces griseus* immobilized on carboxymethylcellulose (CMC-protease; Sigma) suspended in PBS/PVP-40 for various times at 37°C exactly as described by Florman *et al.* 1984. Digested proteins were analysed by SDS–PAGE and radioactivity detected by autoradiography of wet gels for 4 h at 4°C (Florman *et al.* 1984). Binding of CMC-protease-digested zona glycopeptides to proacrosin was investigated using a solid phase assay (see later). Equal amounts (~300000 cts min^{-1} in 100 μl) of ^{125}I-ZPGPs were incubated alone (control) or in the presence of CMC-protease for 1 h, centrifuged to remove beads and recovered supernatants made 10 mM with *p*-aminobenzamidine. Equal amounts were then used to probe immobilized proacrosin and specific binding calculated.

Electrophoresis and western blotting

Proteins were separated in one dimension by reducing or non-reducing SDS–PAGE (Laemmli, 1970) and either transferred to nitrocellulose membranes (0.45 μm) by electroblotting (Towbin *et al.* 1979) or stained with 1% Coomassie Brilliant Blue R-250 in methanol:acetic acid:water (40%:7%:53%, respectively). Blots were blocked with 5% BSA in phosphate-buffered saline pH 7.2 containing 1 mM *p*-aminobenzamidine (PBS/pAB) for 3 h at 23°C and overlaid with one of three different probes; (a) a rabbit anti-boar proacrosin antibody for identification purposes (Jones and Brown, 1987); (b) ^{125}I-fucoidan (200000 cts min^{-1} ml^{-1} in PBS/pAB); (c) ^{125}I-ZPGPs (550000 cts min^{-1} ml^{-1} in PBS/pAB). For b and c, blots were incubated for 1 h, unbound probe removed by washing in PBS (3 times for 5 min with shaking) and bound probe detected by autoradiography with X-ray film (Fuji) at −80°C. Relative molecular masses were calculated by reference to the mobility of known protein standards (Pharmacia 'Rainbow™' markers).

Extraction and purification of sperm proacrosin

Proacrosin was extracted from washed ejaculated boar spermatozoa into 0.25 M sucrose/50 mM benzamidine HCl titrated to pH 3 with 0.1 N HCl (Jones *et al.* 1988). Proacrosin was purified from these acid extracts by the method of Fock-Nüzel *et al.* (1984) or, in later experiments, by electroelution from gels after non-reducing SDS–PAGE. Purified protein was either stored frozen at −20°C or dialysed against 1 mM HCl pH 3 and lyophilized.

Assay for binding of ^{125}I-fucoidan and ^{125}I-ZPGPs to proacrosin

A solid phase competition assay was developed to measure quantitatively the avidity of the interaction between proacrosin and ^{125}I-fucoidan and proacrosin and ^{125}I-ZPGPs. 100 pmoles of purified proacrosin was immobilized onto strips of nitrocellulose (1 cm^2) and blocked with 5% BSA in PBS/pBA for 3 h at 23°C. Strips were rinsed in PBS and blocked for a second time for 1 h with various concentrations (see Results) of saccharides, polysaccharides and synthetic polymers in 0.1 ml PBS/pAB. Samples were drained and probed with 100000 cts min^{-1} of either ^{125}I-fucoidan (0.4×10^6 cts min^{-1} μg^{-1}) or ^{125}I-ZPGPs (1.4×10^6 cts min^{-1} μg^{-1}) in

0.1 ml PBS/pAB as described above for western blots. After washing three times in PBS, bound radioactivity was counted in an LKB gamma counter. Preliminary experiments showed that $t_{\frac{1}{2}}$ for binding of ^{125}I-fucoidan and ^{125}I-ZPGPs was 14 min and 16 min, respectively. In some experiments, proacrosin was reduced and alkylated with 5% mercaptoethanol/10 mM iodoacetamide at 37°C for 3 h. Heat denaturation was performed at 100°C for 5 min. Maximum binding is defined as the amount of probe retained on the nitrocellulose in the presence of the proacrosin target but in the absence of the competing agent. Nonspecific binding is defined as the amount of probe remaining on the nitrocellulose in the absence of target protein and specific binding as the amount of probe bound to target protein minus nonspecific background. All assays were done in duplicate and experiments repeated at least twice.

Fluorescence microscopy

Washed spermatozoa (intact or permeabilized by cold shock) were doubled labelled in suspension with fluorescein isothiocyanate-conjugated zona glycoproteins (FITC–ZPGPs; 0.25 μg ml^{-1}; O'Rand and Fisher, 1987) and rhodamine-conjugated peanut agglutinin (Rh–PNA; 1 mg ml^{-1}; Vector Laboratories). Rh–PNA binds specifically to acrosomal membranes and acrosomal matrix material of permabilized boar spermatozoa and is an accurate marker of cell integrity. 25 μl sperm in PBS/0.2% BSA/2 mM pAB were mixed with 25 μl FITC–ZPGPs and 1 μl Rh–PNA and incubated for 45 min at 23°C. Sperm were washed twice with PBS/0.1% BSA and resuspended in 50 μl PBS/pAB containing 2.5% 1,4-diazobicyclo-(2,2,2)-octane pH 8.6. Samples were examined by phase contrast and epifluorescence optics on a Zeiss Axiophot photomicroscope using differential filters. For competitive inhibition studies, spermatozoa were incubated in the presence of various saccharides and polymers at 100 times the $IC_{50\%}$ concentration shown in Table 1 for 10 min before addition of the fluorescent probe.

Protein sequencing

N-terminal amino acid sequence analysis was carried out on an Applied Biosystems gas phase sequencer (model 470A) equipped with on-line phenylthiohydantoin amino acid derivative analyser (model 120A). The sequencing programme was run as recommended by the manufacturer.

Results

Recognition of proacrosin by ^{125}I-fucoidan and ^{125}I-ZPGP probes

As reported previously and as shown in Fig. 1, ^{125}I-fucoidan and ^{125}I-ZPGPs bind strongly to two proteins at M_r 67×10^3 and M_r 53×10^3 on western blots of pH 3 extracts of boar spermatozoa (Jones and Brown, 1987; Töpfer-Petersen and Henshen, 1987; Jones, 1989). The M_r 67×10^3 protein has not been positively identified but the doublet at M_r 53×10^3 has been shown to represent proacrosin on the basis of (a) its recognition by anti-proacrosin antibodies (Jones and Brown, 1987) and (b) N-terminal sequence analysis which yields Arg-Asp-Asn-Ala-Thr-Cys-Asp-Gly-. This is identical to that reported by Fock-Nüzel *et al.* (1984) for acrosin light chain. The purified preparation of proacrosin was >90% homogenous as judged by staining with Coomassie Blue (Fig. 1) and bound both ^{125}I-fucoidan and

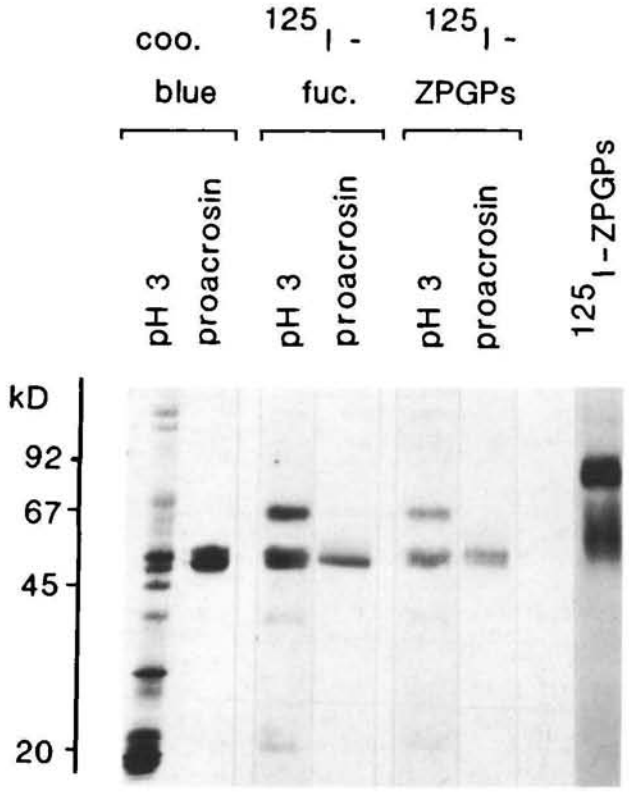

Fig. 1. Detection of proacrosin on western blots using ^{125}I-fucoidan and ^{125}I-ZPGP probes. Proteins in pH 3 extracts of boar spermatozoa or purified proacrosin were separated by SDS–PAGE and either stained with Coomassie Blue R-250 (first panel) or transferred to nitrocellulose paper and probed with ^{125}I-fucoidan (second panel) or ^{125}I-ZPGPs (third panel). Bound probe was detected by autoradiography. The fourth panel shows the composition of the ^{125}I-ZPGP probe.

^{125}I-ZPGPs in a linear fashion over the range 10–200 pmoles target protein in the presence of excess probe (Fig. 2A).

Effects of protease digestion on binding of ^{125}I-ZPGPs probe to proacrosin

Since the ^{125}I-fucoidan probe had been subjected to extensive pronase digestion followed by dialysis and gel filtration on Sephadex G-75, its interaction with proacrosin was presumed to involve reactive groups on the polysaccharide chain and not contaminating glycopeptides. To investigate if binding of ^{125}I-zona probe to proacrosin was mediated *via* its protein or carbohydrate moiety, ^{125}I-ZPGPs were treated with *Streptomyces griseus* protease and the resultant glycopeptides then used as probes in the solid-phase binding assay. As shown in Fig. 2B insert, protease digestion for 120 min caused considerable breakdown of ZPGPs to lower M_r components ranging from approx. 5×10^3 to 40×10^3. These ^{125}I-zona glycopeptides, however, still bound to immobilized proacrosin (Fig. 2B), suggesting that integrity of the protein backbone of ZPGPs is less critical for recognition than reactive groups on the carbohydrate moiety.

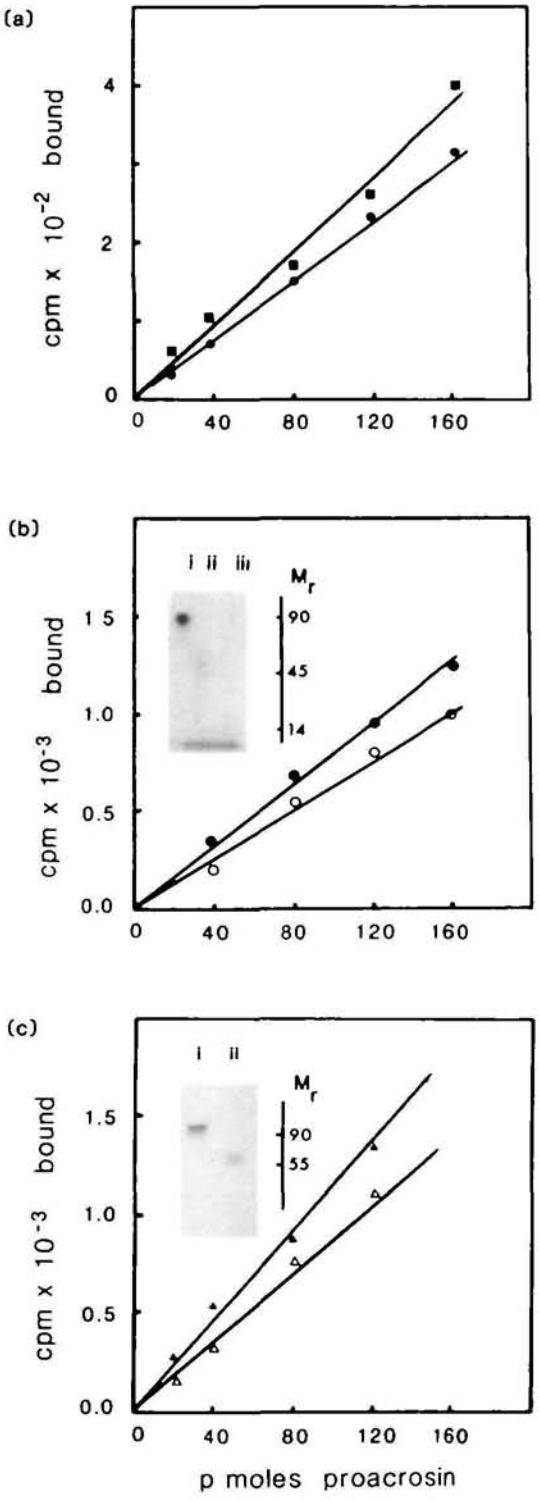

Fig. 2. Specific binding of ^{125}I-fucoidan and ^{125}I-ZPGPs to boar sperm proacrosin. Increasing amounts of proacrosin were dot-blotted onto nitrocellulose and probed with (A), ^{125}I-fucoidan (■) or ^{125}I-ZPGPs (●); (B) intact ^{125}I-ZPGPs (●) or protease digested (120 min) ^{125}I-ZPGPs (○). Insert shows autoradiograph after SDS–PAGE of ^{125}I-ZPGPs incubated with CMC-protease for (i) 0 min, (ii) 30 min, (iii) 120 min; (C) purified ^{125}I-labelled M_r 90×10^3 (△) and M_r $55\alpha+55\beta\times10^3$ (▲) ZPGPs. Insert shows autoradiograph of ^{125}I-labelled purified components after SDS–PAGE. The specific activities of the [^{125}I] M_r 90×10^3 and M_r $55\alpha+55\beta\times10^3$ probes were 143 737 cts min^{-1} μg^{-1} and 132 241 cts min^{-1} μg^{-1} respectively as measured by ethanol precipitation.

Binding of purified M_r 90×10^3 *and* $55\alpha+55\beta\times10^3$ *zona glycoproteins to proacrosin*

To determine if binding of the whole ^{125}I-ZPGP probe to proacrosin was mediated by one or several of its component glycoproteins, purified M_r 90×10^3 and $55\alpha+55\beta\times10^3$ glycoproteins were iodinated and used in solid phase assay described above. As shown in Fig. 2C, both the M_r 90×10^3 and $55\alpha+55\beta\times10^3$ preparations bound to proacrosin in a linear fashion when increasing amounts of target protein were incubated with excess probe. Consistently higher binding was obtained with the M_r $55\alpha+55\beta\times10^3$ glycoproteins that the M_r 90×10^3 component. However, saturation kinetics with these purified ZPGPs proved difficult, suggesting that all 3 may be co-operatively involved in binding to proacrosin. Therefore, in subsequent experiments on the mechanism of proacrosin recognition, we used the whole ^{125}I-ZPGPs probe as this is closer to the biological situation at the cellular level.

Kinetics of binding of ^{125}*I-fucoidan and* ^{125}*I-ZPGPs to proacrosin*

The binding of both ^{125}I-fucoidan and whole ^{125}I-ZPGPs to proacrosin approaches saturation under the conditions described for the solid phase binding assay. 0.5 μg of proacrosin bound a maximum of 32 ng of fucoidan and 0.7 ng of zona glycoproteins (results not shown). Scatchard plot analysis of the binding data yielded a slightly curved function for ^{125}I-fucoidan suggesting the presence on the target protein of several binding sites with different affinities. The apparent K_d for fucoidan was 5.0×10^{-8} M and for ZPGPs it was 1.2×10^{-8} M.

Inhibition of binding of ^{125}*I-fucoidan and* ^{125}*I-ZPGPs by polysaccharides and synthetic polymers*

Preliminary studies using western blotting techniques have suggested that binding of ^{125}I-fucoidan to sperm proacrosin displays some specificity, in the sense that certain polysaccharides can effectively compete with the probe whereas other closely related polymers are unable to do so (Jones and Williams, 1990). To assess quantitatively, under defined conditions, the critical features of the polymer (size, composition, charge density, etc.) that are important for mediating binding of ^{125}I-fucoidan and ^{125}I-ZPGPs to proacrosin, inhibition experiments were carried out with a variety of saccharides, polysaccharides and synthetic polymers. As shown in Fig. 3 and summarized in Table 1, binding of ^{125}I-ZPGPs to proacrosin was inhibited by 'cold' (i.e. non-radioactive) zona glycoproteins, fucoidan, poly(vinyl sulphate), poly(styrene sulphonic acid) and dextran sulphate (M_r 500×10^3 and 5×10^3) but not by chondroitin sulphates A or C, hyaluronic acid, dextran (M_r 500×10^3), glucosamine hydrochloride, poly(vinyl phosphate), glucose-6-sulphate, glucosamine-2,

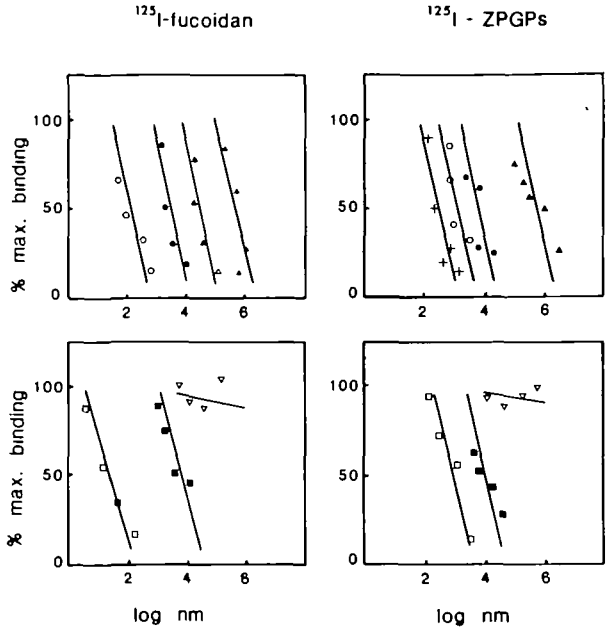

Fig. 3. Inhibition of specific binding of ^{125}I-fucoidan or ^{125}I-ZPGPs to proacrosin by sulphated and non-sulphated polymers. Percentage maximum specific binding is plotted against $\log_{10}$ concentration of competitor. Fucoidan (○); poly(vinyl sulphate) (●); galactan (△); ZPGPS (+); dextran sulphate M_r 500×10^3 (□); dextran sulphate M_r 5×10^3 (■); dextran M_r 500×10^3 (▽).

3-disulphate or any monosaccharide (D(+) glucose, D(+) fucose, L(−) fucose, D(+) mannose, D(+) galactose) or disaccharide (lactose, sucrose) or trisaccharide (raffinose, fucosyllactose) tested. Heparin gave inconclusive results in that although 50% inhibition could be achieved with as little as 2 μM, a reliable dose–response relationship was difficult to obtain. The importance of sulphation and chain length for binding is demonstrated clearly by the dextran/dextran sulphate series. Dextran sulphate M_r 5×10^3 was nearly 10 times less inhibitory than M_r 500×10^3 dextran sulphate while non-sulphated M_r 500×10^3 dextran was unable to compete, even at concentrations as high as 80 mg ml^{-1}. Sodium sulphate (0.2 M) had no inhibitory ability. It should be noted at this point that both the ^{125}I-fucoidan and ^{125}I-ZPGP probes are sulphated (DeAngelis and Glabe, 1987; Shimizu *et al.* 1983; Nakano *et al.* 1990).

Essentially similar results were obtained with inhibition of ^{125}I-fucoidan binding to proacrosin (Fig. 3), the principal differences being that galactan was more effective against this probe while xylan had poor inhibitory activity (Table 1). Some effect was obtained with cold ZPGPs but slopes of inhibition curves were too shallow to obtain an accurate ID_{50} even at the highest concentration tested (1.6 mg ml^{-1} protein).

The above experiments were repeated using total pH3 extracted proteins immobilized on nitrocellulose in place of purified proacrosin. Identical results were obtained to those shown in Fig. 3 in terms of the relative potency of competing agents (results not shown). Assuming that proacrosin contributes to at least 50% of total binding in crude extracts, these results indicate that its purification had not had a deleterious effect on its fucoidan- or zona-binding properties. Furthermore, when qualitative assays were performed on western blots taken from SDS gels containing total pH 3 extracted proteins, binding of ^{125}I-fucoidan and ^{125}I-ZPGPs to both the M_r 67×10^3 and 53×10^3 components were blocked only by 'cold' fucoidan, poly(vinyl sulphate), dextran sulphate (M_r 500×10^3 and 5×10^3), xylan and galactan; $M_r\times10^3$ dextran, chondroitin sulphates A and C and hyaluronic acid were ineffective competitors. These results also reinforce the hypothesis (Jones *et al.* 1988) that the M_r 67×10^3 protein may be related to proacrosin.

Table 1. *Concentration of competitors for 50% inhibition (IC_{50}) of binding of ^{125}I-fucoidan and ^{125}I-ZPGPs to proacrosin*

Competitor (M_r)	IC_{50} ^{125}I-Fucoidan	IC_{50} ^{125}I-ZPGPs
Fucoidan (~100K)	0.1 μM	0.35 μM
Dextran SO_4 (500K)	0.03 μM	1.4 μM
Dextran SO_4 (5K)	8.9 μM	12.0 μM
Dextran (500K)	>160 μM§	>160 μM§
Galactan (~100K)	22.4 μM	>80 μM
Xylan (~10K)	4.3 mM	–
Poly(vinyl sulphate) (~100K)	29.9 μM	7.9 μM
Poly(styrene sulphonate) (~75K)	22.4 μM	12.6 μM
Poly(vinyl phosphate) (~75K)	80.0 μM	>80 μM§
Chondroitin SO_4 A (~30–50K)	>400 μM§	>400 μM§
Chondroitin SO_4 B (~20–30K)	>400 μM§	–
Chondroitin SO_4 C (~30–50K)	>400 μM§	>400 μM§
Hyaluronic acid	>2 mg ml^{-1}§	>2 mg ml^{-1}
ZPGPs	>1.6 mg ml^{-1}§	35.0 μg ml^{-1}§
Monosaccharides*	>400 mM§	>400 mM§
Oligosaccharides†	>400 mM§	>400 mM§
Fucosyllactose	>80 mM§	> 80 mM§
Amino sugars‡	>400 mM§	>400 mM§
Glucose-6-SO_4	>400 mM§	>400 mM§
Glucosamine-2,3-di SO_4	>400 mM§	>400 mM§
Sodium Sulphate	>200 mM§	>200 mM§

* D(+)glucose, L(−) or D(+)fucose, D(+)mannose, D(+)galactose.
† raffinose, sucrose, lactose.
‡ glucosamine, N-acetylglycosamine, galactosamine.
§ where 50% inhibition could not be achieved the values shown are the highest concentrationed tested.
K $\times10^3$.

Effects of ionic strength on binding of ^{125}I-fucoidan and ^{125}I-ZPGPs to proacrosin

Since the above results suggest that the presence and number of negatively charged sulphate groups on fucoidan and ZPGPs are important parameters for binding to proacrosin, the ability of high salt concentrations to perturb the interaction was investigated. It is known that binding of many polyanions to charged matrices is primarily electrostatic in nature, the strength of which is dependent on the ionic strength of the medium (DeAngelis and Glabe, 1987, 1988; 1990). As shown in Fig. 4, at low ionic strength (20 mM sodium phosphate pH7.2), both probes showed appreciable

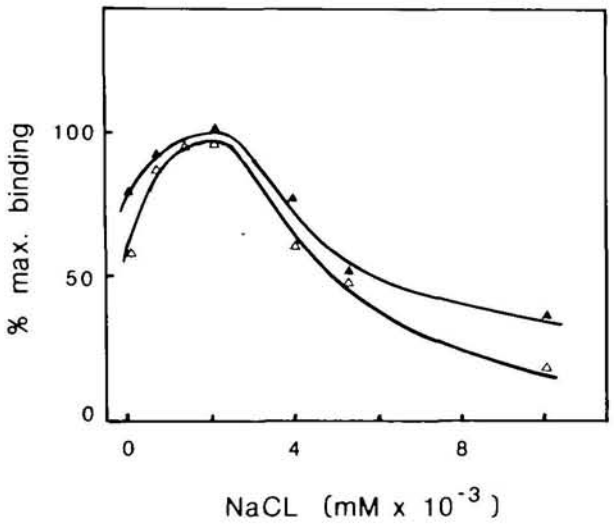

Fig. 4. Effects of ionic strength on specific binding of ^{125}I-fucoidan and ^{125}I-ZPGPs to proacrosin. Iodinated probes were suspended in 20 mM sodium phosphate pH 7.2 to which various concentrations of NaCl were added. Specific binding was measured and expressed as a percentage of the maximum recorded. ^{125}I-fucoidan (△); ^{125}I-ZPGPs (▲).

binding but this increased rapidly to reach a maximum between 150 mM and 200 mM added NaCl and then decreased progressively to low levels at 1.0 M NaCl. ^{125}I-fucoidan binding to proacrosin was half-maximal at 490 mM added NaCl and ^{125}I-ZPGPs binding half-maximal at 595 mM added NaCl. However, if ^{125}I-fucoidan and ^{125}I-ZPGPs were first allowed to bind to proacrosin, then incubation in 1 M NaCl for 30 min displaced only 38 %–48 % of bound probes (results not shown). Between 25 % and 40 % displacement of bound ^{125}I-fucoidan was also obtained with 10 μM 'cold' fucoidan or 3 μM dextran sulphate (M_r 500×10^3) or 6 M urea but not 160 μM dextran (M_r 500×10^3) or distilled water. In total, these results suggest that although electrostatic forces may be involved in the binding of ^{125}I-fucoidan and ^{125}I-ZPGPs to proacrosin other types of bonding must also participate to stabilize the interaction.

Cytochemical labelling of spermatozoa with FITC–ZPGPs

Since the previous experiments had been carried out on proteins immobilized on solid supports, it was important to investigate if FITC–ZPGPs would bind to spermatozoa in suspension and if the interaction was sensitive to inhibition by sulphated polymers. When freshly washed boar spermatozoa were stained with FITC–ZPGPs, fluorescence was observed over the acrosomal domain of only 10 %–20 % of cells. These spermatozoa were judged to be damaged as the same cells were also positive on the acrosome with Rh–PNA; intact spermatozoa (negative with Rh–PNA) did not bind FITC–ZPGPs (Fig. 5). However, if spermatozoa were first permeabilized by cold shock and then stained, >95 % showed fluorescence over the acrosomal domain with both FITC–ZPGPs and Rh–PNA (Fig. 5). No

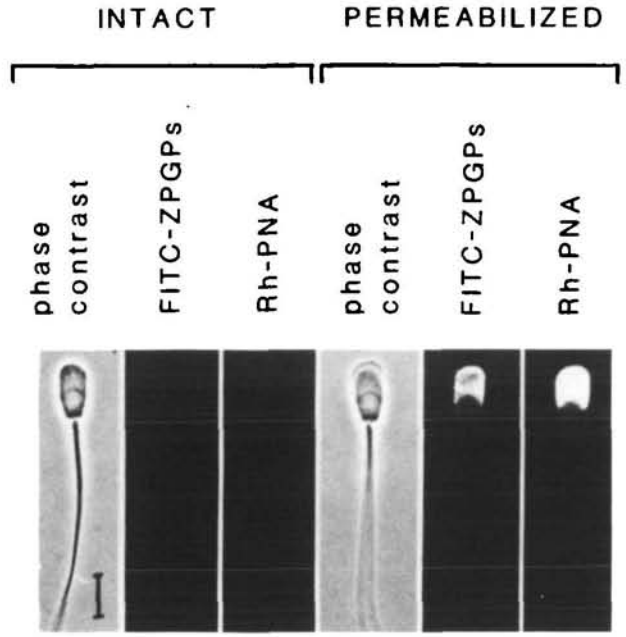

Fig. 5. Cytochemical labelling of spermatozoa with FITC–ZPGPs. Intact or permeabilized (by cold shock) spermatozoa were double labelled in suspension in PBS/0.2 % BSA/2 mM pAB with FITC–ZPGPs and Rh–PNA. Note the ruffled appearance of the acrosome in the permeabilized spermatozoon. Bar=10 μm.

staining was associated with the tail under these conditions. Uptake of the FITC–ZPGPs by permeabilized spermatozoa was reduced to background levels by 10 μM fucoidan or 3 μM dextran sulphate (M_r 500×10^3) or 790 μM poly(vinyl sulphate) but not by 160 mM dextran (M_r 500×10^3) or 0.25 M L(−) fucose or 0.25 M D(+) galactose or 0.25 M lactose or 0.25 M glucose-6-sulphate. Thus, cytochemically, binding of zona glycoproteins to the acrosome of permeabilized spermatozoa takes place in a similar fashion to that found on the solid phase assay system for purified proacrosin.

Discussion

This work has shown that the density and orientation of negatively charged sulphate groups on fucoidan and ZPGPs are critical parameters in mediating recognition and high-affinity binding to boar sperm proacrosin. Superimposed upon these requirements are restrictions of tertiary structure and complementarity with binding sites on the target protein. Therefore, together with its cellular location, these properties strongly support the hypothesis that mammalian sperm proacrosin is biochemically and functionally analogous to bindin from sea urchin spermatozoa. The apparent low specificity of recognition by both proteins would be tolerated by virtue of their intracellular location (within the acrosome) and the fact that they are targeted to their site of action (surface of the egg). Hence, spurious interactions with other cell types are avoided. Species specificity could be imparted on the system by the

requirement for an exact stereochemical 'fit' between aligned sulphate groups on zona glycoproteins and complementary binding sites on homologous proacrosin.

The zona pellucida of mammalian eggs is known to consist of relatively few species of glycoproteins e.g. 3 in the mouse (Bliel and Wassarman, 1980) and 3–4 in the pig and guinea-pig (Hedrick and Wardrip, 1987; Jones and Williams, 1990). In the mouse only one (ZP3) has sperm receptor activity, apparently mediated by terminal galactose or fucose residues on O-linked carbohydrate chains (Bliel and Wassarman, 1987). The nature of the complementary ligand(s) on the surface membrane of mouse spermatozoa remains problematic but Leyton and Saling (1989) have provided evidence for involvement of tyrosine kinases and Bliel and Wassarman (1990) have detected an M_r 56×10^3 glycoprotein from photoaffinity crosslinking studies. This type of binding is clearly different from what we have observed here. First, proacrosin is normally contained within the acrosomal vesicle and only exposed during the acrosome reaction. Thus, it is unlikely to be operative as a primary zona ligand. Second, all 3 of the pig ZPGPs inhibit sperm-zona binding *in vitro* (Berger *et al.* 1989) indicating that receptor activity is not confined to one specific glycoprotein. This may or may not be related to our finding that all 3 ZPGPs also have affinity for proacrosin. The latter point concurs with our previous observations (Brown and Jones, 1987) and the recent data by Nakano *et al.* (1990) that both the M_r $55\alpha+55\beta\times10^3$ and M_r 90×10^3 ZPGP families are sulphated. Nakano *et al.* (1990) have also shown that 60–90 % of bound sulphate can be released from pig ZPGPs by endo-β-galactosidase digestion confirming our view that the carbohydrate moiety forms the 'backbone' structure for poly(sulphate) groups.

The involvement of poly(sulphate) groups in the binding process is shown clearly by the inability of high relative molecular mass (M_r 500×10^3) dextran to inhibit binding of ^{125}I-ZPGPs or ^{125}I-fucoidan probes to proacrosin whereas its sulphated form is very potent in this respect. Since the composition of the competing polymers is very different (e.g. dextran sulphate is a branched polymer of repeating α 1–6 linked glucose units whereas galactan consists of β 1–3 and α 1–4 linked galactose disaccharides; Percival, 1970), it indicates that constituent sugars do not play a direct role in the binding process, except insofar as they provide a repeating polymeric framework. This is demonstrated most clearly by poly(vinyl sulphate), a polymer that lacks any saccharide structure. In the case of ZPGPs, the protease digestion experiments suggest that the polymeric framework is provided primarily by the carbohydrate moiety although the involvement of sulphated amino acids cannot be excluded. Therefore, it might be more accurate to refer to proacrosin as a poly(sulphate) binding protein and not as a fucose- or carbohydrate-binding protein. In passing, it is noteworthy that the $IC_{50\,\%}$ for fucoidan against either probe is close to that for inhibition of sperm binding to pig eggs *in vitro* (i.e. 0.1 μM fucoidan gave 73 % inhibition; Jones *et al.* 1988).

However, it is also apparent that binding is not simply a function of the presence or absence of sulphate groups (glucose-6-sulphate and glucosamine-2,3-disulphate are ineffective competitors), or charge density (chondroitin sulphates, heparin and hyaluronic acid have charge densities equal to or greater than fucoidan; DeAngelis and Glabe, 1987, 1988) or polymer size (although chain length is relevant, e.g. M_r 500×10^3 *versus* 5×10^3 dextran sulphate). Rather, the above parameters appear secondary to the position and alignment of the sulphate groups. A useful precedent illustrating the subtlety of such a recognition system is the binding of heparin to anti-thrombin III (reviewed by Lindahl *et al.* 1986). Heparin consists of an alternating backbone of hexuronic acid (D-glucoronic or L-iduronic acids) and D-glucosamine units joined by 1,4, glycosidic linkages. The anti-thrombin III binding region resides in an internal pentasaccharide containing four-O-sulphates and two-N-sulphates. At least four of these sulphates are required for recognition, especially the O-sulphate on C3 of the central glucosamine. Absence of a sulphate group at this position results in low affinity binding heparin. If a similar situation applies to the interaction of fucoidan and ZPGPs with proacrosin, then it would explain why glycosaminoglycans such as chondroitin sulphates show low affinity binding; their projecting sulphate groups are simply not in the correct spatial configuration.

The role of these charged sulphate groups in the recognition process raises the question of the nature of the primary adhesive forces involved. From a detailed study of the binding of sulphated fucans to bindin, DeAngelis and Glabe (1987, 1988, 1990) concluded that the mechanism was not a simple electrostatic one but required coordinated hydrogen bonding between guanido moieties on arginine residues in the protein and the 3 oxygen atoms of co-planar sulphate groups. The degree of ionization seems important as phosphate groups, whose oxygen atoms have a similar tetrahedral geometry to sulphate, were not inhibitory. Such a mechanism, which would be highly sterically dependent, has been described for the sulphate binding protein from *Salmonella typhimurium* (Pflugarth and Quiocho, 1985) and would explain much of the data for proacrosin. The greater sensitivity to salt inhibition shown by the fucoidan binding to proacrosin relative to bindin (IC_{50}=0.5 M NaCl and 1.2 M NaCl respectively) may be due to the participation of basic residues such as lysines and histidines rather than arginines. It has been shown for example, that fucoidan binding to polylysine or polyhistidine is half-maximal at 0.5 M to 0.75 M NaCl (DeAngelis and Glabe, 1988). The greater resistance to salt shown in the bindin system may be related to the fact that fertilization in sea urchins takes place in sea water (0.5 M–0.6 M NaCl) whereas in mammals oviduct fluid contains only 0.1 M–0.12 M NaCl. Interestingly, there is less than 17 % linear sequence homology between boar proacrosin and sea urchin bindin (Adham *et al.* 1989; Gao *et al.* 1986) suggesting that they have

evolved their poly(sulphate) recognition capacities independently.

Allied to the above problem of the type of binding forces involves, is the number and distribution of sulphate binding sites on the target protein. The finding that 'cold' ZPGPs are poor inhibitors of ^{125}I-fucoidan binding suggests that ZPGP binding sites on proacrosin are more restricted in distribution than fucoidan binding sites, but that the latter overlap the former. Another possibility is that fucoidan inhibits ZPGP recognition by some kind of allosteric mechanism and that the binding sites are completely distinct from each other, bearing in mind that both probes are large molecules. However, the presence of multiple binding sites on proacrosin for fucoidan is supported by Scatchard plot data and by the observation that fucoidan inhibits both amidase and protease activities of β-acrosin (Urch and Hedrick, 1988). These results lend weight to the hypothesis that binding sites for fucoidan are distributed over the surface of the molecule whereas the site(s) for ZPGPs are localized and are more dependent on protein folding. Many examples of the latter phenomenon are known, e.g. substrate and drug binding sites on enzymes. It is difficult to predict where a ZPGP-sulphate 'docking' site might lie but it would seem not to be on the N-terminal 23 amino acid peptide that constitutes the light chain (Töpfer-Petersen and Henshen, 1987) or on the 75 amino acid proline-rich domain at the C terminus. During activation to β-acrosin this proline-rich domain is cleaved by endoproteolysis, yet β-acrosin retains its ability to bind ZPGPs (Urch and Patel, 1991). Latest evidence indicated that ZPGP binding activity is associated with the N-terminal peptide of the heavy chain (Töpfer-Petersen *et al.* 1990). Again an instructive analogy is the heparin binding site on antithrombin III. Fragmentation analysis has shown that the heparin docking site is associated with two S–S linked peptides comprising residues 89–96 and 114–156 (Peterson *et al.* 1987). The lysine residue at position 125 would seem to be especially crucial as shown by site-directed mutagenesis. Tertiary folding of antithrombin III is also important as reduction and alkylation abolishes its capacity to bind to heparin–Sepharose (Ferguson and Findlay, 1983). In total, these results emphasise the importance of complementarity between the orientation of sulphate groups on ZPGPs on the one hand and their docking site on proacrosin on the other. Variations in the stereochemistry of either reactant could conceivably impart species specificity on the system.

Lastly, it should not be construed from the foregoing that we regard proacrosin/acrosin as the only zona ligand in mammalian spermatozoa. Evidence from several laboratories has indicated that there are multiple egg-binding proteins on spermatozoa, e.g. in the mouse, galactosyltransferase (Shur, 1986), tyrosine kinase (Leyton and Saling, 1989), a trypsin-like enzyme (Benau and Storey, 1987) and an M_r 56×10^3 antigen (Bliel and Wassarman, 1990) are all contenders as zona ligands. Such ligands must be present on the surface membrane overlying the sperm head (to account for those species in which acrosome intact spermatozoa bind to the zona, e.g. mouse) and also within the acrosome (to account for those species in which acrosome reacted spermatozoa bind to the zona, e.g. guinea-pig, rabbit). These two levels of recognition are not mutually exclusive and one may override the next depending on the species. A model incorporating these concepts has recently been discussed in detail (Jones, 1990). In this scheme, proacrosin is considered to be a secondary ligand operative after the acrosome reaction. Further work is necessary to establish the behaviour of this molecule on the surface of the zona at the exact point of sperm attachment.

The author records with pleasure numerous stimulating conversations with Dr Bert Urch, UC, Davis, in the course of this work. The assistance of Mr P. Barker (protein sequencing), and Mrs L. Notton and Mrs D. Styles (manuscript preparation) is acknowledged.

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(Accepted 28 January 1991)