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Binding of a 15 kDa glycoprotein from spermatozoa of boars to surface of zona pellucida and cumulus oophorus cells

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Summary. A highly purified 15 kDa glycoprotein isolated from ejaculated spermatozoa was used to raise antisera in female rabbits. An indirect immunofluorescence technique was used to detect the antigen in the seminal vesicle tissue and on the acrosomes of ejaculated, native and capacitated, boar spermatozoa. No immunoreactivity was detected on cells of the seminiferous tubules (spermatogonia, spermatocytes, and spermatids), on spermatozoa in the ductus epididymis and in cells of the epididymal and testicular tissues. These observations support the view that the 15 kDa protein is produced in the seminal vesicle secretory epithelium, and is attached to the sperm plasma membrane during the exposure of spermatozoa to seminal vesicle compounds. The observations that the antigen remained on the acrosome of ejaculated spermatozoa after capacitation and blocked sperm–oocyte binding *in vitro* suggest that the antigen plays a role in sperm–egg interactions. The strong immunoreactivity exhibited by cumulus cells after incubation of antisera with the porcine egg surrounded by cumulus cells shows the possible importance of the 15 kDa glycoprotein for contact of spermatozoa with cells of the cumulus oophorus surrounding the egg.

Keywords: zona pellucida; binding proteins; sperm–egg interaction; gamete recognition; boar; indirect immunofluorescence

Introduction

Gamete interaction has been the subject of intensive research, but the nature and sequence of the reactions in sperm–egg recognition and binding are not fully understood. A frequently used mechanism involves complementary protein–carbohydrate interaction (Macek & Shur, 1988; O'Rand, 1988). The carbohydrate moiety of the glycoproteins of the zona pellucida was determined as the sperm receptors of the zona (see review by Yanagimashi, 1988) that are recognized by the carbohydrate-binding proteins on the sperm surface (Peterson *et al.*, 1983). Significant progress has been made in studying the glycoproteins of the zona pellucida (see review by Wassarman, 1988). The 83 kDa zona pellucida glycoprotein 3 (ZP3) in mice (Bleil & Wassarman, 1980) and a 55 kDa glycoprotein of the zona (Sacco *et al.*, 1984) in pigs have been reported to serve as sperm receptors of the zona. Putative zona-pellucida-binding proteins of spermatozoa have been recognized in various species (O'Rand *et al.*, 1985; Peterson *et al.*, 1985; Jones & Brown, 1987; Töpfer-Petersen & Henschen, 1987; Jones & Williams, 1990; Moos *et al.*, 1990; Hanquing *et al.*, 1991). In boar spermatozoa, the 53 kDa and 33 kDa proteins possessing zona-binding activity were identified as

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acrosin (Jones & Brown, 1987; Töpfer-Petersen & Henschen, 1987) and spermin (Čechová *et al.*, 1990; D. Čechová & E. Töpfer-Petersen, unpublished), respectively. Recently we have searched for low-molecular-mass zona-binding proteins in boar spermatozoa and isolated a 15 kDa glycoprotein with lectin-like activity binding the heat-solubilized zona pellucida (Jonáková *et al.*, 1991).

In the present report, we provide evidence for the presence of this glycoprotein in secretory epithelium of boar seminal vesicle tissue, on the surface of ejaculated, native and capacitated, boar spermatozoa. The reaction of the glycoprotein with the porcine zona pellucida and cumulus cells was demonstrated.

Materials and Methods

Materials

Ejaculated boar, bull and rabbit spermatozoa were collected using an artificial vagina. Both the first and the second phases of normal boar ejaculates were used separately. All treatments involving living boar spermatozoa were performed at 32–37°C. Boar cauda epididymal spermatozoa were obtained from epididymal fluid of slaughtered animals. Spermatozoa were washed free of seminal plasma or epididymal fluid (three times) with phosphate-buffered saline (PBS) followed by centrifugation (5 min at 600 *g*). The erythrocytes were obtained from porcine heparinized blood. The porcine lymphocytes were washed out from the lymph node (submandibular), which was surgically excised and cut out into fragments into PBS. Cell pellets were resuspended in PBS and used for further studies. Porcine ovaries, blood serum and fluids from boar and bull seminal vesicles, prostate and cauda epididymis and tissues from reproductive and other organs of boar were obtained from slaughtered animals, frozen and kept at −70°C. Oocytes stripped from cumulus oophorus and the follicular cells were obtained from thawed mature ovaries using the procedure described by Dunbar *et al.* (1980). Thawed ovaries were homogenized twice using a meat grinder to rupture the follicles and release the oocytes into isolation buffer (10 mmol sodium phosphate l^{-1}, pH 7·4, 125 mmol NaCl l^{-1}, 3 mmol sodium citrate l^{-1}, 2 mmol EDTA l^{-1} and 3 mmol sodium azide l^{-1}). Zona-encased oocytes were recovered by sieving them through nylon screens of decreasing pore size (2000–160 μm) and collecting on a 80 μm screen, and washing with the same buffer. Porcine oocytes with cumulus cells were obtained by puncture of Graafian follicles from thawed mature ovaries (Peterson *et al.*, 1980). Isolation of the 15 kDa sperm glycoprotein (15-SGP) has been described (Jonáková *et al.*, 1991).

Capacitation

Freshly ejaculated boar spermatozoa (1 ml) were diluted with 5 ml of warmed (37°C) sperm buffer (1·5 mmol calcium chloride l^{-1}, 1 mmol magnesium chloride l^{-1}, 95 mmol sodium chloride l^{-1}, 1 mmol dipotassium hydrogen phosphate l^{-1}, 20 mmol Hepes l^{-1}, 5 mmol glucose l^{-1}, 60 mmol sucrose l^{-1}, 5 mmol sodium pyruvate l^{-1} and 0·5% (w/v) bovine serum albumin (BSA), pH 7·4) (Sanz *et al.*, 1992b). After 60 min of incubation at 37°C, 1 ml of swim-up spermatozoa was recovered, diluted 1:1 (v/v) with the same buffer, and centrifuged for 15 min at 180 *g* to remove the seminal plasma. The pellet was resuspended in 10 ml of capacitation medium (1% medium TCM 199 (w:v), 2·3% BSA, 26 mmol sodium hydrogen carbonate l^{-1}, 8 mmol sodium lactate l^{-1}, 1 mmol Hepes l^{-1}, pH 7·4) and incubated for 1 h in a cell culture chamber at 37°C in a 5% CO_2 atmosphere. For an indirect immunofluorescence assay, the spermatozoa were resuspended in the same medium to 2×10^8 spermatozoa ml^{-1}, for sperm–oocyte binding assays to 2×10^6 cells ml^{-1}.

Preparation of antisera and vaginal washings

Antiserum to 15 kDa glycoprotein was prepared by immunizing female rabbits, each with an intracutaneous injection of 1 mg glycoprotein dissolved in 0·1 ml saline mixed with complete Freund's adjuvant. Two months later, rabbits were injected with 200 μg of the antigen in complete Freund's adjuvant. Two weeks after the last injection, the animals were bled through the ear vein. Vaginas of the animals were rinsed with 5 ml of PBS (pH 7·2) to collect vaginal washings. Antiserum and vaginal washings were heat-inactivated for 30 min at 56°C. Organ specificity was checked by preabsorption of antisera to 15 kDa glycoprotein with tissue homogenates of porcine liver, kidney and spleen at a 1:1 (v:v) ratio at 37°C for 1 h, followed by overnight incubation at 4°C. The decrease in titre was checked by ELISA and immunofluorescence.

Enzyme-linked immunosorbent assay (ELISA)

To detect the glycoprotein in blood serum, follicular fluid, seminal plasma and reproductive tract fluids, wells in microELISA plates were primed overnight at 4°C with antigens (1 μg per well) in PBS. After extensive washing (PBS,

1% BSA, 0·05% Tween 20), either vaginal washings or antisera (serial dilutions in PBS-Tween) were added to each well and the plates were incubated at 22°C for 1 h. After thorough washing, the 15 kDa glycoprotein antibodies were reacted with goat anti-rabbit globulin coupled to peroxidase and washed again and the bound peroxidase activity was detected using *o*-phenylene diamine and hydrogen peroxide as substrate. The absorbance was determined at 495 nm using an RM ELISA reader (Dynatech AG, Branch Germany, Denkendorf, Germany). For sperm ELISA (Isojima *et al.*, 1987), the microELISA plates were filled with 50 µl of a suspension of washed boar, rabbit or bull ejaculated spermatozoa (6×10^6 cells ml^{-1}), air dried at room temperature and incubated overnight at 4°C. Protein binding sites of the wells were blocked by blocking buffer (PBS, 1% BSA, 2% normal porcine serum) for 1 h at 22°C. After washing with PBS, 50 µl of serially diluted antiserum to glycoprotein was added to each well. The plates were incubated for 1 h at 22°C. The same procedure was followed as for the detection of the liquid antigens by ELISA.

Fluorescence labelling assays

The indirect immunofluorescence technique was applied to detect the 15 kDa glycoprotein in slices (3 µm thick) of frozen porcine tissues of seminal vesicles, ampullae, vas deferens, head, corpus and tail of epididymis, testes, prostate, urethra, Cowper's gland, liver, kidney, heart, spleen, intestines, lungs, lymphatic gland and muscle. Ejaculated spermatozoa of boar (the first and the second phase of ejaculates), bull or rabbit, boar capacitated and cauda epididymis spermatozoa were smeared on clean glass slides and air dried. The slides were used for assay either directly, or fixed in absolute methanol (30 min) and redried. Cell smears and porcine frozen tissue sections were incubated with a drop of antiserum preabsorbed with the tissue homogenate and diluted 1:20 with PBS for 1 h at 22°C. After washing (0·2% BSA in PBS), porcine anti-rabbit globulin coupled with fluorescein isothiocyanate (FITC) diluted 1:15 was used as the second incubation medium. Normal rabbit serum and antiserum to 15 kDa glycoprotein preabsorbed with the same compound (4 mg ml^{-1}) were used as controls. The slides were observed under the dark-field illumination with an Orthoplan-Leitz microscope (Westlar, Germany) equipped with a halogen-quartz lamp using an FITC interference filter. Living spermatozoa were incubated (10^5 cells ml^{-1}) in tubes with antiserum (1:20 in PBS) for 30 min. Spermatozoa were washed three times (0·2% BSA in PBS) and treated with FITC-conjugated anti-rabbit IgG. A drop of the suspension was applied on a glass slide and examined as described previously.

Coating of 15 kDa glycoprotein on cumulus cells

Porcine oocytes surrounded by cumulus cells were incubated for 30 min at 37°C in PBS containing 200, 100, 50, 10 or 5 µg of glycoprotein ml^{-1}. After washing (PBS, 0·2% BSA), oocytes were incubated for 1 h at 22°C with antiserum to glycoprotein diluted 1:20 in PBS. The isolated porcine erythrocytes and lymphocytes used as a control were treated in the same manner. The procedure described for detection of the spermatozoan antigens by immunofluorescence followed.

Further immunological tests

The sperm agglutination test was performed with ejaculated boar spermatozoa. The semen sample was diluted to 4×10^7 cells ml^{-1}. A volume of 0·3 ml of different dilutions of either vaginal washings or antisera to glycoprotein was mixed with 0·1 ml of sperm suspension and incubated for 2 h at 37°C. In the sperm-immobilization test, the rabbit serum absorbed with three times washed ejaculated boar spermatozoa served as complement. To 0·25 ml of either antiserum or vaginal washings, a mixture of 0·1 ml of boar spermatozoa (2×10^5 cells) and 0·03 ml of complement was added. The control was the inactivated normal rabbit serum and vaginal washings from nonimmunized rabbits. Immunoelectrophoresis was carried out according to Williams (1971).

Sperm–oocyte binding inhibition assays

Oocytes were freshly prepared from ovaries, washed and resuspended as described above. About 50 washed oocytes in 100 µl PBS, pH 7·4, were incubated with either 100 µl PBS or 100 µl of a 0·4 or 0·8 mg ml^{-1} solution of purified 15 kDa glycoprotein in PBS, pH 7·4, for 1 h at 37°C in a 5% CO_2 atmosphere. The protein-incubated oocytes were then washed, by transferring them successively to 100 µl of PBS, pH 7·4. Protein-incubated and PBS-incubated oocytes were then mixed with 100 µl of the suspension of capacitated spermatozoa prepared as described previously and incubated for 1 h at 37°C, as above. After washing the oocytes with PBS (by pipetting) to remove loosely adhering spermatozoa, an aliquot of each suspension was mounted on slides and examined using a phase-contrast microscope.

Results

Preparation and characterization of antisera

To obtain an antiserum against the 15 kDa glycoprotein, rabbits were immunized with a highly purified protein sample showing only one amino-terminal sequence (HAQN). In the reaction of

rabbit anti-15 kDa glycoprotein sera with the antigen solution (0·5%), one precipitation line was detected by immunoelectrophoresis (Fig. 1). The same precipitation line was visible during the reaction of the antisera with boar seminal vesicle fluid. No reaction of the antiserum with the porcine testicular, cauda epididymal and follicular fluid was observed. The reactions with bovine cauda epididymal, ampullar, follicular and seminal vesicle fluids were also negative. In ELISA, the antiserum titre in the reaction with the antigen was 1:800 000. The sperm agglutination test of the antiserum was positive at a titre of 1:128; the sperm immobilization test was negative. The antigen used for immunization was so antigenic that the antibodies against 15 kDa glycoprotein were detected in vaginal washings of the immunized females. It was demonstrated by sperm agglutination with vaginal washings (titre 1:32). Positive reaction of the vaginal washings was proved by a positive reaction in ELISA and by immunoelectrophoresis.

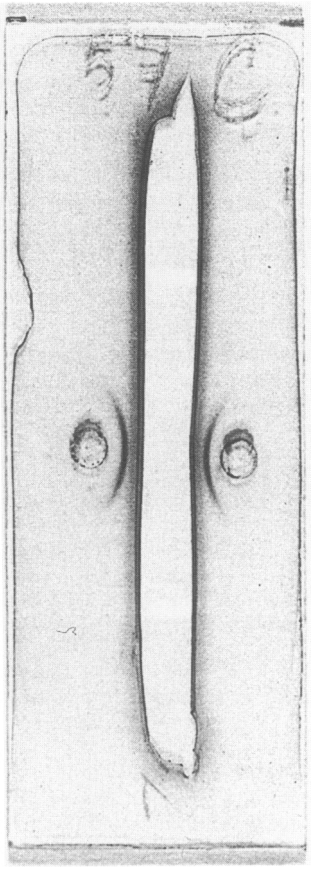

Fig. 1. Immunoelectrophoretic pattern of the reaction of anti-15 kDa glycoprotein sera from boar with homologous antigen (0·5%) solution.

Antigen localization

In ELISA, the antiserum titre was 1:400 000 in the reaction with boar seminal vesicle fluid diluted 1:30 with PBS and 1:13 000 with washed boar ejaculated spermatozoa (1×10^6 cells ml^{-1}) (Table 1). No reaction with boar cauda epididymal spermatozoa was found. The reaction with all other fluids and cells tested was also negative (see Table 1).

When boar ejaculated spermatozoa were labelled with antiserum against 15 kDa glycoprotein, spermatozoa displayed a mostly uniform fluorescence pattern, solely at the sperm head (Fig. 2a). Living, air-dried, unfixed and air-dried, methanol-fixed spermatozoa all exhibited the same pattern when reacted with antiserum. The reaction of antiserum with boar cauda epididymal spermatozoa and with the first spermatozoan fraction of the ejaculate (the spermatozoa not contaminated with seminal vesicle fluid) was negative. No reaction was found with smeared bull and rabbit spermatozoa.

Table 1. Results of ELISA and immunofluorescence assay of antiserum to 15 kDa glycoprotein with spermatozoa, reproductive fluids and tissue sections of boar

Sample	ELISA	Immunofluorescence
Spermatozoa		
Ejaculated, living	n.t.*	+
Ejaculated, air dried	+	+
Capacitated	n.t.	+†
Epididymal	–	–
Fluids		
Seminal vesicle	+	n.t.
Epididymal	–	n.t.
Ampullar	–	n.t.
Blood serum	–	n.t.
Tissue of		
Seminal vesicle	n.t.	+
Prostate	n.t.	–
Epididymis	n.t.	–
Testis	n.t.	–

*n.t.: not tested.
†65–80% of the cells.

Capacitated spermatozoa in smears, both fixed and unfixed, and the living ones exhibited fluorescence labelling after treatment with anti-15-kDa glycoprotein sera (Fig. 2b) in the 65–80% cells.

After application of the antiserum to tissue sections, strong fluorescence of the secretory epithelium in boar seminal vesicle tissue was detected (Fig. 2c). The control with a normal serum was negative. No fluorescence was observed when the antiserum was applied to any other tissue tested (Table 1).

Reaction of the 15 kDa glycoprotein with porcine oocytes and cumulus oophorus cells

After the treatment of the egg with 200, 100 or 50 μg of 15 kDa glycoprotein ml^{-1}, the cumulus cells reacted positively with antiserum to the antigen in indirect immunofluorescence (Fig. 2d). The reactions of the cumulus cells with 10 or 5 μg of 15 kDa glycoprotein ml^{-1} or with cumulus cells not incubated with the antigen were negative. Reactions with normal rabbit serum or with antiserum absorbed with glycoprotein were also negative. If the egg without cumulus cells was used, there was a negligible reaction with the antiserum. As a control, we used porcine erythrocytes and lymphocytes. These nonreproductive cells pretreated with the glycoprotein did not react in immunofluorescence with homologous antiserum. To ascertain whether 15 kDa glycoprotein binds to the zona pellucida, we tested the ability of the glycoprotein to block the sperm binding to oocytes. Oocytes were incubated either in the absence or presence of glycoprotein (two different concentrations were used), washed and then incubated with capacitated spermatozoa for 2 h. After washing, the oocytes were examined under a microscope. In the control group, spermatozoa

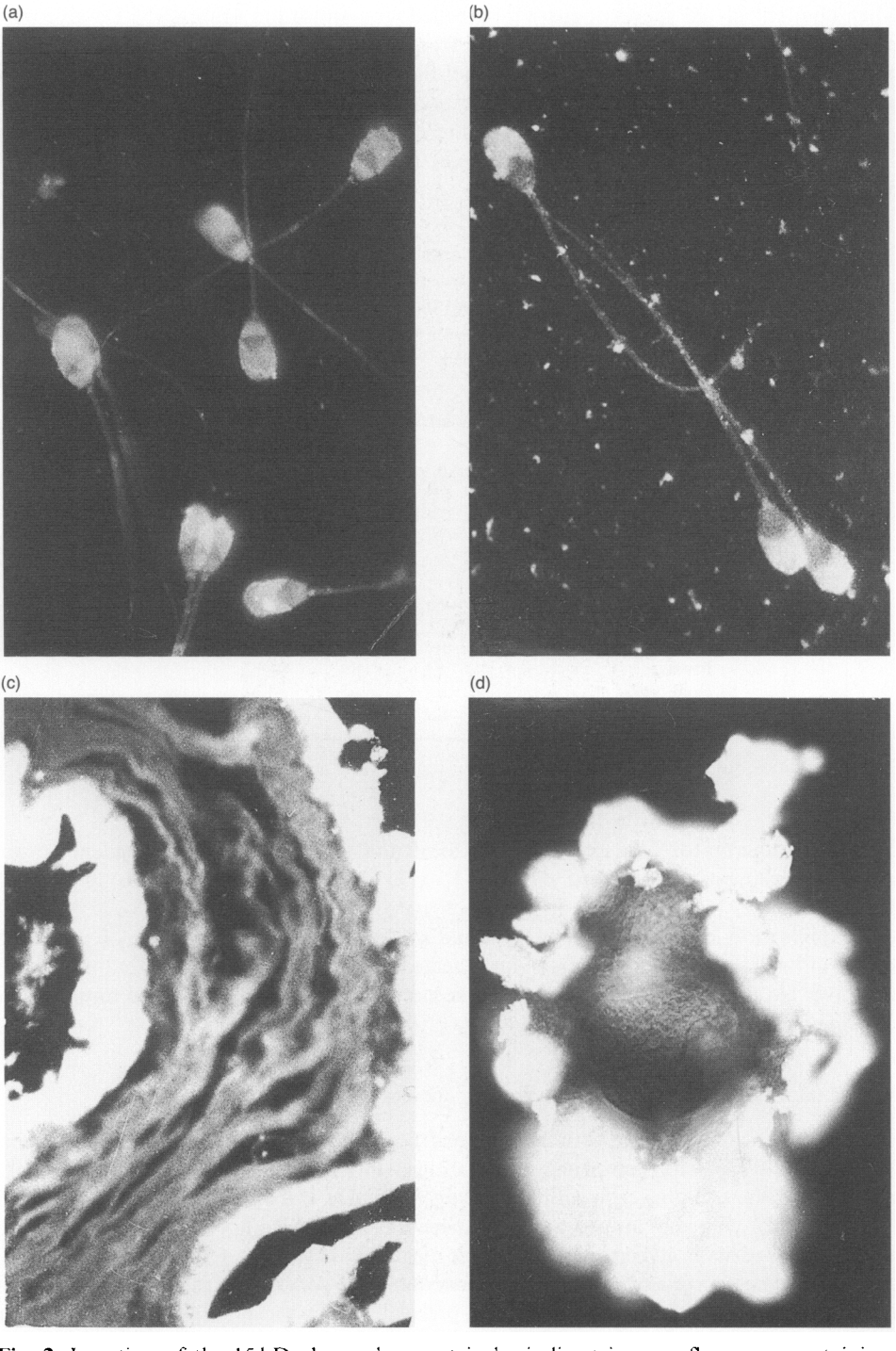

Fig. 2. Location of the 15 kDa boar glycoprotein by indirect immunofluorescence staining: (a) unfixed ejaculated spermatozoa (× 600), (b) methanol-fixed capacitated spermatozoa (× 600), (c) reaction of antisera to 15 kDa glycoprotein with tissue section of boar seminal vesicle (× 200) and (d) porcine zona pellucida surrounded by cumulus cells preincubated with 0·1 mg 15 kDa glycoprotein ml^{-1} solution before reaction with antisera (× 200).

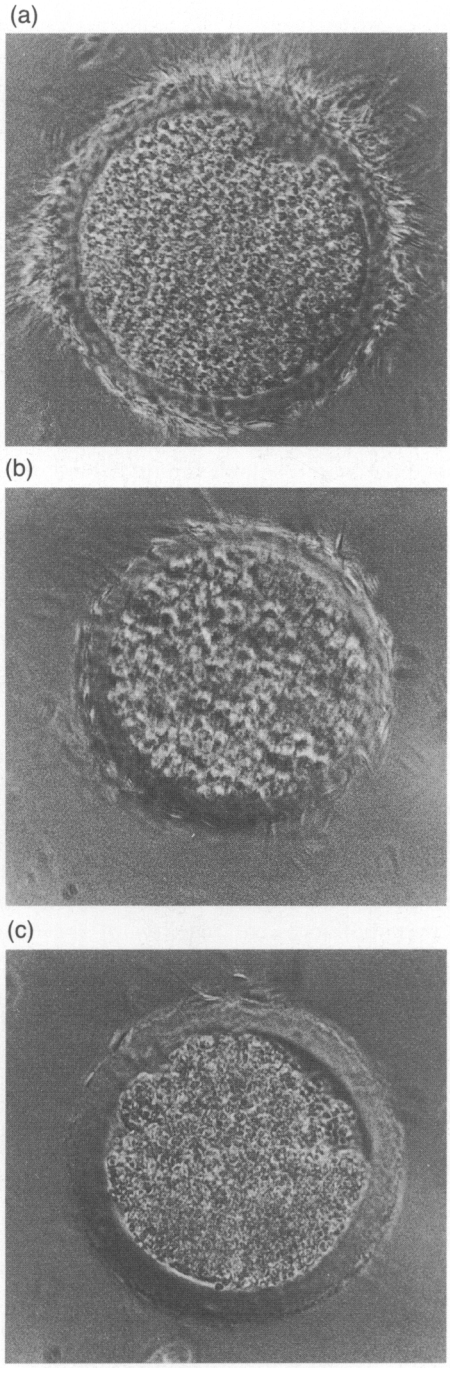

Fig. 3. Sperm–oocyte binding inhibition assays. Freshly isolated porcine oocytes were preincubated in the absence or presence of the 15 kDa boar glycoprotein and then coincubated with capacitated spermatozoa. After washing, the oocytes were examined with a phase microscope: (a) control, without antigen, (b) oocytes preincubated with 0·2 mg antigen ml^{-1} and (c) oocytes preincubated with 0·4 mg antigen ml^{-1}.

were bound to oocytes, whereas the oocytes pretreated with 15 kDa glycoprotein showed weak or no sperm binding, depending on protein concentration (Fig. 3).

Discussion

A zona pellucida-binding protein of low molecular mass has been found in sperm extracts of all mammalian species tested (O'Rand *et al.*, 1985; Töpfer-Petersen *et al.*, 1985; Jones & Brown, 1987; Jones & Williams, 1990; Moos *et al.*, 1990; Hanquing *et al.*, 1991). We have isolated a 15 kDa glycoprotein with lectin-like activity from washed ejaculated boar spermatozoa (Jonáková *et al.*, 1991). The glycoprotein possessed zona pellucida-binding activity that was inhibited by fucoidan. The *N*-terminal amino acid sequence showed a homology with bindin – a major protein of the sea urchin sperm acrosome, suggesting that the 15 kDa glycoprotein, like bindin, might belong to the sperm–egg recognition-mediating protein family.

The zona-binding protein of spermatozoa would be expected to be located on the sperm surface. However, no information was available about where the protein is on the plasma membrane of ejaculated intact spermatozoa as is, for example, the rabbit sperm membrane antigen (O'Rand, 1988), or whether it is on the inner acrosomal membrane, as is bindin, which is present in the sea urchin sperm acrosomal vesicle and functions on the sperm surface after the acrosome reaction (Glabe, 1985). More information was obtained about the distribution of the glycoprotein on boar spermatozoa by preparing highly specific antibodies against the 15 kDa glycoprotein and the antigen was detected on sperm cells and in various body fluids and tissues. A positive reaction occurred when ejaculated boar spermatozoa were tested. The antiserum reacted with the acrosome of both viable and smeared (unfixed and fixed) spermatozoa. The results confirmed that the 15 kDa sperm glycoprotein is distributed on the surface of the sperm plasma membrane covering the acrosome, as is the rabbit sperm membrane antigen (RSA-1) (O'Rand, 1988).

We attempted to determine the origin of the glycoprotein. It was demonstrated that the most extensively studied low-molecular-mass antigen of RSA-1 (O'Rand, 1988) was synthesized in testes (Welsh *et al.*, 1986). The 15 kDa glycoprotein is not a testis lectin: no immunoreactivity was detected on cells of the testicular seminiferous tubules (spermatogonia, spermatocytes and spermatids). Moreover, the immunofluorescent reaction of the testicular tissue was negative. Since neither epididymal spermatozoa (and spermatozoa from the first phase of the ejaculate) nor epididymal tissue gave a positive reaction with serum against 15 kDa glycoprotein, we concluded that the glycoprotein was not synthesized in the epididymal tissues. However, a positive reaction was found with the fluid and the epithelium of the boar seminal vesicle. We therefore concluded that the 15 kDa glycoprotein is synthesized by epithelial cells of the boar seminal vesicle, and that the glycoprotein is one of the major polypeptides that originate from the seminal vesicles and get into the epididymal spermatozoa during ejaculation. In addition, the well-studied, heparin-binding proteins that adhere to epididymal spermatozoa are mainly the products of the seminal vesicles (see review by Miller & Ax, 1990).

The fact that the 15 kDa glycoprotein remained attached to the membrane, even after capacitation, emphasizes the role of this glycoprotein in sperm–zona pellucida recognition events, and that it belongs to the recently described spermadhesins (Sanz *et al.*, 1992a, b). The finding that the 15 kDa glycoprotein was absent from epididymal spermatozoa and from spermatozoa in the first fraction of the ejaculate indicates its supporting role in fertilization rather than an essential one.

The reaction of the 15 kDa glycoprotein with the zona pellucida was confirmed by incubating the pig ovum with the glycoprotein. We used eggs both with cumulus cells and with cumulus cells stripped off. We found that the cumulus cells had a strong affinity for the protein. The immunofluorescent reaction was so intense that detection of the protein attachment to the zona pellucida was problematic. From our previous work, we knew that the protein possessed zona-

binding activity. To confirm the reaction of the protein with the zona pellucida, we used a test of inhibition of egg–sperm interaction. The results were very convincing. The egg that had been pre-treated with the protein did not show any reaction with boar spermatozoa. We therefore conclude that not only the cumulus cells but also the zona pellucida bind the protein to their surfaces. We found that the glycoprotein is not bound to other cell types, such as erythrocytes or lymphocytes. However, the surface of these cells is different from that of the cumulus cells, which are embedded in a matrix of hyaluronic acid; the possibility that cumulus cell can bind other small glycoproteins or lectins equally well cannot therefore be excluded. Recently we have reported (Sanz *et al.*, 1992a) that the 15 kDa protein belongs to the spermadhesin protein family (Sanz *et al.*, 1992b), whose members, although not possessing detectable enzymic activity, show features of serine proteinases and may be involved in both sperm capacitation and sperm–egg recognition and binding events (Sanz *et al.*, 1992a).

Whereas most of the early studies have indicated involvement of the oocyte in the formation of zona pellucida components, the role of the follicle cells on zona compounds remains less clear. It has been postulated that, at least in mice, zona proteins are exposed solely in a oocyte-specific manner (see review by Wassarman, 1988).

Available evidence suggests that follicle (granulosa) cells are involved in the synthesis of zona pellucida material (see review by Sacco, 1990). Some zona proteins were produced by *in vitro* cultured granulosa cells (Maresh & Dunbar, 1987; Timmons *et al.*, 1990). Like these proteins, a sperm receptor of zona that interacts with 15 kDa glycoprotein might be synthesized in cumulus cells and then transferred to the zona pellucida.

It is well known that sperm receptors are present on the zona pellucida. However, the possible presence of sperm receptors also on cumulus cells cannot be ignored. Fully mature eggs released from the ovary are surrounded by cumulus oophorus cellular mass (see review by Yanagimachi, 1988). The viscoelastic extracellular matrix of the cumulus oophorus is the first layer with which the spermatozoon comes in contact. It is possible that the 15 kDa glycoprotein located on the intact ejaculated sperm surface helps the spermatozoa to find the egg surrounded with cumulus cells. Active movement and hyaluronidase digestion may make the path of the cell through the hyaluronic-acid-rich cumulus oophorus to the zona pellucida surface.

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