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Participation of the 39-kDa glycoprotein (gp39) of the vitelline envelope of *Bufo arenarum* eggs in sperm–egg interaction

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Summary

The acquisition of egg fertilizability in *Bufo arenarum* takes place during the oviductal transit and during this process the extracellular coelomic envelope (CE) of the eggs is converted into the vitelline envelope (VE). It has been stated that one of the necessary events leading to a fertilizable state is the proteolytic cleavage of CE glycoproteins in the oviductal pars recta by oviductin, a serine protease. Consequently, there is a marked increase in the relative quantity of glycoproteins with 39 (gp39) and 42 kDa (gp42) in the VE. In the present study, sperm–VE binding assays using heat-solubilized biotin-conjugated VE glycoproteins revealed that both gp39 and gp42 have sperm binding capacity. According to this result, our study was focused on gp39, a glycoprotein that we have previously reported as a homologue of mammalian ZPC. For this purpose, rabbit polyclonal antibodies against gp39 were generated at our laboratory. The specificity of the antibodies was confirmed with western blot of VE glycoproteins separated on SDS-PAGE. Immunohistochemical and immunoelectron studies showed gp39 distributed throughout the width of the VE. In addition, immunofluorescence assays probed that gp39 bound to the sperm head. Finally, as an approach to elucidate the possible involvement of gp39 in fertilization, inhibition assays showed that pretreatment of eggs with antibodies against gp39 generated a significant decrease in the fertilization rate. Therefore, our findings suggest that gp39, which is modified by oviductal action, participates as a VE glycoprotein ligand for sperm in *Bufo arenarum* fertilization.

Keywords: Fertilization, Oviductin, Sperm–egg interaction, Vitelline envelope, ZPC

Introduction

The vitelline envelope is an extracellular coat that surrounds amphibian eggs. This envelope is composed of evolutionarily conserved glycoproteins (Spargo & Hope, 2003), which are biosynthesized and deposited by growing ovarian oocytes during oogenesis (Yamaguchi *et al.*, 1989; Cabada *et al.*, 1996). Like its homologous structure in mammals (zona pellucida, ZP), the vitelline envelope fulfills a significant set of biological functions in amphibians. They include protection of eggs and embryos in early development, modulation of sperm–egg interaction during fertilization, block to polyspermy and participation in the hatching process of the embryo (reviewed in Miceli & Cabada, 1998; Hedrick, 2008).

Numerous reports have provided evidence that the compositional, structural and physicochemical properties of the amphibian egg envelope change during the passage of eggs through the first portion of the oviduct (pars recta, PR) (reviewed in Miceli & Cabada, 1998; Hedrick, 2008). Consequently, the envelope is named differently, according to its structural and molecular state: (1) before passing the PR, when the egg is in the coelomic cavity of the animal, it is called coelomic envelope (CE); and (2) after passing the oviduct and when it is deposited, it is specifically called vitelline envelope (VE). In the first case, eggs surrounded by the CE can be collected from the

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coelom of a female after ligation of the oviducts and subsequent induction of ovulation with homologous hypophysis homogenate (Miceli *et al.*, 1978; Hedrick and Hardy, 1991). When these eggs are inseminated *in vitro*, sperm does not bind to the envelopes and they are not susceptible to sperm lysin. Consequently, the CE is impenetrable to sperm and eggs cannot be fertilized. On the other hand, eggs that have crossed the oviduct or have been treated *in vitro* with an extract or with the secretion fluid of the PR, acquire sperm binding activity (sperm binds to the envelope) and reach a fertilizable state (Miceli *et al.*, 1978; Takamune *et al.*, 1986; Bakos *et al.*, 1990; Katagiri *et al.*, 1999).

One critical factor that leads to acquisition of egg fertilizability is the ultrastructural change of the egg envelope, together with limited hydrolysis of its constitutive glycoproteins, a process regulated by oviductin, a multidomain proteolytic enzyme with trypsin-like activity and exclusively synthesized and secreted in the PR (Lindsay *et al.*, 1999; Hiyoshi *et al.*, 2002; Barrera *et al.*, 2010). The CE is composed of interconnected fibril bundles that form a mesh, with little space between them and arranged perpendicularly and parallel to the surface of the egg. After oviductin action, expansion and diffusion of the fibril bundles could be observed in the VE with a decrease in space between them (reviewed in Hedrick & Nishihara, 1991; Llanos *et al.*, 2006). The CE in *B. arenarum* is composed of eight major glycoproteins: gp122, gp84, gp82, gp55, gp48, gp42, gp39 and gp34, in which the numbers refer to their apparent molecular weight after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Llanos *et al.*, 2006). Comparison of the CE and VE protein patterns revealed that oviductin selectively hydrolyzes gp84 and gp55 of the CE, increasing the relative quantity of gp39 (2.3-fold) and gp42 (1.2-fold), and decreasing the relative quantity of gp48 (4.5-fold) in the VE of fertilizable eggs. The effect of oviductin on the egg envelope has also been examined in other amphibian species. In *Xenopus laevis*, the CE is formed by gp120-112, gp69, gp64, gp43 and gp37 glycoproteins (Gerton & Hedrick, 1986). In this species, oviductin cleaves gp43 of the CE, transforming it into a 'mature' form of 41 kDa (gp41) in the VE (Hardy & Hedrick, 1992; Kubo *et al.*, 1999). SDS-PAGE showed that CE from *B. japonicus* is composed of gp112, gp61–65 and gp40–52 glycoproteins (Takamune *et al.*, 1987). Proteolytic processing of the envelope involves proteolysis of CE glycoproteins gp40–52 and a concomitant increase in a 39-kDa glycoprotein (gp39) and the appearance of another component of 36 kDa (gp36) in the VE (Takamune & Katagiri, 1987).

Based on cDNA sequence analysis, *Xenopus* gp43 has been identified as a mammalian ZPC homologue (Kubo *et al.*, 1997; Yang & Hedrick, 1997). In relation to this, ZPC (gp41) after proteolytic processing possesses the highest sperm binding activity in the VE (Vo & Hedrick, 2000), which has been associated with its *N*-linked glycans with terminal *β*-*N*-acetylglucosamine residues (Vo *et al.*, 2003). In the VE of *B. japonicus*, the gp36–39 components are involved in sperm binding, which is thought to be related to their *N*-acetylgalactosamine and/or sialic acid residues (Omata & Katagiri, 1996). Coincidentally, the VE components of both species involved in sperm binding to the VE are oviductin-modified glycoproteins. Therefore, it has been suggested that proteolytic processing of glycoproteins in the envelope could expose existent sperm binding sites through conformational transformation from the CE into VE (Miceli *et al.*, 1980; Omata & Katagiri, 1996; Katagiri *et al.*, 1999; Vo & Hedrick, 2000).

The molecular principle of the conversion of CE into VE and the acquisition of sperm binding activity in *B. arenarum* are less known. It has been shown that when sperm was incubated with glycoproteins extracted from the VE prior to *in vitro* insemination, the fertilization rate significantly decreased (Valz-Gianinet *et al.*, 1991). These authors suggested that the VE molecules would be able to recognize specific sites on sperm and block fertilization. Moreover, *in vitro* sperm adhesion assays using solubilized egg envelopes showed that VE glycoproteins were able to bind to sperm, whereas sperm binding to CE glycoproteins was not observed (Llanos *et al.*, 2006). It seems likely that *B. arenarum* gp39 could be a putative homologue of the 39-kDa glycoprotein in *B. japonicus*, which is involved in sperm binding to the VE (Omata & Katagiri, 1996). Sequence analysis of three peptides obtained from *B. arenarum* gp39 showed 100% identity with internal sequences of a *B. arenarum* ZPC precursor (Llanos *et al.*, 2006). Bearing in mind that mammalian ZPC glycoproteins have long been associated with sperm binding to ZP (reviewed in Wassarman & Litscher, 2008), this result indicates that gp39 could be a plausible mediator of sperm binding to the VE in *B. arenarum.*

The important increase in gp39 molecules in the VE accompanied by the acquisition of egg fertilizability and its evolutionary relationship with ZPC glycoproteins, made us hypothesize that gp39 is involved in sperm binding during the fertilization process. To confirm this hypothesis, assays for sperm–VE interaction were developed using total biotin-conjugated glycoproteins. Polyclonal antibodies against isolated gp39 were generated to identify and localize gp39-recognition sites on the sperm surface. Finally, biological participation of gp39 in sperm–VE binding was evaluated by analyzing the inhibitory effect on fertilization of the antibodies generated against gp39.

Materials and methods

Experimental animals

Sexually mature female and male specimens of *Bufo arenarum* (Anura: Bufonidae) toads were collected in Tucumán, Argentina, between May and October, and they were kept in a moist chamber at 20–22°C. Animal maintenance and experimental procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (European Communities Council Directive, 1986).

Gamete collection

A total of 16 animals (eight females and eight males) were used. Female specimens were kept in a moist chamber at 23–25°C for 1 day before stimulation. Ovulation was induced by injection of homologous hypophysis homogenate into the dorsal lymphatic sacs at a ratio of 1 hypophysis/ml of Ringer–Tris solution (110 mM NaCl, 1.4 mM $CaCl_2$, 2 mM KCl, 10 mM Tris-HCl, pH 7.6) per animal. About 7–12 h post-stimulation, eggs surrounded by the oviduct-secreted jelly coats were removed from the ovisacs. To obtain dejellied eggs, egg strings were treated with Ringer–Tris solution containing 1% thioglycolic acid, pH 7.0, and then rinsed with several changes of Ringer–Tris solution.

Sperm was obtained by maceration of testes from male *B. arenarum* specimens in Ringer–Tris. The cell suspension was then filtered through a 30-μm-mesh nylon screen and centrifuged at 1600 g at 4°C for 5 min. The supernatant was discarded, the cell pellet was suspended in Ringer–Tris solution and cells were counted in a hemocytometer under an Olympus BX40 microscope.

Envelope isolation and solubilization

For each experimental animal (five females), dejellied eggs were homogenized in Ringer–Tris without $CaCl_2$ in a Potter-Elvehjem homogenizer. Envelopes were isolated by filtering the homogenate through a 30-μm-mesh nylon screen; then, they were thoroughly washed with Ringer–Tris and recovered from the filter. All procedures were carried out at 0–4°C.

Isolated VEs were solubilized by heating at 70–80°C for 30–40 min. Solubilized VEs were centrifuged at 10,000 g at 4°C for 20 min, and the supernatant solutions were dialyzed against 10 mM Ringer-Tris buffer, pH 6.8, at 4°C for 12 h and stored at –20°C until use. Concentration of envelope glycoproteins was determined with the MicroBCA™ Protein Assay kit (Pierce), according to the manufacturer's instructions.

Labeling of VE glycoproteins with biotin

Solubilized VE glycoproteins were labeled with biotin according to Bayer & Wilchek (1980). Briefly, envelope glycoproteins (1 mg/ml in 0.1 M borate buffer, pH 8.8) were incubated at 25°C for 4 h in the presence of 50 μg biotin *N*-hydroxysuccinimide (Sigma-Aldrich Co.). Then, the reaction was stopped by the addition of 10 μl of 0.1 M NH_4Cl. After 10 min of incubation, the solution was dialyzed against 10 mM Tris–HCl buffer, pH 7.2, at 4°C for 12 h to separate unbound biotin molecules. Biotin-labeled glycoproteins were stored at 4°C until use. The purity and integrity of the labeled VE components was analyzed by SDS-PAGE and western blot as described previously (Llanos *et al.*, 2006).

Sperm–VE glycoprotein binding assays

Sperm suspensions (10^7 cells/ml) were centrifuged at 4°C for 10 min at 200 g and pellets were suspended and incubated with biotin-conjugated VE glycoproteins (150 μg of protein/10^6 sperm cells/ml) or Ringer–Tris solution (control). After 1.5 h at 25°C, the sperm was centrifuged at 1500 g at 4°C for 5 min, the supernatant discarded and the cells were washed three times with Ringer–Tris solution by centrifugation at 1500 g at 4°C for 5 min. At this time, sperm pellets were resuspended in 10 μl of ice-cold lysis buffer (1% Triton X-100, 1 mM $NaVO_3$, 1 mM PMSF, 5 mM EDTA, 150 mM NaCl, 10 mM Tris, pH 7.6) and stored on ice for 10 min with 3 × 5 s vortexing. Then, samples and biotinylated standards (Bio-Rad, Hercules, CA) were mixed with 10 μl of sample buffer containing 10% (w/v) of 2-β-mercaptoethanol, incubated at 90°C for 10 min and then subjected to SDS-PAGE on 7.5% gels (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes at 35 V at 4°C during 10 h. Membranes were blocked overnight at 4°C with phosphate-buffered saline (PBS; pH 7.4), containing 2% bovine serum albumin (BSA), and then incubated with peroxidase-conjugated streptavidin (Sigma-Aldrich Co.) at a dilution of 1:1000 at 25°C for 30 min. After extensive washing with PBS–0.02% Tween-20, the color development was carried out with 0.1 mg/ml 3,3′-diaminobenzidine (3,3′, 4,4′-tetra-aminobiphenyl) and 0.01% H_2O_2 in 0.1 M Trizma, pH 7.2 (Sigma-Aldrich Co.). The reaction was stopped by washing several times with distilled water. Membranes were scanned with a Bio-Rad Gel Doc 1000 densitometer and analyzed with Molecular Analyst software (version 1.4.1; Bio-Rad) to establish the molecular weights of the glycoproteins detected. Results of VE glycoproteins and sperm from five different animals were identical.

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Isolation of gp39 from polyacrylamide gels

SDS-PAGE was performed using the Protean® II xi Cell system (Bio-Rad). Molecular weight standards (Bio-Rad) and solubilized VE glycoprotein samples were denatured by boiling them in sample buffer at 95°C for 10 min. Aliquots of 50 μg of total glycoproteins were loaded per lane onto 1.5-mm-thick slab gels with 4% w/v acrylamide stacking and 7.5% w/v acrylamide resolving gels. Runs were carried out at 1.5–2.0 mA/lane using a PS30102 power supply (Sigma-Aldrich Co.). Gels were fixed overnight in 10% isopropanol–10% acetic acid, stained with 0.1% w/v Coomassie brilliant blue R-250 and destained in a 10% methanol–10% acetic acid solution with several changes. The area of the gp39 bands was cut out and the gel slices were stored at 4°C until use.

Preparation of antiserum against gp39

Polyclonal antiserum was prepared by immunizing a white male New Zealand rabbit with gp39 as antigen according to Dunbar (1987). Pre-immune serum was obtained from the animal before the first injection. Polyacrylamide gel pieces supporting gp39 were minced into small pieces and the initial immunogenic emulsion contained 400 μg of protein in 1 ml PBS, pH 7.4, previously mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich Co.). This emulsion was injected in multiple intradermal sites on the back of the animal. A second injection was performed 20 days later, with 400 μg of the immunogen in 1 ml PBS, pH 7.4, emulsified with an equal volume of incomplete Freund's adjuvant (Sigma-Aldrich Co.). Ten days after the second injection, the immune serum was obtained and stored at –70°C after fractioning.

Purification of antibodies

Antibodies of pre-immune and immune serum were purified on a protein A column (Sigma) and the immunoglobulin fractions were identified through absorbance at 280 nm (Harlow & Lane, 1988). The purity of the pre-immune (IgG Ab) and immune (anti-gp39 Ab) antibodies was checked with SDS-PAGE under reducing conditions.

Dot blot and western blot assaying

Reactivity against the antibodies was analyzed with Dot Blot immunoassaying using a Bio-Dot® Microfiltration Apparatus (Bio-Rad). VE glycoproteins (50 μg) were blotted onto a nitrocellulose membrane and assays were performed according to the manufacturer's protocol. The reaction specificity was determined by incubation of the pre-immune serum with VE glycoproteins spotted on the membrane.

Antibody specificity was determined with western blot analysis. Total VE glycoproteins (35 μg), separated by SDS-PAGE using a Mini-Protean® II apparatus (Bio-Rad), were electroblotted onto a nitrocellulose membrane in a Mini Trans-Blot® electrophoretic transfer system (Bio-Rad) at constant voltage (35 V for 10 h). The buffer solution contained 192 mM glycine, 25 mM Tris and 20% methanol, pH 8.3. Membranes were washed, air-dried, stained with Ponceau S and blocked with 2% (v/v) BSA in PBS, pH 7.4. Thereafter, the membrane was incubated at room temperature (RT) for 1 h with diluted antiserum (1:200) as primary antibody. After extensive washing with PBS containing 0.02% Tween-20, the membrane was incubated with 1:1000 diluted biotin-conjugated anti-rabbit IgG as secondary antibody at RT for 1 h. Specific binding of antibodies was visualized by incubation with 1:1000 streptavidin-peroxidase conjugate (Sigma-Aldrich Co.) and for 10 min after washing three times. Color was developed with 0.1 mg/ml 3,3′-diaminobenzidine (3,3′, 4,4′-tetra-aminobiphenyl) and 0.01% H_2O_2 in 0.1 M Trizma, pH 7.2, during 3–5 min and the reaction was stopped with five washes of distilled water. For antibody specificity control, membranes were treated with anti-gp39 serum previously incubated overnight with an excess of gp39 recovered by electro-elution from pieces of polyacrylamide gels according to Barisone *et al.* (2007).

Immunohistochemistry

Eggs recovered from the coelom as described elsewhere (Llanos *et al.*, 2006) and dejellied eggs were fixed overnight at RT in a solution containing 3.7% formaldehyde, 0.25% glutaraldehyde and 0.2% Triton X-100 in PBS, pH 7.4. Samples were then embedded in Histowax (Merck). Deparaffinized sections were rinsed with PBS and blocked with 2% BSA-PBS at RT for 30 min. Subsequently, anti-gp39 Ab prepared at our laboratory and pre-immune rabbit serum diluted 1:50 in blocking solution were added. After 1 h at RT, samples were washed with PBS-Tween 20 (0.02%) and incubated with biotin-labelled anti-rabbit IgG (1:200) (Sigma-Aldrich Co.) at RT for 1 h. Then, samples were thoroughly rinsed with PBS and incubated with alkaline phosphatase-conjugated streptavidin (1:1000; Sigma Aldrich Co.) for 1 h. Following three washes with PBS–Tween-20 (0.02%), alkaline phosphatase activity was measured with a precipitating substrate (SIGMA *FAST*™ BCIP/NBT; 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 0.30 mg/ml nitroblue-tetrazolium, 100 mM Tris buffer, 5 mM $MgCl_2$) (Sigma-Aldrich Co.) during 3–5 min and the reaction was stopped with several washes of distilled water. Finally, the sections were mounted in 50% glycerol–PBS and

the slides were examined under an Olympus BX40 light microscope and photographed with an Olympus C-5060 wide zoom digital camera.

Immunoelectron microscopy

Eggs without jelly coats were fixed by immersion in a solution consisting of 4% paraformaldehyde plus 0.8% glutaraldehyde in 0.1 M PBS, pH 7.4, at 4°C. Dehydration was carried out at increasing ethanol concentrations followed by gradual filtration with LR-White acrylic resin mixed with the solvent as follows: 2:1 (v/v) 100% ethanol:LR-White for 1 h and then 1:1 (v/v) overnight followed by embedding in pure LR-White. Ultrathin sections mounted on nickel grids were treated with 1% BSA buffered in 0.01 M PBS, pH 7.4, and then incubated with anti-gp39 Ab used as primary antibody. After repeated rinses in PBS, sections were incubated with the secondary antibody (goat anti-rabbit IgG; Pelco) conjugated with 10-nm colloidal gold particles. Labelled sections were then post-stained in uranyl acetate and lead citrate before electron microscopic examination. Control sections were treated in the same way but without the primary antibody.

Immunodetection of gp39 bound to sperm

Washed *B. arenarum* sperm was fixed in 4% (v/v) formaldehyde in Ringer–Tris for 10 min to maintain integrity after long incubation periods. Fixed sperm (10^7 cells/ml) was washed with PBS, pH 7.4, and centrifuged at 4°C for 5 min at 1500 *g* three times and the pellet was suspended in a solution of solubilized VE glycoproteins (150 μg of protein/10^7 sperm cells/ml) and incubated at 25°C for 1 h. After incubation, sperm was washed three times with a PBS solution and incubated at 25°C for 1 h with 1% BSA in PBS for the blocking of non-specific sites. Then, it was incubated with a 1:50 dilution of anti-gp39 Ab and pre-immune serum, washed with PBS (3×) and incubated for 1 h with a 1:100 dilution of goat anti-rabbit immunoglobulin G conjugated to biotin (Sigma-Aldrich Co.). After washing with PBS (3×), sperm was further treated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Sigma-Aldrich Co.) at a dilution of 1:1000 at 25°C for 1 h. Then it was examined with an Olympus BX40 microscope equipped with epi-fluorescence and documented using a digital Olympus C-5060 camera. The experiments were performed with VE glycoprotein samples and sperm obtained from five different animals.

Fertilization inhibition assays

Eggs with and without jelly coats (a set of 50–90 eggs) were incubated with anti-gp39 Ab (1 μg/μl) and pre-immune IgG Ab (1 μg/μl) as control in 500 μl of Ringer–Tris solution at 25°C for 45 min in three independent experiments. After three washes, the eggs with jelly coats were inseminated with sperm suspensions (10^6 cells/ml) in 500 μl of 10% Ringer–Tris and incubated at 22–25°C for 30 min. Dejellied eggs were inseminated incorporating 20 mg/ml of egg water in the fertilization medium as described by Barbieri & del Pino (1975). Finally, fertilization medium was washed, eggs were kept in 10% Ringer–Tris solution and the fertilization rate was determined at the 2-cell stage.

Statistical analysis

Results of the fertilization inhibition assays are presented as mean ± SEM and they were analyzed with Student's *t*-test to establish the significance of the percentage of fertilized eggs (with and without jelly coat) pre-incubated with anti-gp39 Ab and pre-immune IgG Ab. In all cases, comparisons were considered significantly different when p-values were < 0.05.

Results

Binding of biotinylated VE glycoproteins to sperm

Acquisition of sperm binding activity of the egg envelope is correlated with its structural and/or molecular alteration when the CE is converted into VE. In order to determine which glycoprotein(s) in a solubilized total VE protein mixture has/have sperm binding abilities, *in vitro* adhesion assays were performed, incubating solubilized biotin-labelled VE glycoproteins with homologous sperm. Initially, we checked that all VE glycoproteins were biotin-conjugated through detection on a nitrocellulose membrane with streptavidin-peroxidase (Fig. 1). Then, sperm was incubated with the biotin-labelled glycoproteins and after this time, they were lysed as described in Materials and methods. Lysed samples were separated on SDS-PAGE and electroblotted onto a nitrocellulose membrane. Visualization of biotinylated VE glycoproteins bound to sperm was carried out with a streptavidin-peroxidase amplification system. Figure 2 shows two biotinylated bands on the membrane at 42 and 39 kDa, while the control membrane, corresponding to samples of lysed sperm without incubation with biotin-labeled VE glycoproteins, does not show any biotin-labelled protein band. These results suggest that gp39 and gp42 in *B. arenarum* are VE glycoproteins with sperm binding capacity.

Antiserum specificity against gp39

To study the egg gp39 VE glycoprotein, polyclonal antiserum was obtained from a rabbit immunized

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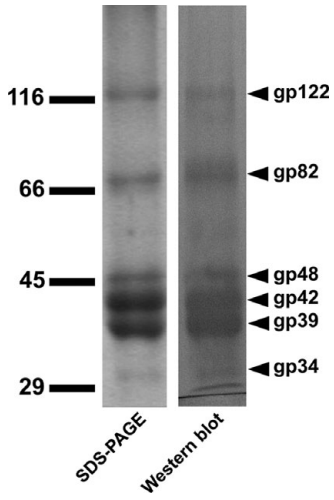

Figure 1 SDS-PAGE stained with Coomassie brilliant blue and streptavidin-biotin western blot of vitelline envelope (VE) glycoproteins conjugated to biotin. Molecular weight standards (in kDa) are indicated on the left.

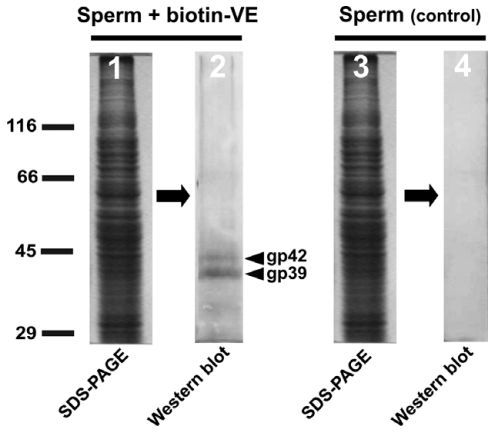

Figure 2 Detection of biotin-conjugated vitelline envelope (VE) glycoproteins bound to sperm. Gel 1: SDS-PAGE of lysate of sperm incubated with biotin-conjugated VE. Membrane 2: Streptavidin–biotin western blot of lysate of sperm incubated with biotin-conjugated VE; gp39 and gp42 glycoproteins are indicated with arrows. Gel 3: SDS-PAGE of sperm lysate. Membrane 4: Streptavidin–biotin western blot of lysate of sperm without incubation with biotin-conjugated VE (control). Molecular weight standards (in kDa) are indicated on the left.

with isolated gp39. Immunobiochemical studies were carried out to determine the reactivity and specificity of the antibodies generated in the immune response against VE glycoproteins. Dot blot assay showed positive reactivity of the antiserum against immunogenic total VE glycoproteins. Negative reactivity against VE glycoproteins was observed using pre-immune serum from an immunized animal (Fig. 3*A*).

When solubilized VE samples were probed in western blot analysis with antiserum against gp39, the antibodies specifically revealed a band of around 39 kDa on the nitrocellulose membrane, corresponding to gp39. Cross-reactivity of the antibodies generated with other VE components was not observed (Fig. 3*B*). Antibody specificity was also demonstrated by the fact that antiserum did not detect the glycoprotein when it was pre-incubated with an excess of gp39 (Fig. 3*B*).

Detection of gp39 on the egg envelope by immunohistochemistry and immunoelectron microscopy

Immunohistochemical studies were carried out to detect gp39 in *B. arenarum* egg sections under different conditions. Anti-gp39 Ab detected antigens in the VE of eggs that had traversed the oviduct and staining was found all over the structure indicating an apparent homogeneous distribution of gp39 (Fig. 4*A*). In comparison, only weak staining of the envelope was observed in eggs collected from the coelom (Fig. 4*C*). A particularly intense color was observed on the outer edge of the CE (Fig. 4*C*). In both cases, envelopes were not stained when egg sections were incubated with pre-immune serum (Fig. 4*B*, *D*). Immunoelectron microscopy studies using anti-gp39 primary antibody and secondary antibody coupled to gold particles revealed that gp39 was localized throughout the entir width of the VE (Fig. 5*A*, *C*).

Immunofluorescent detection of gp39 bound to sperm treated with solubilized VE

To explore the sperm binding activity of gp39, *B. arenarum* sperm was incubated *in vitro* with solubilized VE and afterwards, gp39 was localized on the sperm surface by indirect immunofluorescence with anti-gp39 Ab. As shown in Fig. 6*A*, fluorescence was observed on the entire surface of the sperm head with a more intense labeling at the apical acrosomal region. No fluorescence was observed on control sperm exposed to pre-immune IgG Ab and biotin-conjugated anti-rabbit IgG (Fig. 6*B*, *C*). These results indicate that solubilized gp39 was able to bind to sperm *in vitro* suggesting its participation in the sperm–VE interaction.

Fertilization rates of eggs pretreated with antibodies against gp39

If gp39 is a sperm-binding glycoprotein in the VE, one would expect that the anti-gp39 antibodies should block sperm–egg binding and fertilization, and hence the effect of anti-gp39 antibodies on *in vitro* fertilization rates was examined. Eggs with and without jelly coats (dejellied eggs) were pretreated with anti-gp39

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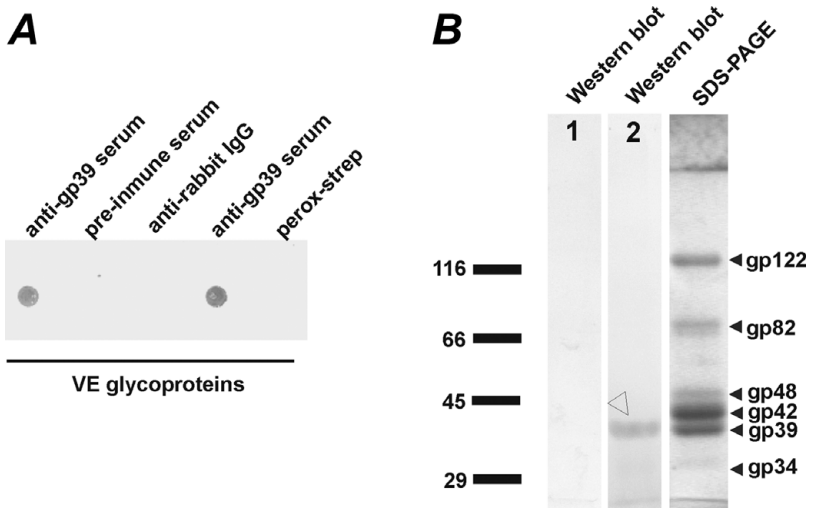

Figure 3 Specificity of antiserum against gp39. (*A*) Dot blot analysis. Total vitelline envelope (VE) glycoproteins were loaded onto a nitrocellulose membrane and incubated with anti-gp39 serum and with pre-immune serum, anti-rabbit biotin-conjugated IgG (secondary antibody) and streptavidin–peroxidase conjugate as negative controls. A positive signal was observed just when VE glycoproteins were exposed to antiserum against gp39. (*B*) Western blot of VE samples showing the specific detection of gp39 (membrane no. 2, indicated with arrow) with anti-gp39 antiserum. Total VE glycoproteins were also probed with anti-gp39 serum pre-incubated with an excess of gp39 (membrane no. 1). Coomassie brilliant blue-stained gel (right) of identical VE sample. Molecular weight standards (in kDa) are indicated as lines on the left of the figure.

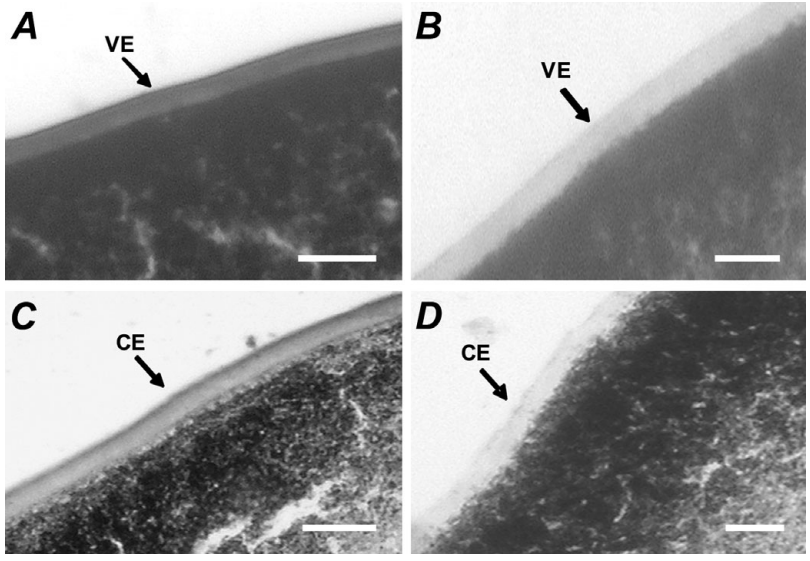

Figure 4 Immunohistochemical detection of gp39 in the vitelline envelope (VE) and coelomic envelope (CE) of *B. arenarum* eggs. Histological sections of oviposited eggs were stained with anti-gp39 antibodies (*A*) or with pre-immune serum (*B*) as described in Materials and methods. Homogeneous staining of the VE shows gp39 detection (*A*). No staining is observed in the control assay with pre-immune serum (*B*). Staining was localized in the CE with anti-gp39 antibodies (*C*) but not with pre-immune serum (*D*) as described in Materials and methods. A more intense stain is seen on the outer surface of the CE (*C*). Scale bars represent 10 μm.

Ab (1 μg/μl), for blocking gp39 sites on the VE, and pre-immune IgG Ab (1 μg/μl) as control. Then, both egg types were fertilized *in vitro.* Fig. 7 shows the percentages of fertilized eggs obtained with the different treatments. Although the fertilization rate of eggs with jelly coats incubated with anti-gp39 Ab (83.0 $\pm$ 3.1%) was lower than that of eggs incubated with pre-immune IgG Ab (87.5 $\pm$ 2.5%), this difference was not significant ($P < 0.05$). However, dejellied eggs incubated with anti-gp39 Ab showed a significant decrease in the fertilization rate (23.2 $\pm$ 3.2%) compared with dejellied eggs incubated with

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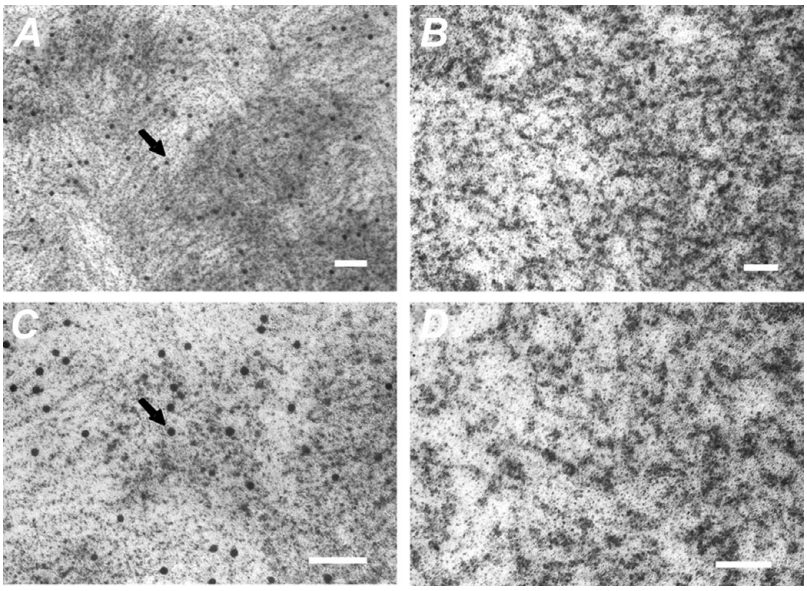

Figure 5 Immunoelectron detection of gp39 in the vitelline envelope (VE) of *B. arenarum* eggs. In immunoelectron microscopy sections of eggs treated with anti-gp39 antibodies and goat anti-rabbit IgG secondary antibodies conjugated to gold particles, gp39 was detected throughout the entire width of the VE (*A*) and (*C*) electron micrographs; arrows. VE filaments are devoid of gold particles in control sections (*B*) and (*D*) electron micrographs. Scale bars represent 100 nm.

pre-immune IgG Ab as control (68.3 ± 7.6%). In addition, the difference in fertilization rate between dejellied eggs pre-incubated with pre-immune IgG Ab and those pre-incubated in Ringer–Tris (74.5 ± 5.1%) was not significant ($P < 0.05$).

Discussion

Fertilization is essentially a process mediated by the interaction of specific and complementary molecules present on the surface of both gametes. This cell–cell interaction begins with sperm–egg recognition and then follows with penetration of the extracellular egg coat and fusion of the gamete plasma membranes. One of the main functions of the egg envelope is to provide binding sites for sperm during fertilization. However, the molecular principle of the sperm–VE interaction and the conversion of the CE into VE in *B. arenarum* have not been completely elucidated to date. The present study focuses on gp39, a glycoprotein of the *B. arenarum* egg envelope, regarding its participation in sperm–egg interaction. In this species, sperm does not bind or penetrate the CE, but in contrast it readily binds to and penetrates the VE. Changes in the egg envelope from a non-binding and non-penetrable state, to a binding and penetrable state are caused by molecular modifications of certain envelope glycoproteins. These molecular modifications involve proteolytic cleavage of CE glycoproteins by the protease oviductin. Consequently, gp84 and gp55 glycoproteins are present in the CE but not in the VE of fertilizable eggs. Furthermore, the relative quantity of gp42 and gp39 molecules in fertilizable egg envelopes increased 1.2- and 2.3-fold, respectively, and the relative gp48 concentration decreased 4.5-fold after conversion of the CE into the VE (Llanos *et al.*, 2006).

In vitro sperm binding assays have shown that the solubilized gp39 component of the *B. arenarum* egg VE has the ability to bind sperm. However, the binding process between sperm and the VE could be mediated by more than one glycoprotein. Biotin-labelled gp39 and gp42, bound to sperm, were extracted after solubilization with a mild detergent and detected on a nitrocellulose membrane after electrotransference by incubation with streptavidin-peroxidase conjugate. These two glycoproteins are the most abundant components of the VE (Llanos *et al.*, 2006). Nevertheless, the binding of other quantitatively minor components of total VE to sperm can not be discarded. Barisone *et al.* (2007) obtained comparable results and they cloned a *B. arenarum* cDNA that encodes the protein precursor homologue of ZPC. The authors believe that this cDNA encodes an equivalent glycoprotein for gp42 assuming that the proteolytic processing of its glycoprotein precursor in the CE is similar to that proposed in *Xenopus* (Kubo *et al.*, 1999). In this latter species, oviductin specifically cleaves a single component of the CE, transforming the gp43 glycoprotein into gp41

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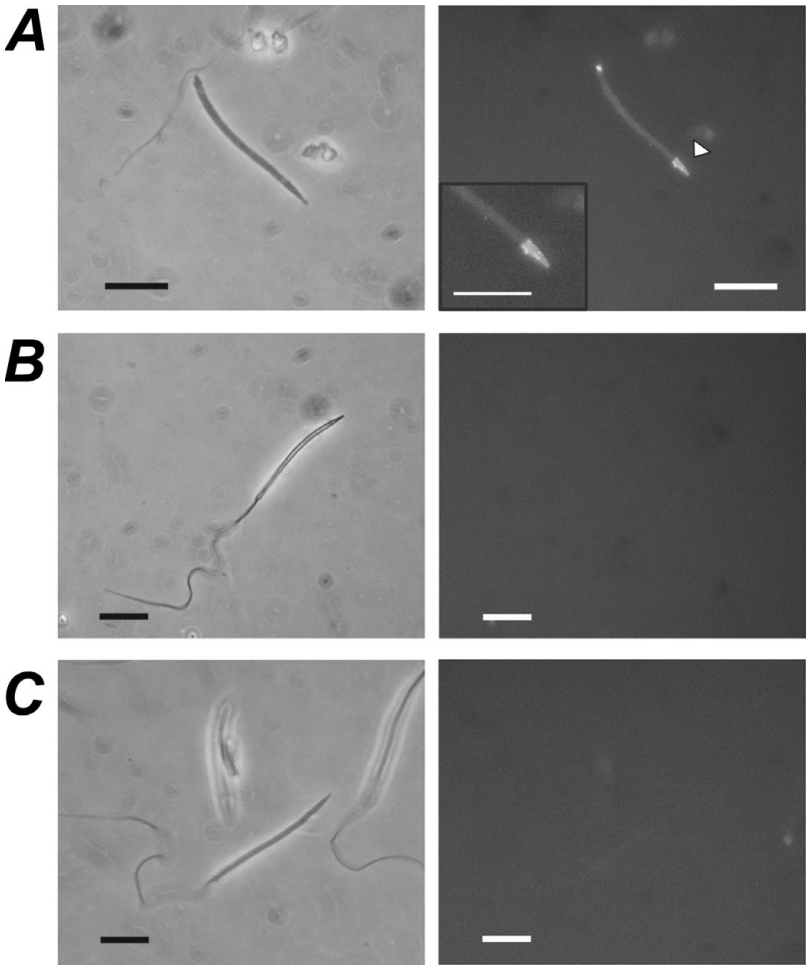

Figure 6 Sperm binding to the gp39 component of the vitelline envelope (VE). Detection of gp39 in *B. arenarum* sperm pre-incubated with solubilized VE glycoproteins was carried out using purified anti-gp39 antibodies with indirect immunofluorescence. Detection was performed with streptavidin–FITC. Images are representative of five independent experiments. (*A*) Sperm–VE incubated with anti-gp39 antibodies (Ab). (*B*) Sperm–VE incubated with pre-immune IgG Ab. (*C*) Sperm–VE incubated with anti-rabbit secondary antibody. Observe the fluorescent labeling on the sperm head indicating sperm binding to gp39. Inset in (A): sperm apical region. Scale bars represent 10 μm.

(Hardy & Hedrick, 1992), which acts as a sperm binding ligand in the VE (Vo & Hedrick, 2000). However, the corresponding molecular conversion in *Bufo* is apparently more complex than in *Xenopus* because oviductin hydrolyzed more than one CE component. In fact, the molecular principle of the transformation of CE into VE in *Bufo* is still not fully understood and further research on proteolysis of glycoproteins in the egg envelope is necessary to elucidate details of the molecular increase and decrease in VE glycoproteins. As a first step to identification of the VE molecules modified by oviductin, the sequence of internal gp39 peptides was examined in previous studies (Llanos *et al.*, 2006). Results showed that gp39 could be a homologous glycoprotein of mammalian ZPC. Taking into account this evidence and the current findings, some molecular relationship between gp39 and gp42 could not be discarded. However, to further prove this idea, information about the amino acid sequence of gp42 and structural analysis of its carbohydrates are necessary. In *X. laevis*, Tian *et al.* (1997) suggested that the main function performed by two molecularly related glycoproteins (gp69 and gp64) is that of a ligand in sperm binding. Coincidentally, Infante *et al.* (2004) reported that, similar to *B. arenarum*, two VE glycoproteins in *Discoglossus pictus* (gp63 and gp75) with similar protein sequence were able to bind sperm *in vitro.*

To further study the sperm-binding activity of gp39, polyclonal antiserum against this glycoprotein was prepared by immunizing a rabbit with *B. arenarum* gp39 isolated with SDS-PAGE. Western blot analysis clearly demonstrated the high specificity of the anti-gp39 antiserum obtained. It is interesting, that the

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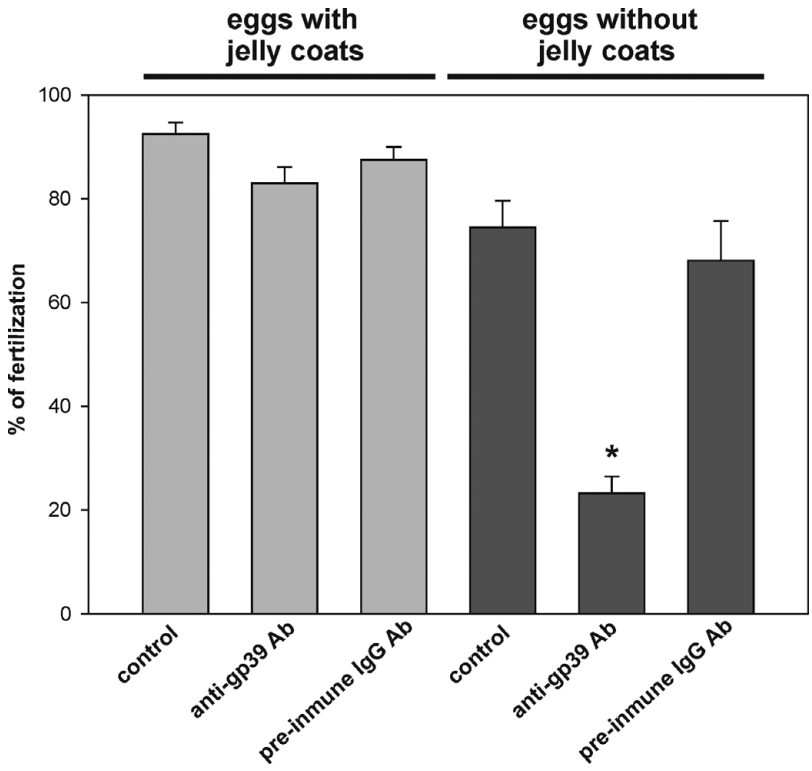

Figure 7 Effect of anti-gp39 antibodies on the fertilization rate. Pre-incubation of eggs without jelly coats with anti-gp39 antibodies (1 $\mu g/\mu l$) caused a significant diminution (*) in the fertilization rate with respect to controls ($p < 0.05$). Each bar represents the mean ± SD, $n = 3$ (no. of experiments).

antibodies did not show cross-reactivity with other VE components. This could indicate that the reactive gp39 epitopes with higher antigenicity are specific for this molecule. This phenomenon is probably more related to the amino acid sequence or some conformational determinant than to the carbohydrate ratio, even though the immune response against carbohydrate structures is characterized by the production of antibodies with low affinity and the ability to recognize related structures. Absence of cross-reactivity is not surprising, because several authors have mentioned the presence of immunodominant determinants unique to each individual component in constitutive glycoproteins of the ZP as well as specific reactivity of polyclonal antibodies against glycoproteins purified from the ZP (Timmons *et al.*, 1987; Hasegawa *et al.*, 1991). However, cross-reactivity of antibodies among extracellular coat components of different species has been demonstrated (Maresh & Dunbar, 1987; Takamune *et al.*, 1987).

The ability of anti-gp39 antibodies to detect this glycoprotein in both types of egg envelopes (CE and VE) was tested by indirect immunostaining of histological sections. Presumably, the antibodies would also be able to detect gp39 preglycoprotein forms on the CE that were there already before oviductin-mediated processing. Although the CE and VE were immunostained with antibodies raised against gp39 some differences could be observed. When the same concentration of antibodies and developer were used, a higher concentration of gp39 was seen in the VE. This finding is in agreement with reported SDS-PAGE observations that showed an increase in the relative concentration of gp39 in the VE (Llanos *et al.*, 2006). Apparently, gp39 or its preglycoprotein forms seem to be heterogeneously distributed in the CE with predominance on the external region of the envelope. However, reactivity of the antibodies showed a homogeneous distribution in the VE, which was confirmed by immunolocalization studies using electron microscopy. The spatial distribution of gp39 or its preglycoprotein forms in the external portion of the CE could be indicative of structural differences observed between the CE and VE in *B. arenarum* (Mariano *et al.*, 1984; Llanos *et al.*, 2006).

Previous studies have suggested that *B. arenarum* gp39 could be involved in the mechanism of sperm-binding to the egg envelope (Llanos *et al.*, 2006). The current study assayed the ability of gp39 to bind to sperm. For this purpose, *in vitro* sperm binding assays were performed using solubilized VE and anti-gp39 antibodies. Binding of *B. arenarum* gp39 to the sperm surface was clearly observed through an intense fluorescent label at the anterior end of

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the sperm head. This label coincided with the most apical region of the sperm acrosome. These results demonstrate that the binding activity is conserved after heat solubilization of the VE glycoproteins, which is in agreement with similar assays by other authors (Barisone *et al.*, 2002). Most of the knowledge of the functional aspects of extracellular coat glycoproteins during fertilization has emerged from the murine model. The ZP of mouse eggs is composed of three glycoproteins called ZPB (Mr ~200 kDa), ZPA (Mr ~120 kDa) and ZPC (Mr ~83 kDa). ZPA and ZPC are postulated as sperm receptors in the ZP. Evidence in mice suggests that acrosome-intact sperm first binds to ZPC, completes the acrosome reaction, then binds to ZPA, penetrates the ZP, and eventually fuses with the egg plasma membrane (reviewed in Wassarman & Litscher, 2008). In amphibians, VE glycoprotein homologues of ZP glycoproteins are also involved in sperm–egg interaction. In *X. laevis*, sperm binding has been attributed to ZPC component gp41 (Vo & Hedrick, 2000), which is generated after oviductin-mediated processing of its preglycoprotein form (gp43) when the CE is converted into the VE. Sperm-binding activity was associated to a lesser extent to gp69 and gp64 (ZPA), two related glycoproteins (regarding their amino acid sequence) with sperm-ligand activity (Tian *et al.*, 1997; Vo & Hedrick, 2000). A similar situation has been described in *B. japonicus;* gp36–gp39 glycoproteins that appear in the VE after oviductin action on the CE participated in the sperm–envelope binding (Omata & Katagiri, 1996). It is important to remark that information obtained from the protein sequence showed that *B. arenarum* gp39 could be a member of the ZPC glycoprotein family (Llanos *et al.*, 2006). Even if the amino acid sequence of *B. japonicus* gp39 is unknown to date, it seems likely that the molecular process for generation of the 39-kDa glycoprotein of the *B. japonicus* egg envelope by digestion of oviductin is similar for both species. This idea is supported by the fact that comparison of the amino acid sequence of the active protease domain of *B. arenarum* oviductin and its orthologous enzyme in *B. japonicus* showed more than 90% sequence identity (Barrera *et al.*, 2010). In view of these findings, we consider *B. arenarum* gp39 a candidate for sperm binding to the VE during fertilization.

To confirm the participation of *B. arenarum* gp39 as a mediator of sperm binding to the VE, we determined the fertilization rate of *B. arenarum* eggs treated with anti-gp39 antibodies. The percentage of fertilization of eggs with jelly coats previously incubated with the same antibody concentration as dejellied eggs did not significantly diminish. In contrast, pre-incubation of dejellied eggs with anti-gp39 antibodies led to a marked decrease in fertilization rate (approximately 80%) compared with controls. In this case, the antibodies presumably had easier access to the VE due to the absence of jelly coats. Thus, sperm binding sites in gp39 could be blocked efficiently impeding sperm–egg binding. These results are consistent with those obtained by *in vitro* detection of gp39 on sperm surface as mentioned above. Barisone *et al.* (2002) achieved inhibition of fertilization of dejellied eggs in *B. arenarum* using anti-VE polyclonal antibodies. In *B. japonicus*, binding of 36–39-kDa components to sperm was blocked by specific antibodies inhibiting fertilization (Omata & Katagiri, 1996). Because fertilization was partly impeded in our experiments, we believe that, even though gp39 plays a major role in sperm binding, certain sites on other VE glycoproteins (like gp42) or other molecules able to compensate the blockage of the binding sites on gp39 are involved in the global process of the early gamete binding. The supramolecular model proposed for sperm–ZP interaction in mice involves the three-dimensional structure of the ZP in sperm binding (reviewed in Hoodbhoy & Dean, 2004). This model can easily be applied to amphibians. It is known that the CE is impenetrable to sperm, but this condition changes when oviductin produces a limited proteolysis of CE components which leads to envelope reorganization and conversion into the VE. Additionally, comparison of SDS-PAGE profiles of the CE and the VE revealed different concentrations of certain components. This effectively indicates the possibility that these molecular changes produce a global transformation of the envelope to interact with sperm. Furthermore, several reports have provided evidence that VE glycoproteins in *Xenopus* and *Bufo* interact in a synergistic manner to promote sperm–envelope binding (Vo & Hedrick, 2000; Barisone *et al.*, 2007). Thereby, the current study has validated the binding capacity of gp39 to sperm confirming its relevant participation in sperm–egg interaction.

The present work describes one of the functional consequences of proteolytic activity of oviductin on the egg envelope in *B. arenarum.* According to the results, gp39 is a VE glycoprotein that plays an active role during sperm–egg interaction. These findings, together with the fact that gp42 is another glycoprotein with sperm binding capacity, suggest that for binding to the VE sperm requires the interaction with glycoproteins previously modified by proteolytic activity of oviductin during the transit of the eggs through the oviductal PR. Therefore, our *in vitro* observations support the idea that selective proteolysis of envelope glycoproteins by oviductin affects the molecular conformation of the envelope so that the actual binding sites for sperm become exposed.

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