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Xenopus laevis Sperm–Egg Adhesion Is Regulated by Modifications in the Sperm Receptor and the Egg Vitelline Envelope

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The biochemical and ultrastructural changes in the envelope of the *Xenopus laevis* egg that occur during oviposition and fertilization have been thoroughly studied (Hedrick, J. L., and Nishihara, D. M., *Methods Cell Biol.* 36, 231–247, 1991; Larabell, C. A., and Chandler, D. E., *J. Electron Microsc. Tech.* 17, 294–318, 1991). However, the biological significance of these changes with respect to gamete interaction has been unclear. In the current study, it was found that changes in the envelope are directly responsible for regulating sperm–egg adhesion, an initial step of fertilization. As a result of these transformations, sperm bind only to unfertilized oviposited eggs, not to oocytes or coelomic eggs. In addition, they do not bind to fertilized eggs. The molecular and cellular basis of the regulation of the sperm binding process was investigated in the context of our recent findings that two structurally related envelope glycoproteins, gp69/64, serve as sperm receptors during fertilization (Tian, J.-D., Gong, H., Thomsen, G. H., and Lennarz, W. J., *J. Cell Biol.* 136, 1099–1108, 1997). Although the purified gp69/64 glycoproteins isolated from the oocyte or coelomic egg envelopes exhibited sperm binding activity, when these proteins are part of the intact oocyte or coelomic egg envelopes, they are not accessible to either anti-gp69/64 antibodies or to sperm. During the conversion from the coelomic to the vitelline envelope, the gp69/64 sperm receptors become exposed on the surface, an event that correlates with proteolytic cleavage of gp43 and accompanying ultrastructural alterations in the envelope. Conversely, after fertilization, when the vitelline envelope of the egg is converted to the fertilization envelope of the zygote, limited proteolytic cleavage of the sperm receptor results in loss of sperm binding activity. In addition, formation of a fertilization layer on top of the structurally altered VE adds another physical block to sperm binding. These results provide new insights into structure–function relationships between envelope components of the anuran egg, and provide further evidence supporting the key role of gp69/64 as sperm receptors during *X. laevis* fertilization.

INTRODUCTION

Modifications in egg cell surface molecules are known to play an important role in developmental regulation of sperm binding. In mouse and sea urchin gametes, sperm–egg interactions are regulated primarily through modifications of the sperm receptor on the egg surface during fertilization. Maturing mouse oocytes acquire sperm binding activity shortly after they begin to form the zona pellucida, a coat that contains the sperm receptors, ZP3 and ZP2. The sperm binding activity reaches its highest by the time the eggs are fully grown and mature. During fertilization, both ZP3 and ZP2 are modified and lose their sperm binding activities (Bleil and Wassarman, 1980; Moller and Wassarman, 1989). In the sea urchin, the egg receptor for sperm is present on the surface of unfertilized eggs, but disappears upon fertilization, presumably because it is degraded by proteases released from cortical granules (Partin *et al.*, 1996). In anuran amphibians, a very interesting feature of the egg envelope is that it undergoes substantial biochemical and ultrastructural modifications during oviposition and again during fertilization. However, how these modifications regulate the function, especially the sperm binding properties of the egg envelope, has not been elucidated, mainly due to the lack of knowledge of the molecular basis of the interaction of anuran sperm with the egg envelope.

The modifications that occur in the envelope of the anuran oocyte and egg have been especially well characterized

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in the South African clawed frog, *Xenopus laevis* (see Hedrick and Nishihara, 1991; Larabell and Chandler, 1991, for reviews). The *Xenopus* egg envelope is formed during oogenesis. The six glycoproteins comprising the oocyte envelope (OE), namely gp120, 112, 69, 64, 43, and 37, are synthesized by the oocyte itself (Yamaguchi *et al.*, 1989). After ovulation, the hormonally matured oocyte is released into the coelomic cavity. During this process little change in composition of the envelope proteins has been detected, yet the coelomic egg envelope (CE) differs somehow in structure from the OE, probably caused by detachment of follicule cells and retraction of egg surface microvilli from the envelope (Grey *et al.*, 1977; Larabell and Chandler, 1989, 1991). When the coelomic egg subsequently passes through the pars recta region of the oviduct, the CE is converted by oviductal factors to the vitelline envelope (VE) (Grey *et al.*, 1977; Gerton and Hedrick, 1986a; Larabell and Chandler, 1989; Bakos *et al.*, 1990a,b; Hardy and Hedrick, 1992). This conversion involves alteration in the ultrastructure of the envelope and reduction in the molecular weight of gp43 from 43 to 41 kDa (gp41). This change is believed to be caused by proteolytic cleavage of the C-terminus of gp43 by oviductin, a unique serine protease secreted by the oviduct (Hardy and Hedrick, 1992). These ultrastructural and biochemical changes somehow convert the envelope from a sperm-impenetrable state to a sperm-penetrable state. During final passage through the oviduct the egg acquires several layers of jelly coat and emerges as a mature, oviposited egg.

After fertilization, cortical granule contents released from the fertilized egg cause the transformation of the VE to the fertilization envelope (FE) (Gerton and Hedrick, 1986b; Lindsay and Hedrick, 1989, 1995). During the early phase of conversion the VE hardens, becoming more resistant to heat, proteases, and reducing reagents (Wolf, 1974; Wolf *et al.*, 1976; Urch and Hedrick, 1981; Bakos *et al.*, 1989). The ''hardening'' reaction presumably results from a conformational change of the envelope (Schmell *et al.*, 1983; Bakos *et al.*, 1989; Larabell and Chandler, 1988b), rather than crosslinking of the envelope components as is the case in the sea urchin (Foerder and Shapiro, 1977; Hall, 1978). The envelope hardening is immediately followed by a limited hydrolysis of two structurally related glycoproteins, gp69 and gp64, at their C-termini, reducing their apparent molecular weights to 66 and 61 kDa, respectively (Gerton and Hedrick, 1986b; Lindsay and Hedrick, 1989). At least two proteases are thought to be involved: a 30-kDa trypsin-like protease released from the cortical granules and another 45-kDa chymotrypsin-like protease found in the perivitelline space. The former presumably activates the later protease, which in turn causes the cleavage of gp69/64 (Lindsay and Hedrick, 1989, 1995). In the meantime, a lectin released from the cortical granules diffuses across the VE and binds to its ligand in the inner jelly layer just outside of the altered VE (VE*), forming the so-called ''fertilization layer'' (F-layer) (Nishihara *et al.*, 1986; Mozingo and Hedrick, 1996). As a result of the cortical granule exocytosis, another two glycoproteins are added to the FE, one with a mass of 40–45 kDa and another with a high, undetermined M_r. This fertilization envelope, like the oocyte envelope, is impenetrable to sperm (Grey *et al.*, 1976).

Recently this detailed knowledge of the ultrastructural and compositional properties of the egg envelope and the mechanisms involved in the envelope conversions has been complemented by the demonstration that the gp69/64 glycoproteins in the VE function as sperm receptors mediating sperm–egg binding (Tian *et al.*, 1997). Four major lines of evidence support this conclusion: (1) purified gp69/64 proteins competitively inhibit binding of sperm to eggs; (2) polyclonal antibody against gp69/64 inhibit sperm–egg binding as well as fertilization; (3) agarose beads covalently coated with gp69/64 proteins exhibit sperm binding activity; and (4) treatment of unfertilized eggs with crude collagenase results in proteolytic modification of only the gp69/64 components of the VE and abolishes sperm–egg binding. The identification of this pair of *Xenopus* sperm receptor glycoproteins has enabled us to investigate in more detail the sperm binding activity of the egg surface at different developmental stages. The results of these investigations provide considerable insight into how the conversions of the CE to VE and the VE to FE affect the binding activity of the sperm receptor.

MATERIALS AND METHODS

Gametes

X. laevis were purchased from Nasco Biological Supply Co. (Fort Atkinson, WI). Oocytes were isolated from ovaries according to Colman (1984) and defolliculated by a combination of a brief (0.5 hr) treatment in 0.5% collagenase (type I, Sigma Chemical Co., St. Louis, MO) in OR2 medium (82.5 m*M* NaCl, 2.5 m*M* KCl, 1.0 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 1.0 m*M* Na_2HPO_4, 5.0 m*M* Hepes, 50 μg/ml gentamicin, pH 7.8) to loosen the ovarian tissue and subsequent manual removal of the follicular layer with forceps. Isolated oocytes were transferred to a fresh petri dish and washed with fresh OR2 medium on a slow rocker for 3 × 0.5 hr to remove the remaining follicle cells. Isolated oocytes were cultured in OR2 medium at 18°C overnight before being used in the sperm binding assay. Oocyte after these steps were free of follicle cells.

Oviposited eggs were obtained as described by Wolf and Hedrick (1971). Female frogs were injected in the dorsal lymph sac with 600–700 IU of human chorionic gonadotropin (Sigma Chemical Co.). After 9–10 hr eggs were stripped from the females. To fertilize the eggs, a small piece of testis was chopped and macerated in 0.3 × MR (pH 7.8) and the sperm suspension was applied onto the eggs on a dry dish. Then additional solution was added to just cover the eggs. After fertilization, embryos were allowed to develop in 0.1× MMR (Marc's modified Ringer solution: 100 m*M* NaCl, 2 m*M* KCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* Hepes, pH 7.4) at 18°C. Coelomic eggs were obtained by surgical ligation of the oviduct prior to the induction of ovulation (Bakos *et al.*, 1990; Hedrick and Hardy, 1991). To remove jelly from unfertilized or fertilized eggs, the eggs were exposed for 3–5 min to 45 m*M* β-mercaptoethanol in MR solution (modified Ringer: 100 m*M* NaCl, 1.8 m*M* KCl, 1.0 m*M* $MgCl_2$, 2.0 m*M* $CaCl_2$, 5.0 m*M* Na–Hepes) adjusted to pH 8.5. The solubilized jelly in the supernatant was decanted, and the eggs

were gently rinsed with several changes of MR (pH 6.5). Eggs were artificially activated by incubation in solutions containing 2 μM of calcium ionophore A23187 (Sigma). The ionophore was washed away 10 min after activation.

Gamete Binding Assay

The gamete binding assay was performed as previously described (Tian *et al.*, 1997). The jelly extract used in the binding assays was prepared according to a procedure described by Heasman *et al.* (1991) with minor modification. Freshly laid eggs were incubated in 0.3× MR (pH 7.8) in a ratio of 8 ml solution per 3 g (net weight) of eggs in a culture dish on a rocker plate at medium speed (15 cycles/min). After 45 min of incubation at 20°C, the solution containing factors extracted from the egg jelly was recovered from the culture dish (usually 60% of the originally added volume). This jelly extract was used fresh or stored in a −20°C freezer for several months. The sperm suspension used for the binding assay was prepared by chopping and macerating a freshly excised testis in jelly extract or 0.3× MR (pH 7.8) as specified. The mixture was transferred into a microcentrifuge tube and tissue debris was removed by centrifugation at 100*g* for 3 min at 4°C. The concentration of mature sperm (as determined by morphology) in the supernatant was measured with a hemocytometer. The sperm suspension was then diluted to various concentrations with jelly extract or 0.3× MR as specified.

The binding assays were performed by adding groups of 20 oocytes, coelomic eggs, dejellied unfertilized or fertilized eggs or embryos to 0.5 ml of the sperm suspension (1.0×10^7 sperm/ml) and incubating for 15 min at 18–22°C. Then each group of sperm-treated eggs or oocytes was washed by allowing them to fall through a 15-cm-tall tube filled with MR buffer (pH 6.5). The eggs or oocytes with bound sperm quickly fell to the bottom of the well, while unbound free sperm settled much more slowly. The unbound sperm in the buffer could therefore be separated from those bound to the eggs by swiftly aspirating the buffer above the eggs. The eggs were further washed with three or more changes of MR buffer. Finally, the gametes were fixed and stained in 3% formaldehyde in MR buffer (pH 6.5) containing the DNA dye Hoechst 33342 (0.2 mg/ml, Molecular Probes, Inc., Eugene, OR) and viewed with an Axioskop-fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) with a Plan-Neofluar 5× or 10× objective. Images were recorded on Kodak 400 film (Eastman Kodak Co.). To measure sperm binding to fertilized eggs or embryos, eggs were fertilized with sperm and then dejellied. The sperm binding assays were carried out 30 min after fertilization for fertilized eggs and at the 2- or 4-cell stage for embryos.

Purification of Envelope Proteins and the Sperm Receptors

Total envelope proteins from OE, CE, VE, or FE were purified using a sieving method initially described by Wolf *et al.* (1976). Oocytes, coelomic eggs, dejellied oviposited eggs, or fertilized eggs were lysed by passing them through an 18- to 19-gauge needle. The lysate was poured through a 100-μm nylon screen and the envelopes retained on the screen were washed with ice-cold Tris–DeBoers solution (110 m*M* NaCl, 1.3 m*M* $CaCl_2$, and 1.3 m*M* KCl, 10 m*M* Tris–HCl, pH 7.5) until visually free from contaminating particulate material. The clean envelopes were washed from the screen into a centrifuge tube and collected by centrifugation at 4°C for 5 min at 5000*g*. For sperm–egg binding competition assays using solubilized total envelope proteins, the envelope pellet was dissolved in 0.3× MR (pH 7.8) by heating and repeated vortexing at 80°C for 10 min, followed by centrifugation for 2 min at 14,000*g* to remove particulate material.

To purify gp69/64, the isolated envelopes were incubated at 95°C for 2 min in 2% SDS and then centrifuged at 16,000*g* for 5 min to remove any particulate material. The supernatant was subjected to 7.5% SDS–PAGE (Laemmli, 1970). The 69- and 64-kDa bands on the gel were visualized by staining with 0.3 *M* $CuCl_2$ for 5 min and washing with distilled water several times (Harlow and Lane, 1988). The two bands was excised from the gel and destained in destain buffer (0.25 *M* EDTA, 0.25 *M* Tris, pH 9.0). Then, the gel slices were subjected to electroelution in SDS–electrophoresis buffer at 8–10 mA per elution tube for 4–6 hr, followed by electrodialysis at 1 W constant power for 4 hr against electrophoresis buffer not containing SDS (Bleil and Wassarman, 1980). The individual proteins were then dialyzed in distilled water at 4°C for 48 hr and then lyophilized. Prior to use the proteins were dissolved in water.

Polyclonal Antibodies

Rabbit antisera were raised against purified gp69 and gp64. Antibodies were partially purified by ammonium sulfate precipitation and DEAE–matrix chromatography (Harlow and Lane, 1988), followed by preadsorption with other VE proteins (gp120/112, gp41, and gp37) that had been covalently linked to Affigel-15 (Bio-Rad Laboratories, Melville, NY). This polyclonal anti-gp69/64 antibody specifically recognized the two glycoproteins in both their denatured form and their native form in the VE (Tian *et al.*, 1997).

Western Blot and Immunofluorescent Staining

Purified total envelope proteins were separated on 7.5% SDS–PAGE (Laemmli, 1970) and electroblotted onto membranes. Western blotting was carried out using polyclonal anti-gp69/64 antibody (1:250 dilution) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) as secondary antibody and the LumiGLO detection method (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

To determine the reactivity of the polyclonal antibody with the surface of live oocytes, coelomic eggs, or dejellied unfertilized or fertilized eggs, these cells were incubated with the polyclonal anti-gp69/64 (1:1 dilution) in MR (pH 6.5) for 30 min at 18–20°C, then washed three times with MR, and fixed in 3% formaldehyde in MR for 2 hr at room temperature. Fixed eggs were then washed 3 × 20 min in MR and blocked for 1 hr in MR solution containing 5% BSA (Fraction V, Sigma). At the end of this period, FITC-labeled goat anti-rabbit IgG secondary antibody (Sigma) was added to the blocking solution at a final concentration of 10 μg/ml. After another 30 min, the eggs were washed thoroughly with MR and viewed with a Nikon Diaphot fluorescence microscope (Nikon, Inc., Melville, NY) with a 4× objective.

^{125}I Labeling of VE

Dejellied unfertilized eggs or water solubilized total envelope proteins were radioiodinated using Iodo-beads iodination reagent (sodium *N*-chlorobenzenesulfonamide, Pierce, Rockford, IL) according to the manufacturer's instructions. To increase the efficiency of labeling of the intact egg surface, an excess of beads was used in order to ensure that the beads were in close contact with

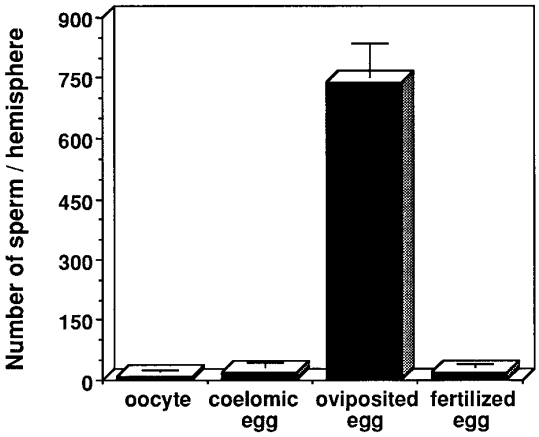

FIG. 1. Measurement of the number of sperm bound to one hemisphere of an oocyte, coelomic egg, dejellied oviposited egg, or dejellied fertilized egg (0.5 hr after fertilization). The number of bound sperm per hemisphere was determined by focusing stepwise through the depth of the top half of the egg, and counting sperm heads. (For details of the assay see Tian *et al.*, 1997.) Bars indicate the standard deviation (SD) with $n = 15$ eggs.

the egg surface. After iodination, the eggs were rinsed three times in ice-cold 10 m*M* Tris–DeBoers solution (pH 7.5). Envelope proteins were prepared from these eggs as described previously (Tian *et al.*, 1997). Proteins were analyzed by 7.5% SDS–PAGE and ^{125}I-labeled proteins were detected by autoradiography.

Protein Determinations

The bicinchoninic acid (BCA) kit (Pierce Chemical Co., Rockford, IL) was used to determine protein concentrations. Bovine serum albumin (BSA) was used as a standard.

RESULTS

Xenopus Sperm–Egg Binding Is Developmentally Regulated

Using the quantitative gamete binding assay, the interaction between *Xenopus* sperm and eggs at the following developmental stages was studied: fully grown stage VI oocyte, coelomic egg prior to entry into oviduct, oviposited matured egg, and fertilized egg (0.5 hr after fertilization). It was found that sperm bound only to unfertilized oviposited eggs, not to oocytes, coelomic eggs, or fertilized eggs (Fig. 1; see also Fig. 4A). It was calculated that approximately 1500 ± 200 sperm could bind to an unfertilized egg (1.2–1.3 mm in diameter) in the presence of jelly extract and a saturating amount of sperm ($\geqslant 1.0 \times 10^7$ sperm/ml). Compared with this level, the average number of sperm bound to the oocyte, coelomic egg, or fertilized egg was negligible. This was also true when the binding assay was performed in 0.3× MR (pH 7.8) instead of in jelly extract.

The Sperm Receptor (gp69/64) or Its Modified Form Is Present in OE, CE, VE, and FE

We recently reported that the gp69/64 glycoproteins function as a sperm receptor in the VE and that proteolytically processing of them *in situ* by crude collagenase treatment abolishes sperm binding (Tian *et al.*, 1997). Given these findings we asked if the lack of sperm binding to the envelopes of oocytes, coelomic eggs, or fertilized eggs was due to the absence or modification of these receptor proteins. Purified total glycoproteins isolated from the OE, CE, VE, and FE were separated by SDS–PAGE, transferred, and immunoblotted with polyclonal anti-gp69/64 antibody. It has been shown that this antibody recognizes only gp69 and gp64, not any of the other VE proteins (Tian *et al.*, 1997). The result of the Western blot shown in Fig. 2 indicates that the anti-gp69/64 antibody also recognized the 69- and 64-kDa proteins in OE and CE, as well as the 66- and 61-kDa proteins in the FE. It was clear that there was no detectable molecular weight change of gp69 or gp64 in the transition from OE to CE or from CE to VE. This result confirmed the identity of the 69- and 64-kDa bands in OE and CE, and provided additional proof that gp66 and gp61 in the FE are indeed derived from gp69 and gp64 in the VE (Gerton and Hedrick, 1986b).

Inhibitory Effects of the Receptor Proteins Isolated from OE, CE, VE, or FE on Sperm–Egg Binding

In view of the above observations we asked if the sperm receptor proteins purified from the OE, CE, or FE were functional in mediating sperm binding as effectively as the gp69/64 proteins isolated from the VE (Tian *et al.*, 1997). Using the gamete binding competition assay we determined if

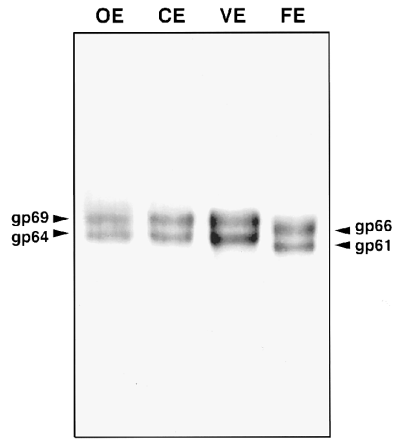

FIG. 2. Western blot showing the presence of gp69/64 glycoproteins in the OE, CE, and VE, and the modified forms, gp66/61, in the FE. Total envelope glycoproteins (1.5 μg) purified from different sources were separated by 7.5% SDS–PAGE and electroblotted onto a nitrocellulose membrane. Polyclonal anti-gp69/64 was used as primary antibody for the immunoblot. See Materials and Methods for details.

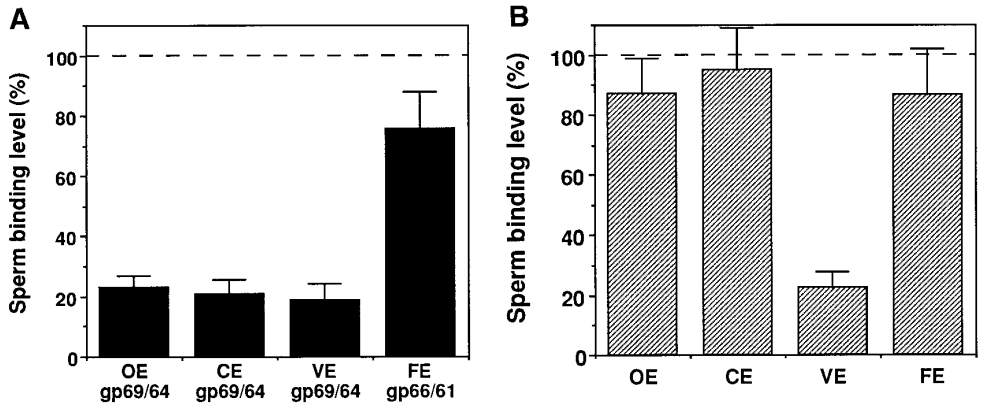

FIG. 3. Inhibitory effects of envelope glycoproteins on sperm–egg binding. (A) Purified gp69/64 glycoproteins (5 μg/ml) from OE, CE, VE, and gp66/61 from FE were used as competitors in the sperm binding competition assays. (B) Heat-dissolved total envelope proteins (50 μg/ml) isolated from OE, CE, VE, or FE were used as competitors. The sperm binding level in the presence of equal concentration of BSA (5 μg/ml in A or 50 μg/ml in B) was used as control and designated as 100% (- - -). Bars indicate SD with $n = 15$ eggs.

gp69/64 proteins purified from OE or CE, or gp66/61 from FE could inhibit sperm–egg binding as effectively as the gp69/64 from VE. Dejellied eggs were mixed with sperm preincubated with equal concentrations of gp69/64 purified from the OE, CE, and VE, or gp66/61 purified from the FE in the presence of jelly extract. The results of the sperm binding competition assay are shown in Fig. 3A. It was found that gp69/64 from OE or CE inhibited sperm–egg binding almost as effectively as gp69/64 from VE. In contrast, gp66/61 was far less effective in inhibiting sperm–egg binding than gp69/64.

We also tested the ability of heat-dissolved total proteins of the OE, CE, VE, and FE to inhibit sperm–egg binding. The envelopes can be dissolved in water by heating for a short time. This treatment has been shown to dissociate the envelopes into soluble supramolecular complexes that are large enough to be excluded from Sepharose 4B that has an exclusion limit of 5×10^6 for globular proteins, but is insufficient to completely dissociate the envelope into individual proteins. After this treatment, the properties of these complexes are similar to the intact envelopes from which they were derived, such as the surface iodination profiles (Nishihara *et al.*, 1983). We found that the heat-dissolved total proteins of the VE were inhibitory; but the heat-dissolved total OE, CE, or FE proteins did not have significant inhibitory effects on sperm–egg binding (Fig. 3B). This observation, coupled with the findings of Nishihara *et al.* (1983), established that the heat-dissolved total envelope proteins behave similarly to the intact envelopes with respect to sperm binding (see Fig. 1).

Anti-gp69/64 Antibody only Recognizes the Surface of VE, not OE, CE, or FE

The observation that the purified sperm receptor inhibited sperm–egg binding *in vitro* but was not functional in the intact OE, CE, or FE or in the solubilized form of these envelopes suggested that the presence of other envelope glycoproteins or differences in the structures of the envelopes at various developmental stages could affect the function of the sperm receptor. To further test this hypothesis, we used the anti-gp69/64 antibody to determine the accessibility of the sperm receptor in the envelopes of oocytes, coelomic eggs, and fertilized eggs. As shown in Fig. 4B, the polyclonal anti-gp69/64 bound only on the surface of the envelope of the oviposited eggs, not to the surface of oocytes, coelomic eggs, or fertilized eggs. Thus, the antibody staining properties agree well with the sperm binding properties shown in Fig. 4A. Since the anti-gp69/64 antibodies have been shown to be capable of inhibiting sperm–egg binding (Tian *et al.*, 1997), the simplest interpretation is that they do so by binding to the sperm binding sites (see Discussion). Therefore, the inability of the antibodies to recognize the surface of the oocytes, coelomic eggs, or fertilized eggs suggests that the functional sperm binding sites involving gp69/64 are present and accessible only on the surface of the VE; they are not accessible on the OE, CE, or FE. This presumably is the reason why sperm cannot recognize or bind to the envelopes at those stages.

^{125}I Labeling of VE Surface

Further evidence that gp69/64 is readily accessible on the egg surface was obtained in an experiment in which the surface of dejellied eggs or water "solubilized" total VE proteins were labeled with ^{125}I. When the labeled proteins were analyzed by SDS–PAGE and autoradiography, it was found that the 69- and 64-kDa bands were the two most intensively labeled bands, even though they are quantitatively minor components of the total VE proteins. In contrast, gp41 was very poorly labeled (Fig. 5), even though it is the most abundant component in the VE and can readily be labeled by ^{125}I when separated from other VE proteins (data not shown). Radioiodination of

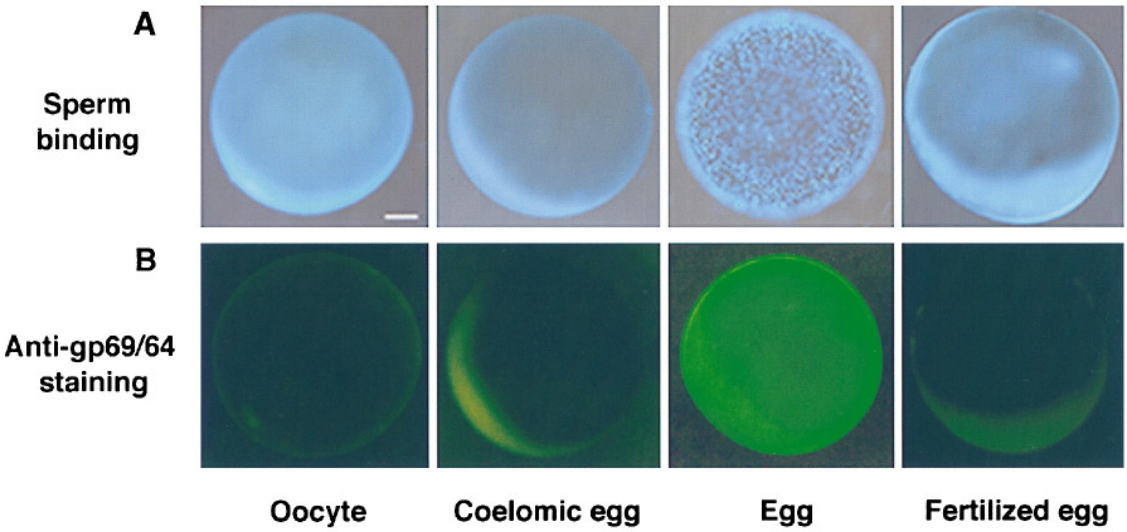

FIG. 4. (A) Fluorescence micrographs of sperm binding to an oocyte, coelomic egg, dejellied oviposited egg, and fertilized egg (0.5 hr after fertilization). The sperm heads were stained with Hoechst 33342 (0.2 mg/ml) and appeared in the images as bright dots on the surface of the top, darker animal hemisphere the egg. The vegetal hemisphere appeared brighter due to autofluorescence. Because of the size of the egg (1.2–1.3 mm in diameter), not all of the sperm are in focus. Bar, 0.2 mm. (B) Fluorescence micrographs showing polyclonal anti-gp69/64 antibody staining of an oocyte, coelomic egg, dejellied oviposited egg, and a dejellied fertilized egg. The vegetal hemisphere appears brighter due to autofluorescence, not because of a higher level of staining by the antibody. See Materials and Methods for details.

water-solubilized total VE proteins showed identical labeling profile to labeled intact VE, again suggesting that the VE glycoproteins exist as complexes in the aqueous solution that are similar to their organization in the intact envelope.

Mechanisms of Blocking Sperm Binding Following Fertilization

Upon fertilization, cortical granule exocytosis results in the conversion of gp69/64 to gp66/61 and the formation of the F-layer outside of the structurally altered VE (see Introduction). Following these alterations, sperm are no longer able to bind to the fertilized eggs (see Fig. 1). To investigate the contribution of each of these two changes to the loss of sperm binding, oviposited eggs were activated by addition of calcium ionophore A23187 under different conditions so that only one change, i.e., either the formation of the F-layer or the cleavage of gp69/64 occurred. It has been shown that high salt solutions, such as that in full strength DeBoers solution (1× DB, 110 m*M* NaCl, 1.3 m*M* KCl, 1.3 m*M* $CaCl_2$, to pH 7.2 with $NaHCO_3$), effectively inhibits the proteolytic processing of gp69/64 that occurs during egg activation, while not affecting F-layer formation as a result of binding of cortical granule lectin to its ligand in the jelly (Lindsay and Hedrick, 1989). Therefore, to determine if the formation of the F-layer by itself results in a block to sperm binding, oviposited eggs with jelly were preincubated in 1× DB for 30 min and then activated by addition of ionophore to a final concentration of 2 μM. Activation of the eggs was evident by the occurrence of the cortical contraction. Approximately 30 min after activation, when the F-layer had completely formed, the eggs were dejellied and assayed for sperm binding in 0.3× MR (pH 7.8). The result of the sperm binding assay shown in Fig. 6A (bar 2) indicates that sperm do not bind to eggs that have a F-layer, even though the gp69/64 glycoproteins were not processed, as shown by SDS–PAGE analysis of the envelope proteins collected from these eggs (Fig. 6B, lane 2). A control group of eggs in 1× DB that was not activated showed normal sperm binding activity (Fig. 6A, bar 1) and contained unpro-

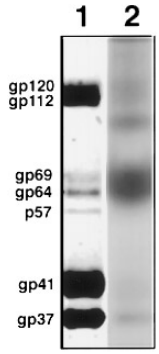

FIG. 5. ^{125}I surface labeling profile of the glycoproteins in the intact VE. Lane 1, VE proteins separated by 7.5% SDS–PAGE and stained with Coomassie blue. The proteins are identified on the left. Lane 2, autoradiograph of radioiodinated VE surface proteins. A minor contaminating band below gp112 (not visible in Coomassie blue-stained gel) was also labeled.

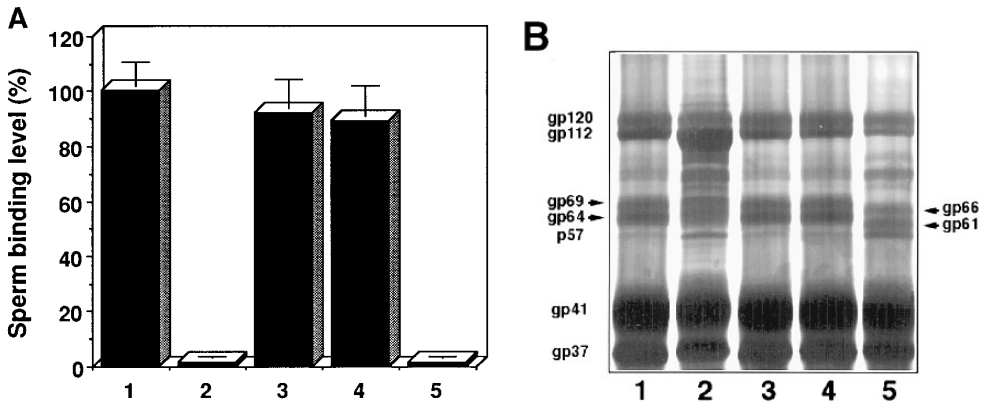

FIG. 6. (A) Effects of F-layer or hydrolysis of gp69/64 on sperm binding. The experimental design was described in the text. Eggs were activated by addition of 2 μM (final concentration) of ionophore A23187 under different conditions. Thirty minutes after activation, the eggs were washed with 0.3× MR (pH 7.8) and tested for sperm binding activity. Bars indicate relative sperm binding levels to: 1, unactivated, mature, dejellied eggs; the average number of sperm bound to each egg in this group (1300 ± 180) represents approximately the normal binding level and is set as 100%. The relative levels of sperm binding to other groups was obtained by comparison to this number; 2, eggs with intact jelly preincubated in 1 × DB (pH 7.2) for 0.5 hr and then activated and dejellied; 3, 4, and 5, dejellied eggs activated in 1× DB, 2 mM of chymostatin in 0.05× DB, or 0.05 × DB (without protease inhibitor), respectively. Bracketed lines represent SD with n = 20 eggs. (B) SDS–PAGE analysis of total envelope proteins from the same samples in (A). The gel was stained by silver.

cessed gp69/64 (Fig. 6B, lane 1). When an aliquot of the activated eggs was incubated with the polyclonal anti-gp69/64 antibody and then FITC-conjugated anti-IgG, and examined with fluorescence microscopy (as described in Fig. 4), it was found that the surfaces of these eggs were not recognized by the anti-gp69/64 antibody, another indication that the F-layer had formed and blocked access to gp69/64 on the envelope surface.

Next, we studied the effect of the proteolytic processing of gp69/64 on sperm–egg binding. The formation of F-layer was prevented by removing the egg jelly coat before activation, which removes the ligand for the cortical granule lectin. It has been shown that the protease that causes the cleavage of gp69/64 can be activated only in low salt buffer, such as 0.05× DB (pH 7.2), but not in high salt buffer, i.e., 1× DB, and that the cleavage of gp69/64 can be inhibited by protease inhibitors such as chymostatin (Lindsay and Hedrick, 1989). Therefore, we measured the sperm binding levels of eggs activated under these conditions and found that dejellied eggs activated in 1× DB or in the presence of 2 mM of chymostatin (in 0.05× DB) still retained high levels of sperm binding activity (Fig. 6A, bars 3 and 4); under these conditions gp69/64 was not cleaved (Fig. 6B, lanes 3 and 4). However, when activated in 0.05× DB, the eggs lost more than 98% of their sperm binding activity (Fig. 6A, bar 5) compared with unactivated eggs, and gp69/64 was processed to gp66/61 (Fig. 6B, lane 5).

Sperm Detachment from Egg Surface during Egg Activation

Having established that the truncation of gp69/64 alone is able to abolish the sperm binding to the egg envelope, we next studied the process of sperm detachment from dejellied eggs. In this experiment, a high concentration of sperm (10^7 sperm/ml) was incubated with dejellied eggs in 0.3× MR (pH 7.8) for 20 min to allow maximal sperm binding. Without jelly extract, sperm will not fertilize dejellied eggs. At the end of this period, free unbound sperm were washed away and the eggs were separated into two groups. One group was switched to 1× DB solution (pH 7.2), conditions under which hydrolysis of the sperm receptor will not occur (Lindsay and Hedrick, 1989). The other group of eggs was placed in 0.05× DB solution (pH 7.2) to allow the hydrolysis of gp69/64 when the eggs are activated. The activation of the eggs was achieved by addition of 2 μM of ionophore A23187 to both groups at the same time because ionophore A23187 is more effective than sperm in synchronously activating eggs. After addition of ionophore, an aliquot of eggs from each group was taken at different time points during a 40-min period, quickly washed with 1× DB solution and fixed immediately in ice-cold 3% formaldehyde, and the number of sperm remaining bound on each egg was determined. It was found that sperm gradually detached from the dejellied eggs activated in 0.05× DB, but remain attached to the eggs in 1× DB over the time of the experiment (Fig. 7).

DISCUSSION

In *X. laevis*, the ultrastructural and biochemical properties of the egg vitelline envelope have been well characterized, and yet very little has been known about the exact role of the envelope in sperm–egg interaction during fertilization. In this study, the function of the envelope with

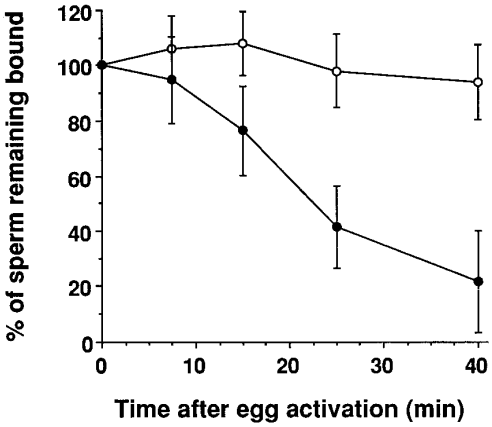

FIG. 7. Kinetics of detachment of sperm from dejellied eggs after egg activation. Sperm (10^7 sperm/ml) were incubated with dejellied eggs in 0.3× MR (pH 7.8) for 20 min. Then, the eggs were divided into two groups: one group was washed with 1× DB, pH 7.2 (open circles); the other was washed with 0.05× DB (filled circles), to remove unbound sperm. After the wash, the egg in both groups was simultaneously activated by addition of 2 μM of ionophore A23187. At different time points, an aliquot of eggs was removed to determine the number of sperm remaining bound on the egg surface (see text). The average number of sperm bound on the dejellied eggs (1500 ± 200 sperm/egg) immediately before activation was normalized as 100%. Bracketed lines represent SD with n = 15 eggs.

respect to its sperm binding activity was addressed, with emphasis on understanding its molecular and cellular basis and how it is regulated. Using the gamete binding assay, it was found that sperm binding activity of the egg envelope is developmentally regulated. Sperm bind only to unfertilized oviposited eggs, not to oocytes, coelomic eggs, fertilized eggs, or to developing embryos. The changes in sperm binding activity of the envelope and the timing appear to correlate well with the compositional and structural changes of the envelope that have been shown to occur during the transitions from CE to VE and from VE to FE (reviewed in the Introduction).

Recently, we established that a pair of related glycoproteins, gp69/64, that comprise a small fraction of the total protein of the VE function as sperm receptors (Tian *et al.*, 1997). In the current study, we investigated how the biochemical and ultrastructural changes in the envelope affect receptor-mediated sperm binding activity to the envelope at different developmental stages. It was found that the isolated, heat-dissolved total proteins from the VE inhibited sperm binding, while the total proteins from the OE, CE, or FE did not. However, Western blot analysis established that the receptor proteins, gp69 and gp64, were present in the OE and CE, and the truncated forms, gp66 and gp61 in the FE. Furthermore, when the gp69/64 was isolated in pure form from either the OE or CE, it was found to be almost as active as gp69/64 isolated from the VE in inhibiting sperm–egg binding. In contrast, gp66/61 isolated from the FE did not inhibit sperm binding. Since the gp69/64 in the OE and CE appear to have the same masses as the gp69/64 in the VE, and the purified proteins from OE or CE are almost equally functional as those from the VE in inhibiting sperm binding, we conclude that the gp69/64 glycoproteins in the OE or the CE are already fully mature and functional. As discussed earlier, in heat-dissolved envelopes the envelope components still remain aggregated and retain similar structural relations as in the intact envelopes (Nishihara *et al.*, 1983). The apparent masking of sperm binding activity of the receptor in both the heat-dissolved and in the intact OE or CE suggest that the sperm binding activity of the receptors is regulated by other envelope glycoproteins in these envelopes . The previously described polyclonal antibody that specifically recognizes gp69/64 glycoproteins in both their denatured states and native states in the VE can block sperm binding to the unfertilized oviposited eggs and fertilization (Tian *et al.*, 1997). Since the two glycoproteins together contribute only approximately 4% to the total mass of the envelope proteins, the blocking of sperm binding by the antibody seemed not likely to be caused by nonspecific masking of the envelope surface. This conclusion was also supported by an electron microscopy study of the VE surface labeled with colloidal-gold-conjugated anti-gp69/64 antibody (data not shown). Therefore, we used the polyclonal anti-gp69/64 antibody as a probe to assess the accessibility of the sperm binding sites on the surfaces of the various envelopes. It was found that the surface of the oocyte, coelomic egg, or the fertilized egg could not be stained by the polyclonal anti-gp69/64 antibody, suggesting that although the glycoproteins are present, functional binding sites are not accessible on the egg surface at those stages. The only stage when the surface could be stained was the oviposited egg before fertilization, which also is the only time period in which sperm binding was observed. These results suggest that in the frog a novel mechanism, namely, control of accessibility of the sperm receptor to sperm, regulates sperm–egg adhesion. In the mouse or the sea urchin, this type of regulation of gamete interaction has not been detected.

Given these findings we next turned to the question of what component in the OE or CE prevents gp69/64 from functioning in sperm binding. During the CE to VE transition, the only biochemical change detected is the conversion of gp43 to gp41, the most abundant component (~43% of the mass) of the envelope (Gerton and Hedrick, 1986a; Hardy and Hedrick, 1992). This modification seems to trigger a reorganization of the envelope glycoproteins that can be detected by changes in ^{125}I-labeling of the egg surface glycoproteins. The gp43 in the CE was intensely labeled (data not shown, but see Nishihara *et al.*, 1983), whereas the labeling of the corresponding gp41 in the VE was very weak (see also Gerton, 1986). In contrast to the cryptic behavior of gp41 in the VE, we observed that gp69/64 was readily accessible and was the most intensely labeled band, even though this pair of proteins are quantitatively minor

components (~4% in mass) of the total VE proteins. The reorganization of the envelope has also been detected by other means, such as dye binding, solubility, chemical modification, deformability studies (Larabell and Chandler, 1988a, 1989; Bakos *et al.*, 1990a,b), and by direct ultrastructural studies of the envelopes. Using quick-freeze, deep-etch, rotary-shadow electron microscopy, it was observed that the loosely bundled large and long fibers that run throughout the CE surface are dispersed and become a dense network of long, swirling fibers seen on the VE surface (Larabell and Chandler, 1988a,b). It has been postulated that the proteolytic processing of gp43 might involve the cleavage of the protein domain that holds the fibers in bundles in the CE to allow these bundles to intermix with the other smaller fibrils (Larabell and Chandler, 1988a). Similar ultrastructural and macromolecular changes during the CE to VE conversion also occur in other anuran species studied, such as *Bufo japonicus*, *Bufo arenarum*, and *Rana japonica*, (Katagiri *et al.*, 1982; Mariano *et al.*, 1984; Yoshizaki and Katagiri, 1981; Takamune and Katagiri 1987). Given the similarity between these changes, it is possible that they play a similar role in converting the egg envelope from a sperm-nonreceptive form to a sperm-receptive form.

Recently, it has been proposed that in *B. japonicus*, the 36- to 39-kDa components in the vitelline coat may possess sperm binding activity (Omata and Katagiri, 1996).To determine if gp41 in the VE of *X. laevis* is also involved in sperm binding, we purified gp41 by SDS/PAGE and electroelution and tested its ability to inhibit sperm–egg binding (Tian *et al.*, 1997). It was found that the gp41 protein purified in this way did not show significant inhibitory effect compared to gp69/64. However, we have not yet been able to exclude the possibility that gp41 lost its biological activity during the purification procedure. We also found that the short glycopeptides generated by extensive Pronase digestion of gp41 did not inhibit sperm-egg binding. Another line of evidence against gp41 functioning as a sperm binding protein comes from egg surface iodination studies (Nishihara *et al.*, 1983). It was found that gp43 in the CE, the precursor of gp41, was very effectively labeled by cell-surface-specific labeling reagents, such as lactoperoxidase or IODO-GEN procedures, while gp41 in the VE was not labeled. This observation suggests that gp41 is not exposed on the cell surface or is masked by other VE components. Although we found no convincing evidence that gp41 in *X. laevis* functions as a surface receptor for sperm, based on Western blot analysis gp41 seems to share some epitopes in common with gp69/64, (Tian *et al.*, 1997). The function of these common epitopes remains to be determined. Also, the molecular relationship between gp41 in *X. laevis* and the 36- to 39-kDa VC components in *B. japonicus* needs to be determined. Given the fact that gp41 is the most abundant component in the VE, it must play an important structural role in the envelope, and therefore, it is not surprising that covalent modification of gp43 has a dramatic influence on sperm accessibility of gp69/64 in the envelope.

The loss of sperm binding upon fertilization or activation of eggs can be attributed to two events: the formation of the F-layer and the proteolytic processing of the sperm receptor, gp69/64. However, our findings indicate that either event alone is sufficient to abolish sperm binding. When eggs with jelly were activated under ionic conditions that prevented processing of gp69/64, but allowed formation of the F-layer, sperm binding did not occur. Similarly, sperm did not bind when eggs were dejellied and then activated under ionic conditions that allowed processing of gp69/64 but, because of the absence of jelly, did not form the F-layer. However, when dejellied eggs were activated in a high salt buffer (1× DB), or in low salt buffer (0.05× DB) but with chymostatin added to prevent the hydrolysis of gp69/64, sperm binding was observed because the eggs contained an intact gp69/64 not masked by a F-layer.

In addition, we found that the proteolytic processing of gp69/64 during fertilization not only abolished sperm binding, but also promoted sperm detachment (Fig. 7). The sperm detachment process seems to be preceeded by the proteolytic processing of the sperm receptor, which is believed to be triggered by ultrastructural alteration and hardening of the egg envelope. Based on electron microscopic examination, the transformation of VE to the altered FE is complete within 5 min after insemination (Larabell and Chandler, 1988b). The envelope hardening caused by the overall structural change occurs somewhat later but still is completed by 10 min after egg activation (Lindsay and Hedrick, 1989). The proteolytic processing of gp69/64 starts after the reorganization of the envelope is almost complete, and when the corresponding zymogen is activated by a protease cascade in the perivitelline space (Lindsay and Hedrick, 1989; 1995). Lindsay and Hedrick (1989) reported that the hydrolysis of gp69/64 occurs between 10 and 25 min after egg activation, with $t_{50\%}$ of about 13.5 min. In our experiments sperm detachment was not observed within the first 10 min after egg activation. In agreement with the observations discussed above, we believe that this period corresponds to the time required for the early events to occur, including transformation and hardening of the envelope, and the activation of the protease cascade. After 10 min, the bound sperm gradually fell off the eggs and by approximately 20–25 min, 50% of the bound sperm had detached from the activated eggs. Collectively, the formation of the F-layer blocks access of unbound sperm to the VE, and the processing of the sperm receptor causes detachment of already bound sperm. Together, these two processes could form a very effective block to polyspermy. Hydrolysis of similar envelope proteins have been found to occur during fertilization of other anuran amphibians, such as gp65/61 to gp62/58 in *B. japonicus* and gp69 to gp64 in *Rana pipiens* (Lindsay *et al.*, 1988); perhaps the same dual process to prevent polyspermy described in *X. laevis* also functions in these other anuran species.

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