Binding of pig sperm receptor in the zona pellucida to the boar sperm acrosome

N. Yonezawa¹, Y. Hatanaka², H. Takeyama¹ and M. Nakano^{1,2}

¹Department of Chemistry, Faculty of Science, and ²Graduate School of Science and Technology, Chiba University, 1–33 Yayoi-cho, Inage-ku, Chiba-shi, Chiba 263, Japan

Pig zona pellucida (ZP) contains three families of glycoproteins: PZP2, PZP3 α and PZP3 β . PZP3 α mediates the binding of the ZP to spermatozoa. In this study, the binding site of pig ZP on boar spermatozoa and the zona-binding proteins of boar spermatozoa were studied using chemically modified zona glycoproteins or anti-pig ZP antiserum. Endo- β -galactosidase-digested PZP3 α (E β G-PZP3 α), which is deficient in sulfated N-acetylpolylactosamine, as well as solubilized ZP, bound to the acrosomal region of acrosome-damaged or partially acrosome-reacted spermatozoa. However, they did not bind to acrosome-intact or fully acrosome-reacted spermatozoa. Solubilized ZP did bind to the acrosomal cap released upon acrosome reaction. In western blot analyses, E β G-PZP3 α bound to the sperm proteins with molecular masses similar to proacrosin–acrosin and the binding was inhibited by fucoidan and anti-pig acrosin antiserum. These results suggest that the binding site of solubilized pig ZP and E β G-PZP3 α on spermatozoa is located mainly in the acrosomal matrix and on the membranous compartments in the acrosome and suggest that E β G-PZP3 α binds to proacrosin–acrosin. The binding of E β G-PZP3 α to proacrosin–acrosin may be involved in the binding of the ZP to the acrosome of partially acrosome-reacted spermatozoa.

Introduction

The zona pellucida (ZP) surrounding mammalian oocytes plays an important role in species-specific recognition between gametes. Pig ZP components can be separated by gel filtration column chromatography into two main fractions of molecular mass 90 kDa and 55 kDa, PZP2 and PZP3, respectively (Nakano *et al.*, 1987). PZP2 is cleaved into 25 kDa and 65 kDa fragments at a specific site upon fertilization (Hatanaka *et al.*, 1992), but its function in gamete recognition has not been determined. PZP3 displays sperm receptor activity and consists of two distinct polypeptides, PZP3 α and PZP3 β (Hedrick and Wardrip, 1987; Yurewicz *et al.*, 1987). Heterogeneities of PZP3 in charge and size are due to the presence of sulfated N-acetyllactosamine repeats in acidic N- and O-linked oligosaccharides. Separation of PZP3 α and PZP3 β is successful only after removal of sulfated N-acetylpolylactosamine by digestion with endo- β -galactosidase (Yurewicz *et al.*, 1987). Therefore, in this study the purified PZP3 α and PZP3 β following endo- β -galactosidase digestion are referred to as endo- β -galactosidase-digested PZP3 α (E β G-PZP3 α) and endo- β -galactosidase-digested PZP3 β (E β G-PZP3 β), respectively. Among the endo- β -galactosidase-digested zona components, only E β G-PZP3 α inhibits sperm–zona binding in a competition assay *in vitro* (Sacco *et al.*, 1989). Although the partial amino acid sequences of E β G-PZP3 β reported by Yurewicz *et al.* (1987, 1992) are very similar to the sequence of the mouse

sperm receptor ZP3, E β G-PZP3 β does not display sperm receptor activity.

The acrosomal status of spermatozoa that bind to ZP varies according to species. Acrosome-reacted spermatozoa bind to ZP in guinea-pigs (Yanagimachi, 1981), whereas in mice and humans, acrosome-intact spermatozoa bind to ZP, which then induces the acrosome reaction (Wassarman, 1988; Liu and Baker, 1990). In pigs the acrosomal status of spermatozoa that bind to the ZP has not been clarified. Solubilized pig ZP has also been reported to induce acrosome reaction in ejaculated spermatozoa, but the increase in the percentage of acrosome-reacted spermatozoa is small (Berger *et al.*, 1989). Jones *et al.* (1988) hypothesized that in pigs, partially acrosome-reacted spermatozoa bind firmly to the ZP and then undergo acrosome reaction. Thus, the mechanisms of sperm–zona binding and initiation of acrosome reaction may vary according to species.

In this study, we investigated the binding site of pig ZP on spermatozoa and the sperm proteins that bind to E β G-PZP3 α , which is deficient in sulfated N-acetylpolylactosamine.

Materials and Methods

Purification of zona glycoproteins

Pig ZP was isolated from ovaries and then solubilized at 70°C for 30 min in distilled water as described by Nakano *et al.* (1990). Solubilized ZP was digested with endo- β -galactosidase (Seikagaku Corp., Tokyo) as described by Yurewicz *et al.* (1987)

Fig. 1. Purification of glycoproteins from pig zona pellucida (ZP). Solubilized pig ZP was digested with endo- β -galactosidase and then fractionated on reverse-phase HPLC. (a) Elution profile of endo- β -galactosidase-digested PZP3 β (E β G-PZP3 β) (peak 1), E β G-PZP3 α (peak 2) and E β G-PZP2 (peak 3). Dashed line represents the acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid (b) SDS-PAGE (11% gel) under nonreducing conditions of peak 1 (lane 1), peak 2 (lane 2) and peak 3 (lane 3) in (a). Molecular weight markers are indicated on left; ft: dye front. (c) SDS-PAGE (10% gel) under nonreducing conditions of fractions on gel filtration column chromatography of solubilized ZP. The PZP2 fractions (lanes 2 and 3) and the PZP3 fractions (lanes 6, 7 and 8) were used in this study. Proteins were stained with Coomassie brilliant blue in both (b) and (c).

and then fractionated into endo- β -galactosidase-digested PZP2 (E β G-PZP2), E β G-PZP3 α and E β G-PZP3 β , by reverse-phase HPLC at 35°C on a Nucleosil 300-7C18 column (4 mm $\times$ 150 mm) (Chemco Scientific Co., Ltd, Tokyo) with three steps of a linear gradient of acetonitrile (0–19 min: 0–37%; 19–49 min: 37–45%; 49–54 min: 45–80%) in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml min⁻¹. E β G-PZP3 β , E β G-PZP3 α and E β G-PZP2 were eluted at concentrations of acetonitrile of about 38%, 40% and 60%, respectively (Fig. 1a). PZP2 and PZP3 were purified from solubilized ZP by gel filtration chromatography as described by Nakano *et al.* (1990). The zona glycoproteins used in this study were homogeneous, as shown in Fig. 1b, c.

Preparation of fluorescein isothiocyanate-labelled proteins

Solubilized ZP, E β G-PZP2, E β G-PZP3 α or E β G-PZP3 β (0.5 mg of each) was incubated with 400 nmol fluorescein isothiocyanate l⁻¹ (FITC) at 25°C for 6 h in 1 ml PBS (for solubilized ZP) or 115 mmol NaHCO₃ l⁻¹, pH 8.5 (for zona components). The reaction was stopped by the addition of glycine. Free FITC was removed by successive overnight dialyses against 10 mmol sodium phosphate l⁻¹ and 300 mmol NaCl l⁻¹, pH 7.0, and then 10 mmol sodium phosphate l⁻¹ and 150 mmol NaCl l⁻¹, pH 7.0 and finally distilled water. FITC-labelled proteins were quantitated by amino acid analysis as described by Nakano *et al.* (1987).

FITC-labelled proteins (10 µg each) were extensively digested with pronase (1 µg) in 0.1 ml PBS at 37°C for 18 h. After adding 3 ml 10 mmol sodium phosphate l⁻¹ and 0.1% SDS, pH 7.0, relative fluorescence intensity to free FITC of known concentration was measured at an emission wavelength of 515 nm, with the excitation at 480 nm. The molar ratios of FITC incorporated into solubilized ZP, EßG-PZP2, EßG-PZP3α and EßG-PZP3β were estimated as 2.2, 6.2, 6.8 and 4.0, respectively.

Cytochemical staining of boar spermatozoa with zona components and peanut agglutinin

Freshly ejaculated spermatozoa or cryopreserved epididymal spermatozoa were washed three times by centrifugation at 800 g for 3 min in 5 ml of culture medium (medium A: 20 mmol Tris l⁻¹, 130 mmol NaCl l⁻¹, 5 mmol KCl l⁻¹, 1 mmol MgCl₂ l⁻¹, 3 mmol CaCl₂ l⁻¹, 5 mmol glucose l⁻¹, 3 mmol sodium pyruvate l⁻¹, 2% (w/v) BSA, pH 7.4) (Peterson *et al.*, 1981) and incubated at 37°C for 1 h for capacitation (sperm concentrations were 10⁸–10⁹ ml⁻¹). The spermatozoa were then incubated with 20 µg FITC-labelled solubilized ZP ml⁻¹ or 50 µg FITC-labelled zona components ml⁻¹ dissolved in medium A for 1 h at 37°C in the presence or absence of 10 µg rhodamine-conjugated peanut agglutinin (R-PNA) ml⁻¹ (sperm concentration was 2 × 10⁷ ml⁻¹). Spermatozoa were washed three times with medium A, fixed with 1.2% (w/v) glutaraldehyde in 150 mmol cacodylate l⁻¹, pH 7.2 (Shams-Borhan and Harrison, 1981) on a cover glass at room temperature for 50 min, washed three times with medium A and mounted onto a slide glass. Samples were examined by phase contrast and fluorescence microscopy on a BH2-QRFL fluorescence microscope (Olympus, Tokyo). The effects of sulfated polysaccharides on the binding of solubilized ZP or EßG-PZP3α to spermatozoa were examined by preincubating spermatozoa in medium A containing 2 mg fucoidan ml⁻¹, 3.2 mg dextran sulfate ml⁻¹ or 10 mg chondroitin sulfate B ml⁻¹ (Sigma, St Louis, MO) for 30 min at 37°C and then incubating with FITC-labelled solubilized ZP or FITC-labelled EßG-PZP3α. The concentrations of polysaccharides were considered to be adequate for the inhibition of the binding of FITC-labelled solubilized ZP to permeabilized spermatozoa (above 1 mg fucoidan ml⁻¹ and 1.5 mg dextran sulfate ml⁻¹; Jones, 1991) as was the concentration of chondroitin sulfate (above 10 mg ml⁻¹; Jones, 1991) for 50% inhibition of binding of solubilized ZP to proacrosin.

Alternatively, capacitated spermatozoa were incubated with 80 µg unmodified solubilized ZP ml⁻¹ or 20 µg each of unmodified zona components (PZP2, EßG-PZP2, PZP3, EßG-PZP3α and EßG-PZP3β) ml⁻¹ instead of the FITC-labelled proteins described above. After washing and fixation, the cover glass was blocked with medium A at 37°C for 1 h. Zona glycoproteins bound to spermatozoa were visualized using anti-pig ZP antiserum diluted to 1:100 with medium A as a first antibody and FITC-conjugated goat anti-rabbit IgG (Wako Pure Chemicals, Tokyo) diluted to 1:1000 as a second antibody. Anti-pig ZP antiserum recognizes all the components of pig ZP (Noguchi *et al.*, 1994).

Detection of zona-binding protein in boar sperm extracts by using anti-pig ZP antiserum or biotinylated EßG-PZP3α

Cryopreserved epididymal spermatozoa were washed and capacitated as described above. The capacitated spermatozoa were washed with PBS once to remove BSA in medium A, suspended in SDS sample buffer (Laemmli, 1970) and heated at 100°C for 3 min. After centrifugation at 2000 g for 3 min, the soluble fraction (10⁴ spermatozoa per lane) was subjected to SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA) as described by Towbin *et al.* (1979). The membranes were blocked with 3% BSA in Tris-buffered saline (TBS), incubated with 2 µg solubilized ZP ml⁻¹ or 1 µg of each of the zona components ml⁻¹ in TBS containing 1% BSA for 3 h at 37°C and then washed three times (for 15 min each time) in TBS containing 0.05% Tween 20 (T-TBS). After incubation with anti-pig ZP antiserum diluted to 1:200 with TBS containing 1% BSA for 2 h at room temperature, the membranes were washed three times (15 min each) in T-TBS and then incubated with peroxidase conjugated anti-rabbit IgG (Wako Pure Chemicals, Tokyo) diluted to 1:1000. After washing, colour was developed with 4-chloro-1-naphthol. Rabbit anti-boar acrosin antiserum (kindly donated by T. Baba, University of Tsukuba, Ibaraki) diluted to 1:1000 was used as a first antibody to detect proacrosin and acrosin.

EßG-PZP3α was biotinylated as described by Töpfer-Petersen and Henschen (1988) and tested as follows. Biotinylated EßG-PZP3α (0.3 µg) was subjected to SDS-PAGE followed by transfer to PVDF membrane. The membrane was blocked as described above and then incubated with streptavidin–peroxidase (Sigma, St Louis, MO) diluted to 1:1000 with TBS containing 1% BSA (w/v) at room temperature for 30 min. After washing, colour was developed with 4-chloro-1-naphthol and biotinylated EßG-PZP3α was strongly stained. Acid extract from freshly ejaculated spermatozoa was prepared as described by Takada *et al.* (1994) and subjected to SDS-PAGE and then transferred to PVDF membrane. The membrane was blocked as described above, preincubated in the presence or absence of anti-acrosin antiserum diluted to 1:400 at room temperature for 1 h. After washing three times (15 min each) with T-TBS, the membrane was incubated with 1 µg biotinylated EßG-PZP3α ml⁻¹ in TBS containing 1% (w/v) BSA at room temperature for 1 h and washed a further three times (15 min each) with T-TBS. Incubation with streptavidin–peroxidase and colour development were performed as described above.

Results

Evaluation of the acrosomal status of boar spermatozoa

It was necessary to evaluate acrosomal status precisely so that the binding site of the ZP on spermatozoa could be examined. We evaluated acrosomal status by combining the fluorescent staining of spermatozoa with R-PNA (Jones, 1991) with the phase contrast observation of spermatozoa fixed with glutaraldehyde in cacodylate buffer (Shams-Borhan and

Fig. 2. Cytochemical staining of boar ejaculated spermatozoa with fluorescein isothiocyanate (FITC)-labelled solubilized zona pellucida (ZP) and rhodamine-conjugated peanut agglutinin (R-PNA). Acrosome-intact (a, b and c), acrosome-damaged or partially acrosome-reacted (d, e and f) and fully acrosome-reacted (g, h and i) spermatozoa were double stained with FITC-labelled solubilized ZP and R-PNA. The acrosomal status of the spermatozoa was evaluated by phase contrast image (a, d and g) and fluorescent staining pattern with R-PNA (c, f and i). Staining with FITC-labelled solubilized ZP is shown in (b), (e) and (h). Scale bar represents 10 μ m.

Harrison, 1981). Acrosome-intact spermatozoa showed a clear line at the apex of the acrosome and were not stained with R-PNA (Fig. 2a, c). Acrosome-damaged or partially acrosome-reacted spermatozoa showed a fuzzy, rippled or swollen acrosome and were stained with R-PNA on the acrosomal region (Fig. 2d, f). The apical ridge of fully acrosome-reacted spermatozoa was absent and the acrosomal region of acrosome-reacted spermatozoa was not stained with R-PNA, but the equatorial segment was sometimes stained (Fig. 2g, i). After capacitation for 1 h, the acrosomes of about 70% of freshly ejaculated spermatozoa and 40% of cryopreserved epididymal spermatozoa were intact.

Analysis of the ZP-binding site on spermatozoa by cytochemical staining with solubilized ZP or ZP components

FITC-labelled solubilized ZP and FITC-labelled E β G-PZP3 α inhibited the sperm-egg binding as effectively as unmodified solubilized ZP and E β G-PZP3 α in a competition assay performed *in vitro* as described by Noguchi *et al.* (1992).

FITC-labelled solubilized ZP bound to the acrosomal region only when the acrosomal region stained positively with R-PNA (Fig. 2e, f). The acrosome region of about 90% of acrosome-damaged or partially acrosome-reacted spermatozoa were stained with FITC-labelled solubilized ZP ($n = 500$). The

Fig. 3. Cytochemical staining of acrosomal cap with fluorescein isothiocyanate (FITC)-labelled solubilized zona pellucida (ZP). Acrosome reaction was induced by incubating ejaculated boar spermatozoa in medium A without BSA and containing 5 μmol ionophore A23187 l^{-1} at 37°C for 1 h, and then incubated with FITC-labelled solubilized ZP. Most acrosomal caps were lost into the supernatant during washings, but several were found and observed. (a) Phase contrast, (b) fluorescent staining with FITC-labelled solubilized ZP.

percentage of acrosome-damaged or partially acrosome-reacted spermatozoa stained with FITC-labelled solubilized ZP and the fluorescence intensity in the acrosomal region were not changed in the absence of Ca^{2+} (data not shown). The staining of acrosome-intact spermatozoa with FITC-labelled solubilized ZP was as faint as that of spermatozoa incubated without FITC-labelled solubilized ZP (Fig. 2b). Staining with FITC-labelled solubilized ZP was also observed in acrosomal caps released upon acrosome reaction (Fig. 3), but not on the inner acrosomal membrane of fully acrosome-reacted spermatozoa (Fig. 2h). When FITC-labelled fetuin or FITC-labelled IgG was incubated with capacitated spermatozoa as a control, the postacrosomal region and midpiece in some spermatozoa were faintly stained, but no significant staining was observed in the acrosomal region.

Approximately 92% of acrosome-damaged or partially acrosome-reacted spermatozoa were stained with FITC-labelled E β G-PZP3 α in the acrosomal region ($n=100$) (Fig. 4e), whereas 33% and 19% of spermatozoa were stained with FITC-labelled E β G-PZP2 and FITC-labelled E β G-PZP3 β , respectively ($n=100$), and the fluorescent intensities were much lower than that with FITC-labelled E β G-PZP3 α . Similar results were obtained by indirect immunofluorescent staining of sperm-bound zona glycoproteins using anti-pig ZP antiserum. In addition, the indirect immunofluorescent staining of sperm-bound PZP3 and PZP2 showed the same pattern on spermatozoa as E β G-PZP3 α and E β G-PZP2, respectively.

Acrosome-intact and fully acrosome-reacted spermatozoa were not stained with solubilized ZP or zona components when R-PNA was omitted from the reaction mixture, indicating that the inability of solubilized ZP or zona components to bind to acrosome-intact and fully acrosome-reacted spermatozoa is not due to the binding of peanut agglutinin to the oligosaccharides of zona glycoproteins or to sperm glycoproteins.

There were no differences in the binding sites of zona glycoproteins of freshly ejaculated and cryopreserved epididymal spermatozoa.

Effects of sulfated polysaccharides on the binding of FITC-labelled solubilized ZP, or FITC-labelled E β G-PZP3 α , to the acrosome

The binding of FITC-labelled solubilized ZP to the acrosomal region of acrosome-damaged or partially acrosome-

reacted spermatozoa was inhibited by fucoidan or dextran sulfate, competitors for the zona-binding of proacrosin, but very little by chondroitin sulfate B (Fig. 4a–d). The binding of FITC-labelled E β G-PZP3 α to the acrosome was also inhibited by fucoidan or dextran sulfate but very little by chondroitin sulfate B (Fig. 4e–h).

Proacrosin and acrosin-binding of E β G-PZP3 α

The zona-binding proteins of capacitated epididymal spermatozoa were detected by immunostaining of zona glycoproteins with anti-pig ZP antiserum. Solubilized ZP, PZP3 and E β G-PZP3 α bound to the sperm proteins of apparent molecular mass 56 kDa, 52 kDa, 42 kDa and 38 kDa (Fig. 5a, lanes 2, 4 and 5). Other than those proteins, E β G-PZP3 α bound to 32 kDa and 18 kDa proteins (lane 5). E β G-PZP3 β did not show significant binding to sperm proteins (lane 7). PZP2 faintly recognized the 56 kDa, 52 kDa, 42 kDa and 38 kDa proteins (lane 8). The binding of solubilized ZP and E β G-PZP3 α to the 56 kDa, 52 kDa, 42 kDa and 38 kDa proteins was reduced in the presence of 1.5 mg fucoidan ml^{-1} (lanes 3 and 6). Moreover, anti-acrosin antiserum recognized the proteins with similar mobilities to the zona-binding proteins (lane 9). The effect of anti-acrosin antiserum on the binding of biotinylated E β G-PZP3 α to the sperm proteins was investigated to determine whether the zona-binding proteins are proacrosin and acrosin. Biotinylated E β G-PZP3 α bound to the 56 kDa, 52 kDa and 42 kDa sperm proteins that were also detected by anti-acrosin antiserum (Fig. 5b, lane 2) and to low molecular mass (20–16 kDa) proteins in the acid extract from freshly ejaculated spermatozoa (lane 3). The binding of biotinylated E β G-PZP3 α to the 56 kDa, 52 kDa and 42 kDa proteins was inhibited by the preincubation of the membrane with anti-acrosin antiserum but the binding to the low molecular mass proteins was not inhibited (lane 4).

Discussion

It was found that solubilized ZP binds inside the acrosome and to the acrosomal cap released upon acrosome reaction. Binding of solubilized ZP to acrosome-intact or fully acrosome-reacted spermatozoa could not be detected by the methods used in the study reported here. Jones (1991) reported that FITC-labelled pig zona glycoproteins do not bind to intact spermatozoa but bind to the acrosomes of permeabilized spermatozoa and the results reported here are consistent with this. These findings suggest that the binding site of the ZP is localized mainly in the acrosomal matrix and on the membranous compartments in the acrosome.

Among the zona components, only E β G-PZP3 α showed significant binding to the acrosome. The binding of solubilized ZP and E β G-PZP3 α to the acrosome was inhibited by fucoidan and dextran sulfate. Western blot analyses of zona-binding proteins suggested that E β G-PZP3 α , as well as solubilized ZP, binds to proacrosin–acrosin, which is a major zona-binding protein of boar spermatozoa. Proacrosin–acrosin may mediate the binding of E β G-PZP3 α , as well as that of solubilized ZP, to the acrosome of partially acrosome-reacted spermatozoa. Jones *et al.* (1988) proposed, taking the binding of proacrosin–acrosin

Fig. 4. Effects of sulfated polysaccharides on the staining of acrosome-damaged or partially acrosome-reacted spermatozoa with fluorescein isothiocyanate (FITC)-labelled solubilized zona pellucida (ZP) or FITC-labelled endo- β -galactosidase-digested PZP3 α (E β G-PZP3 α). Spermatozoa were preincubated in the absence of competitors (a, e) or in the presence of fucoidan (b, f), dextran sulfate (c, g) or chondroitin sulfate B (d, h) and then stained with FITC-labelled solubilized ZP (a–d) or FITC-labelled E β G-PZP3 α (e–h). Scale bar represents 10 μ m.

to ZP into consideration, that in pigs acrosome-intact spermatozoa or spermatozoa in the initial stage of acrosome reaction encounter the ZP. The spermatozoa in an intermediate state between acrosome-intact and fully acrosome-reacted bind firmly to the zona surface. Then, after the spermatozoa remove the acrosomal cap at the surface of the zona, the fully acrosome-reacted spermatozoa penetrate the zona matrix.

When pig eggs were incubated with capacitated spermatozoa and then washed vigorously by pipetting, acrosomal caps were left on the surface of the ZP (Yonezawa *et al.*, unpublished).

A possible reason for the failure to detect the binding of solubilized ZP or E β G-PZP3 α to acrosome-intact or fully acrosome-reacted spermatozoa is that the affinity of the solubilized form of the ZP for acrosome-intact and fully acrosome-reacted spermatozoa is too low to detect the binding. Although solubilized pig ZP induces acrosome reaction in ejaculated boar spermatozoa, the increase in the number of acrosome-reacted spermatozoa is quite low in comparison with that in mice (Berger *et al.*, 1989). We reported that native ZP structure is necessary for the completion of acrosome reaction in a fertilization assay *in vitro* (Yoshizawa *et al.*, 1994). Native ZP structure might be necessary for the binding of the ZP to acrosome-intact spermatozoa.

We also showed that an inhibitor of β -N-acetylhexosaminidase reduces the rate of *in vitro* fertilization in pigs using cumulus-enclosed eggs, but that it changes the rate very little when cumulus-free eggs are used (Takada *et al.*, 1994). β -N-acetylhexosaminidase purified from boar sperm acrosome possesses cumulus dispersion activity (Takada *et al.*, 1994). Although it is not yet clear whether acrosomal enzymes play an essential role in cumulus dispersion in pigs and the

acrosomal status of fertilizing boar spermatozoa on the surface of ZP is not yet known, taking the involvement of proacrosin–acrosin sperm–ZP binding and the involvement of acrosomal enzymes in cumulus dispersion into consideration, we propose that some boar spermatozoa initiate the acrosome reaction before penetration into the cumulus oophorus and disperse cumulus cells by using their acrosomal enzymes, and that partially acrosome-reacted spermatozoa bind to the ZP and that this induces the completion of the acrosome reaction.

The apparent binding of E β G-PZP3 α , which does not contain sulfated *N*-acetylpolylactosamine, to proacrosin–acrosin suggests the involvement of a core region of sugar chains of PZP3 α other than sulfated *N*-acetylpolylactosamine in the binding of the ZP to proacrosin–acrosin. Noguchi *et al.* (1992) reported that neutral sugar chains isolated from PZP3 inhibit sperm–oocyte binding in a competition assay *in vitro*, whereas acidic sugar chains do not. Acidic carbohydrate chains of pig zona glycoproteins are sulfated at the C-6 position of GlcNAc residues in *N*-acetylglucosamine (Noguchi and Nakano, 1992; Hokke *et al.*, 1993). In the study reported here, a sulfated polymer of a neutral sugar, fucoidan and dextran sulfate, inhibited the binding of solubilized ZP or E β G-PZP3 α to the acrosome, whereas a sulfated polymer containing an amino sugar (chondroitin sulfate B) did not inhibit the binding. This finding suggests that the sulfated *N*-acetylpolylactosamine of pig zona glycoproteins is not essential for the species-specific binding of the ZP to spermatozoa, and that if it is involved in binding, it has a subsidiary effect.

The inhibition of proacrosin–acrosin binding of E β G-PZP3 α by fucoidan may result from competition for common binding sites or the hindrance of access of E β G-PZP3 α to its binding site on proacrosin–acrosin. Töpfer-Petersen *et al.* (1990)

Fig. 5. Identification of zona-binding proteins in boar spermatozoa using (a) anti-pig zona pellucida (ZP) antiserum or (b) biotinylated endo- β -galactosidase-digested PZP3 α (E β G-PZP3 α). (a) Solubilized ZP (lanes 2 and 3), PZP3 (lane 4), E β G-PZP3 α (lanes 5 and 6), E β G-PZP3 β (lane 7) or PZP2 (lane 8) were incubated with capacitated epididymal sperm proteins on polyvinylidene difluoride (PVDF) membranes and then the zona glycoproteins bound to sperm proteins were detected with anti-pig ZP antiserum. In lanes 3 and 6, sperm proteins on PVDF membranes were incubated with solubilized ZP (3) or E β G-PZP3 α (6) in the presence of 1.5 mg fucoidan ml⁻¹. Proacrosin and acrosin were detected by anti-acrosin antiserum (lane 9). Anti-pig ZP antiserum and peroxidase-conjugated anti-rabbit IgG antibody did not stain sperm proteins (not shown). Lane 1, protein staining with Coomassie brilliant blue; molecular weight markers are indicated on the left. (b) Biotinylated E β G-PZP3 α was incubated with acid extract from ejaculated spermatozoa on PVDF membranes preincubated in the absence (lane 3) or presence (lane 4) of anti-acrosin antiserum. Lane 1: Coomassie brilliant blue staining; lane 2: immunostaining of proacrosin and acrosin with anti-acrosin antiserum. Arrowheads on the right of lane 4 indicate the protein bands recognized with both anti-acrosin antiserum and biotinylated E β G-PZP3 α . Molecular weight markers are indicated on the left. Biotinylation of E β G-PZP3 α was checked by western blot with streptavidin-peroxidase (lane 6). Lane 5: Coomassie brilliant blue staining of biotinylated E β G-PZP3 α .

reported that the binding site of solubilized ZP was on the amino-terminal 15 kDa part of the heavy chain. One of the important regulatory steps for species-specific recognition between gametes may be binding of ZP to proacrosin-acrosin as reported by Williams and Jones (1993). Studies on the molecular mechanism of binding of E β G-PZP3 α to proacrosin-acrosin would give further insight into the molecular basis of species-specific interaction of gametes.

The authors thank S. Miura and K. Sonohara, Chiba Prefectural Livestock Experiment Station, for kindly supplying the ejaculated boar spermatozoa; and K. Hozumi for technical assistance. This study was supported in part by a Saneyoshi Foundation and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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