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Immunohistochemical localisation of gp69/64 molecules in *Xenopus* egg envelopes in relation to their sperm binding activity

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Summary

A monoclonal antibody (6964M) was generated against the envelope component gp69/64 of *Xenopus laevis* eggs. On indirect immunofluorescence using this antibody, the positive reaction was seen on the surface of both vitelline envelope (VE) and coelomic envelope (CE). On immunoelectron microscopy, gp69/64 was preferentially distributed on the thick bundles forming the edge of the tunnel openings on CE, and this distribution pattern was fundamentally inherited by VE. Counting the number of immunogold particles indicated that VE has about twice as many particles as CE, with a 3–4 times higher density at the animal pole than vegetal pole. The number of sperm bound to CE was small, being approximately one-twentieth of the number of sperm bound to VE. An extremely small number of sperm (< 2 per animal hemisphere) was found to bind to VE* of activated eggs as a background. The sperm binding to CE was inhibited by pretreatment of the envelopes with 6964M or in the presence of purified gp69/64 from VE on insemination, confirming that sperm binding is mediated by gp69/64 exposed on the CE surface. In spite of at most a 2-fold increase in the amount of exposed gp69/64, the sperm binding increased about 20-fold upon CE-to-VE conversion, suggesting that the increase in the amount of exposed gp69/64 is itself insufficient to explain the increase in the number of bound sperm.

Keywords: Coelomic envelope, Sperm binding, Sperm receptor, Vitelline envelope, *Xenopus laevis*

Introduction

Sperm binding to the egg envelope is one of the most important events in the process of gamete membrane fusion, because the binding includes direct cell–extracellular matrix (sperm–envelope) interactions as the recognition step between the gametes. For example, cross-hybridisation and polyspermy are effectively blocked at the step of sperm binding to the egg envelope. The mammalian egg envelope zona pellucida is composed of three species of glycoproteins – ZP1, ZP2 and ZP3 (reviewed by Wassarman, 1988) – and the carbohydrate moiety of mouse ZP3 is known to play a role as an egg receptor for the acrosome-intact sperm (reviewed by Wassarman, 1999). In fact, site-directed mutagenesis of the C-terminally located Ser residues in mouse ZP3 results in a loss of sperm receptor activity (Chen *et al*., 1998), and targeted mutagenesis of mouse ZP3 gene followed by rescue with human ZP3 gene results in the recovery of accessibility and fertilisability by mouse sperm but does not result in the gain of accessibility by human sperm (Rankin *et al*., 1998), supporting the notion that the saccharide portion of ZP3 is involved in mouse sperm–zona binding. The involvement of carbohydrate moieties is also true for sperm–egg interaction in the pig (Yonezawa *et al*., 1995), bivalves (Focarelli & Rosati, 1995), sea urchins (Dhume & Lennarz, 1995; Dhume *et al*., 1996), ascidians (reviewed by Hoshi, 1986; Honegger, 1992; Baginski *et al*., 1999) and amphibians including *Bufo arenarum*

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(Valz-Gianinet *et al*., 1991), *B. japonicus* (Omata & Katagiri, 1996) and *Xenopus laevis* (Tian *et al*., 1997*a*; Vo & Hedrick, 2000).

Among non-mammalian vertebrates, *Xenopus* provides a valuable system for the investigation of sperm–envelope binding, because this frog is the only non-mammalian vertebrate for which a whole set of three ZP homologues has been documented, i.e, gp37 (Kubo *et al*., 2000), gp69/64 (Tian *et al*., 1999) and gp43/gp41 (Kubo *et al*., 1997; Yang & Hedrick, 1997) as homologues of mammalian ZP1 (ZPB), ZP2 (ZPA) and ZP3 (ZPC), respectively. In addition, an envelope component as a hatching enzyme substrate, designated ZPAX, has recently been cloned (Lindsay *et al*., 2001). Unlike in the mouse, the ZP2 homologue gp69/64 possesses the role of the receptor for sperm as a result of its exposure on the surface of the vitelline envelope (VE) by the proteolytic processing of the coelomic envelope (CE), gp69/64 being reportedly hidden from the surface of CE (Tian *et al*., 1997*a*, 1997*b*, 1999). This CE-to-VE conversion is caused by the processing of the predominant component of CE gp43 at Arg61 and Arg373 into VE gp41 (Kubo *et al*., 1997, 1999) with a trypsin-type protease, oviductin, secreted from the pars recta during the passage of coelomic eggs down this portion of the oviduct (reviewed by Katagiri, 1987). The inaccessibility of CE to sperm binding is apparently correlated with the observed lack of gp69/64 on the CE surface upon immunostaining of live eggs with polyclonal antibodies against gp69/64 (Tian *et al*., 1997*b*). On the other hand, Vo & Hedrick (2000) have recently reported that both gp41 and gp69/64 independently possess ligand activity for sperm binding, and that the major ligand is gp41. Although the evaluation of the ligand activity of gp41 is quite controversial, probably due to whether the activity was measured with dejellied eggs (intact VE) (Tian *et al*., 1997) or envelope components artificially coupled to glass (Vo & Hedrick, 2000), it is acceptable in some degree at least that gp69/64 is one of the ligands for sperm binding.

In the present study we established a monoclonal antibody (6964M) against gp69/64 by immunising mice with the antigen purified from VE. By indirect immunofluorescence staining of live eggs with 6964M, we found that the antigen is exposed on the surface not only of VE but also of CE, contrary to the report by Tian *et al*. (1997*b*). The monoclonal antibody was also used for evaluating the exposure level of gp69/64 on the surface of the egg envelopes by immunoelectron microscopy techniques. By precisely counting the number of sperm bound to the egg envelopes, we show here that gp69/64 exposed on the CE surface is indeed accessible by sperm, to a significant but lesser extent than that on VE.

Materials and methods

Materials

Sexually mature males and females of *X. laevis* were obtained from a dealer (Copacetic, Aomori, Japan) and maintained at 20 °C. Protein concentration was estimated with bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL) with bovine serum albumin or mouse monoclonal IgM (Sigma, St Louis, MO) as a standard. Type I collagenase from *Clostridium histolyticum* was purchased from Sigma.

Procurement of gametes and isolation of egg envelopes

Sperm were obtained as described previously (Katagiri *et al*., 1999). Ovulation was induced by injection of human chorionic gonadotropin (hCG) as described previously (Kubo *et al*., 1997). Coelomic eggs were obtained from the body cavity of females 5–6 h after the injection (Kubo *et al*., 1999). Uterine eggs were collected into De Boer's solution (DB; 110 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4) by squeezing the hCG-injected females 4 days after priming with pregnant mare serum gonadotropin, and the jelly layer was removed with 45 mM 2-mercaptoethanol in DB (pH 8.9) (Hedrick & Hardy, 1991). CE and VE were prepared from the coelomic and dejellied uterine eggs, respectively, by the sieving method according to Hedrick & Hardy (1991). Eggs bearing VE* (the residual envelope after extraction of the cortical granule lectin-precipitated F-layer from FE) were prepared by electric shock of uterine eggs at 80 V AC current for 10 s in 0.05 DB followed by dejellying with 20 mM dithiothreitol in Ca-free 0.05 DB (adjusted to pH 9.0 with NaOH) and elimination of the F-layer with 5 mM EDTA-containing Ca-free 0.05 DB for 10 min according to Yoshizaki (1994). Type I collagenase-digested VE was prepared according to Tian *et al.* (1997*a*). VE* and type I collagenase-digested VE were collected by the sieving method.

Sperm binding assay

Coelomic eggs, dejellied uterine eggs, activated eggs bearing VE*, or type I collagenase-digested uterine eggs were inseminated, fixed, and stained with a Hoechst dye as described previously (Katagiri *et al*., 1999). The number of sperm specifically bound to the envelope surface except VE was directly counted under a light microscope equipped for epifluorescence (Axiophoto, Carl Zeiss, Oberkochen, Germany). When hundreds of sperm were bound to egg envelopes (especially VE), the sperm number counted on a 0.05 mm^2 area was multiplied by the ratio of the actual

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number of bound sperm on the entire animal hemisphere of CE and that on an area of 0.05 mm^2 of CE with the highest number of bound sperm. The result was regarded as the actual sperm number bound to the animal hemisphere of the egg envelope.

Competitive inhibition of sperm binding to egg envelopes with purified envelope components

VE gp69/64 was purified by SDS-PAGE (Laemmli, 1970) with a 10–20% acrylamide gradient gel for two-dimensional gel electrophoresis (Daiichi Fine Chemicals, Tokyo, Japan). Following staining of the edge of the gel with Coomassie brilliant blue R-250 (CBB), the region corresponding to gp69/64 was excised from the remaining gel, and gp69/64 was recovered from the gel by electroelution. The purity of the envelope components was checked by SDS-PAGE.

For competitive inhibition of sperm binding with envelope components, sperm (1×10^7) were incubated with various concentrations of envelope components in 250 µl of 0.33 DB for 10 min at room temperature, and the suspension mixed with 250 µl of 0.33 DB containing 10 coelomic eggs. The concentrations of envelope components were expressed as the final concentration on insemination. After 30 min of incubation, the eggs were washed, fixed, and stained for counting the number of bound sperm as described previously (Katagiri *et al*., 1999).

Preparation of monoclonal antibodies

The immunisation of mice with the electrophoretically purified gp69/64 and fusion of the lymph node cells with myeloma PAI were carried out as described previously (Kotani *et al*., 1993; Kubo *et al*., 1999). Hybridoma cells were screened by Western blotting. An aliquot of the culture supernatant containing an anti-gp69/64 monoclonal antibody (6964M) from the established clones was used for determination of the isotype of the antibody as described previously (Kubo *et al*., 1999). Ascites were produced in pristane-primed nude mice, and monoclonal antibodies were purified by gel filtration with an FPLC system equipped with a Superdex 200 column (1.0 × 30 cm; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Western blotting

Egg envelopes (8 µg each for CBB staining or 0.1 µg each for Western blotting) were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) with a 7.5% polyacrylamide gel (Daiichi Fine Chemicals, Tokyo, Japan) followed by electroblotting onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Nippon Millipore, Tokyo, Japan) according to Towbin *et al*. (1979). The blotted membrane was blocked with 5% skim milk in PBS for 1h and incubated with the culture supernatant diluted 5-fold with 1% skim milk in phosphate-buffered saline (PBS) for 1 h followed by incubation with secondary antibody as described previously (Kubo *et al*., 1999). A chemiluminescent detection system (Amersham Pharmacia Biotech) was used for detection according to the manufacturer's protocol.

Surface immunostaining of egg envelopes

Coelomic eggs and uterine eggs bearing VE, VE* or type I collagenase-digested VE (10 eggs each) were blocked with 1% bovine serum albumin (BSA) in DB for 30 min, and incubated for 30 min in 0.5 ml of the culture supernatant which was diluted 5-fold with the blocking medium or mouse monoclonal IgM (1 µg/ml) as a control, followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM $F(ab')_2$ (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 100-fold with the blocking medium in the dark for 30 min. The labelled live eggs were washed thoroughly with DB, and examined with an Axiophoto equipped with an epifluorescence apparatus.

Transmission electron microscopy (TEM)

Coelomic eggs and uterine eggs were blocked and incubated with the purified antibody 6964M (4 µg/ml) as described above. The eggs were then incubated with 50-fold diluted gold-conjugated goat anti-mouse IgM (15 nm gold; British Biocell, Cardiff, UK) for 30 min, followed by fixation with 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4). They were processed for TEM observation as described previously (Katagiri *et al*., 1999).

Scanning electron microscopy (SEM)

Coelomic eggs and uterine eggs incubated with 6964M and the immunogold followed by glutaraldehyde fixation as described above were washed with PBS. In order to distinguish between the animal and vegetal hemispheres of the eggs, each egg in a drop of PBS placed on a piece of Parafilm was marked with a tiny cut around the vegetal pole with a razor blade under a dissection microscope. The marked eggs were post-fixed with 1% osmium tetroxide in PBS on ice for 30 min, dehydrated with a graded ethanol series, and then critical point dried with isoamyl acetate as a transitional fluid. The specimens on a sample stage were coated with osmium (~1 nm thick) with an osmium plasma coater NL-OPC80A (Nippon Laser & Electronics Lab., Nagoya, Japan), and examined with

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an S-4500 scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 5 kV for secondary electron image and back-scattered electron image with a YAG detector (Suzuki *et al*., 1994).

Results

Generation of monoclonal antibodies specific to gp69/64

On Western blots using 6964M (IgM), the reactivity was restricted to gp69/64 from CE and VE but not to the N-terminally truncated forms, gp66/61 and gp65/60, generated during FE formation and by type I collagenase digestion of dejellied uterine eggs, respectively (Fig. 1), indicating that 6964M specifically recognises the portion within the N-terminal 27 amino acids of gp69/64. As the reactivity of 6964M was not diminished but rather strengthened by periodate oxidation of the antigen (not shown), the epitope of 6964M was regarded to reside in the peptide moiety of the N-terminal peptide.

In addition to the doublet band of gp69/64, 6964M also reacted with a faint band having a higher Rf value (~48 kDa) in VE (Fig. 1). This minor band was not detected by CBB staining, indicating its very small quantity in the envelope preparations. Since VE was physically fragile compared with CE, presumably this additional minor band represents an artifact lacking the C-terminal region of gp69/64 which was produced during envelope preparation by the sieving method.

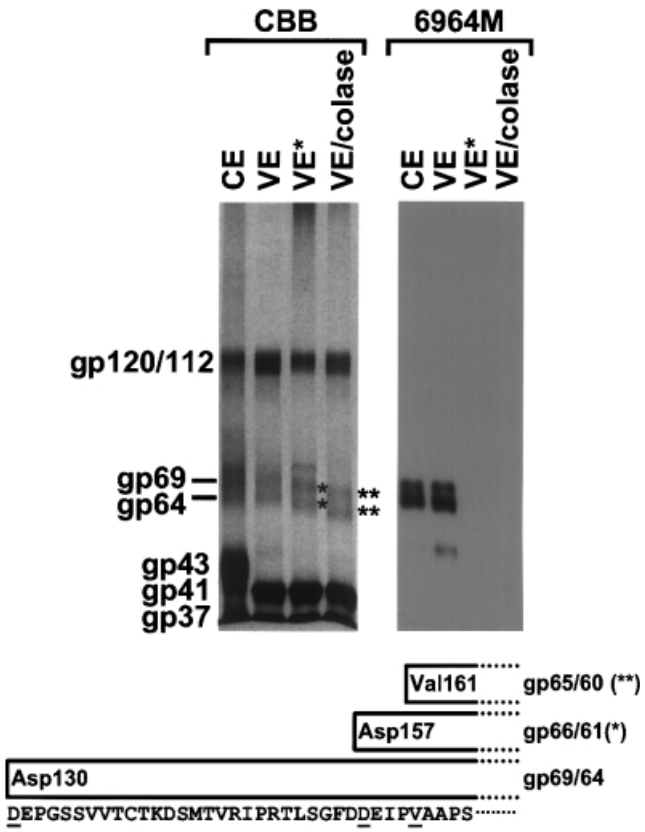

Figure 1 Specificity of monoclonal antibodies against gp69/64. SDS-PAGE profiles of coelomic envelope (CE), vitelline envelope (VE) and vitelline envelopes from activated eggs (VE*) and type I collagenase-digested eggs (VE/colase; 8 µg each) are shown on a 7.5% polyacrylamide gel by Coomassie brilliant blue (CBB) staining. These envelopes (0.1 µg each), electrophoresed and transferred to a PVDF membrane, were probed with the culture supernatant of 6964M followed by chemiluminescent visualisation. 6964M reacts with gp69/64 of CE and VE but not with the N-terminally deleted gp69/64, indicating that it specifically recognises the N-terminal peptide comprising 27 amino acids of gp69/64. The faint band with a higher Rf value immunoreactive to the antibody is assumed to be a truncated form of gp69/64 artifactually deleted on the C-terminal region. The lower illustrations show the N-terminal regions of gp69/64 and its derivatives (gp66/61 and gp65/60 with single and double asterisks, respectively) according to Tian *et al*. (1999).

Exposure of gp69/64 on both CE and VE

By using the monoclonal antibody 6964M, we examined localisation of gp69/64 on the surface of envelopes of live eggs. Contrary to the results of Tian *et al*. (1997*b*) using polyclonal antibodies against gp69/64, fluorescence was seen not only on VE (Fig. 2*C*) but also on CE (Fig. 2*A*). Consistent with the result of Western blotting (Fig. 1), 6964M did not give fluorescence on the surface of VE* (Fig. 2*E*). In addition, no labelling with 6964M was observed in the uterine eggs pretreated with type I collagenase (not shown). Thus, we concluded that a specific epitope in gp69/64 recognised by our 6964M was exposed on the surface of both CE and VE.

Immunoelectron microscopic analyses of egg envelopes

The surface exposure of gp69/64 on CE and VE was further examined by immunoelectron microscopy. Live eggs were pretreated with 6964M and then with a colloidal gold-labelled secondary antibody, followed by fixation with glutaraldehyde for TEM observation. Consistent with the results obtained by immunofluorescence microscopy (Fig. 2), 6964M specifically labelled the surface of both VE and CE (Fig. 3). Especially in CE, the gold particles were deposited around the tunnels formed among criss-crossed fibrous bundles (Fig. 3*A*). No gold particle was observed inside the envelopes or in the perivitelline space (Fig. 3).

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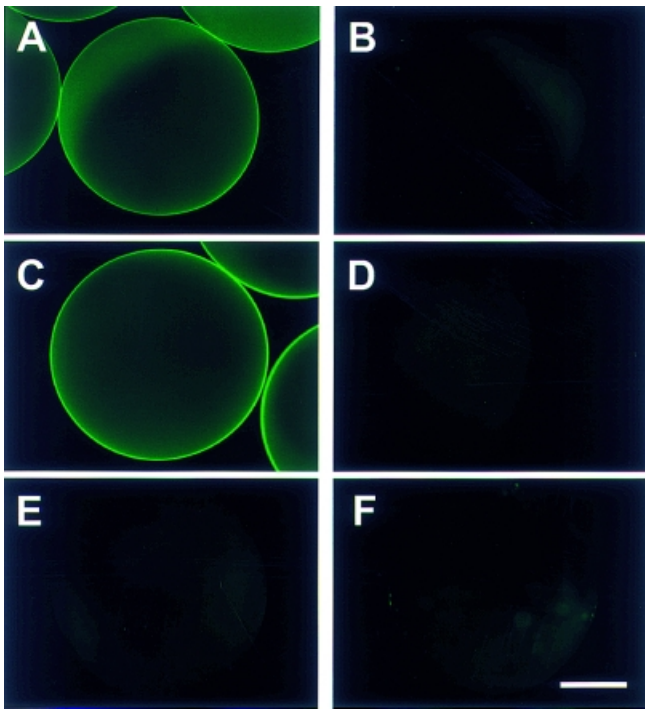

Figure 2 Detection of gp69/64 on CE and VE by indirect immunofluorescence microscopy. Coelomic eggs (*A*, *B*), dejellied uterine eggs (*C*, *D*) and activated eggs followed by dejellying and eliminating the F-layer (*E*, *F*) were incubated for 30 min in 6964M culture supernatant diluted 5-fold with the blocking medium (*A*, *C*, *E*) or mouse IgM (1 µg/ml in the blocking medium; *B*, *D*, *F*). After incubation in FITC-labelled anti-mouse IgM, the live eggs were microscopically observed. gp69/64 is exposed on the surface not only of VE but also of CE. The failure of labelling on the surface of VE* is consistent with the loss of reactivity of the antibody against the N-terminally truncated form of gp69/64 (gp66/61) (cf. Fig. 1). The scale bar in (*F*) represents 300 µm.

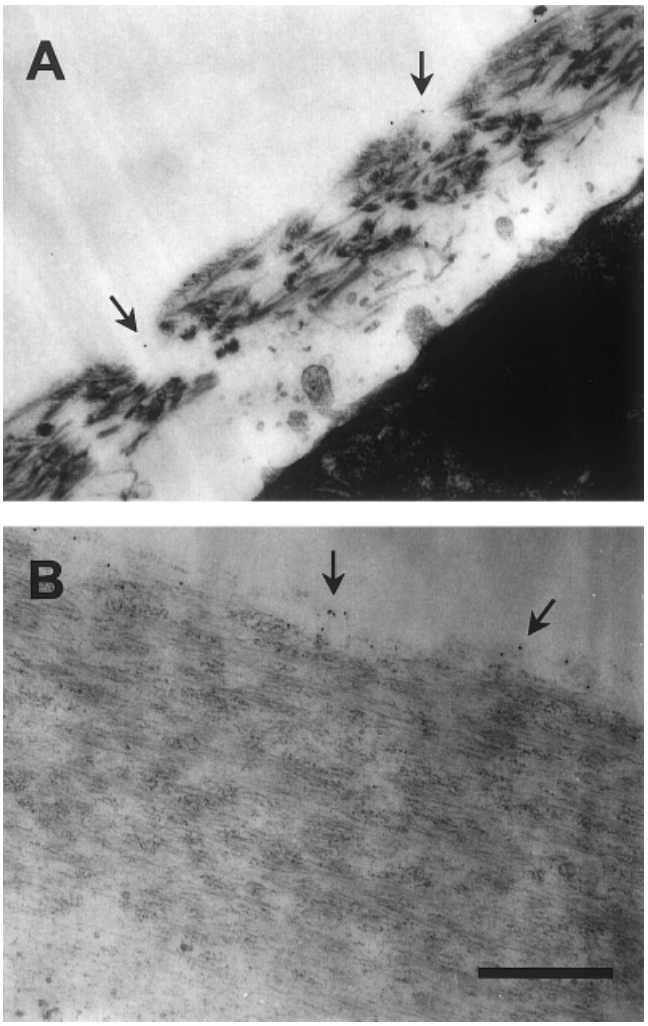

Figure 3 Surface distribution of gp69/64 on CE and VE by TEM. Coelomic eggs (*A*) and dejellied uterine eggs (B) were incubated with 6964M (4 µg/ml) and then with 15 nm colloidal gold-labelled anti-mouse IgM (diluted 50-fold). The eggs fixed with glutaraldehyde were processed for observation by TEM. Gold particles (arrows) were distributed on the surface of the egg envelopes. Especially in CE, the particles were preferentially found at the opening of the tunnels. The scale bar in (*B*) represents 1 µm.

The distribution of the gold particles on the envelope surface was further analysed by a scanning electron microscope equipped with a YAG detector. As described by Grey *et al.* (1977), Larabell & Chandler (1989) and Hedrick & Nishihara (1991), CE shows a very coarse net- or mat-like surface comprising meshworks of large fibres and bundles which are randomly permeated by tunnels (Fig. 4*A*, *C*). Consistent with the TEM observation (Fig. 3*A*), most of the gold particles were preferentially distributed on the bundles along the edge of the tunnel openings (Fig. 4*B*, *D*), and the density of the gold particles was obviously higher around the animal pole than around the vegetal pole (Fig. 4*B*, *D*).

In the case of VE, the tunnels observed in CE are lost and individual fibres of fibrous bundles are dispersed rather uniformly (Fig. 4*E*, *G*), as reported by Grey *et al.* (1977) and Larabell & Chandler (1988). The distribution of the gold particles on the VE surface was not random; rather they tended to be distributed in circles reminiscent of the tunnel openings observed in CE (Fig. 4*F*, *H*). Gold particles were more densely distributed around the animal than the vegetal pole, as for CE (Fig. 4*F*, *H*).

The distribution of gp69/64 on the envelope surface was quantified by counting the number of the gold particles within an area of 1.5 µm^2 at both the animal and vegetal poles of CE and VE (Table 1). Upon CE-to-VE conversion, the particle number increased by 1.8-fold around the animal pole and 1.3-fold around the vegetal pole. As the values were almost the same, oviductin was supposed to exert its digesting effect evenly on the entire surface of CE during the passage of coelomic eggs through the pars recta. When the

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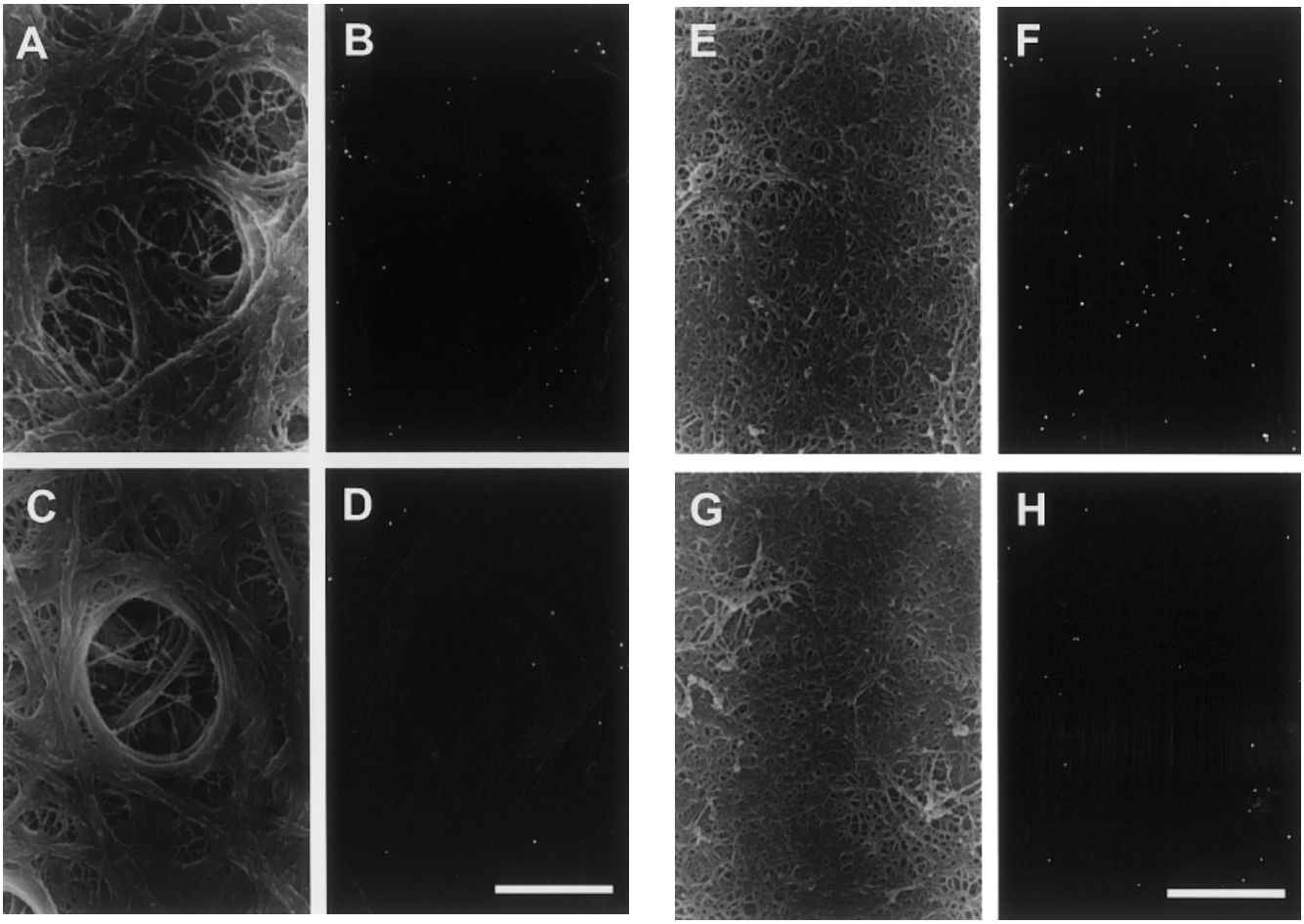

Figure 4 Surface distribution of gp69/64 on CE and VE by SEM. Following successive incubation with 6964M and a colloidal gold-labelled secondary antibody, coelomic eggs around the animal pole (*A*, *B*) and the vegetal pole (*C*, *D*) and dejellied uterine eggs around the animal pole (*E*, *F*) and the vegetal pole (*G*, *H*) were observed by SEM as secondary electron images (*A*, *C*, *E*, *G*) and back-scattered electron images (*B*, *D*, *F*, and *H*). On CE around the animal pole, gp69/64 is preferentially distributed on the thick bundles forming the edge of the tunnel openings (*B*). The distribution pattern is fundamentally inherited by VE (*F*) in spite of the disappearance of the tunnels (*E*). gp69/64 was 3–4 times more densely distributed around the animal pole than the vegetal pole. The scale bars in (*D*) and (*H*) represent 600 nm.

Table 1 Quantification of the number of the gold particles on the envelope surface

Sample	No. of eggs examined	Average particle no.[a]	VEA/ CEA	VEV/ CEV	CEA/ CEV	VEA/ VEV
CEA	3	21.4 ± 5.2			3.10	
CEV	3	6.9 ± 4.8				
VEA	3	37.8 ± 5.9	1.77			4.25
VEV	3	8.9 ± 2.4		1.29		

CEA, CE around the animal pole; CEV, CE around the vegetale pole; VEA, VE around the animal pole; VEV, VE around the vegetal pole.
[a]± SD

particle number was compared between the areas around the animal and vegetal poles, more particles were found around the animal pole; that is, 3.1-fold for CE and 4.3-fold for VE (Table 1). However, there was no apparent difference in the density of the gold particles above and below the equator. The density of the particles decreased rather gradually from the animal to the vegetal pole (not shown), which, however, does not necessarily imply the absence of minor heterogeneity in the density of the gold particles along the animal–vegetal axis.

Sperm binding to CE and VE

Coelomic eggs pretreated with 6964M or mouse IgM were inseminated, and the bound sperm were counted (Table 2). A significant number (~80) of sperm were bound to the animal hemisphere of the control IgM-

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Table 2 Inhibition of sperm binding to CE with 6964M

Treatment	No. of eggs examined	No. of bound sperm[a]	% bound sperm
IgM (20 µg/ml)	10	74.1 ± 12.9	100
6964M (20 µg/ml)	10	1.5 ± 1.1	2.0
6964M (2 µg/ml)	10	2.5 ± 1.7	2.3
6964M (0.2 µg/ml)	9	12.2 ± 12.0	16.5

Coelomic eggs were treated with a monoclonal antibody (6964M) or control mouse IgM, washed with 0.33 De Boer's solution, and inseminated for counting the number of sperm bound to animal hemispheres of eggs.
[a] ± SD.

Table 3 Competitive inhibition of sperm binding to CE by gp69/64

Treatment	No. of eggs examined	No. of bound sperm[c]	% bound sperm
BSA (100 µg/ml)[a]	10	62.6 ± 19.5	100
Sol.VE[b] (100 µg/ml)[a]	8	4.0 ± 3.5	6.4
Sol.VE[b] (10 µg/ml)[a]	9	6.4 ± 4.5	10.2
gp69/64 (50 µg/ml)[a]	9	6.1 ± 4.8	9.7
gp69/64 (10 µg/ml)[a]	8	9.9 ± 7.8	15.8

Sperm were pretreated with solubilised VE or purified gp69/64, and added to coelomic eggs for counting the number of bound sperm.
[a]The concentrations of envelope components and BSA were expressed as the final concentration on insemination.
[b]Heat-solubilised VE.
[c] ± SD.

Table 4 Determination of the conversion ratio for estimation of the total number of the bound sperm on the whole animal hemisphere

Area	No. of sperm									Average no. of sperm[a]	Conversion ratio (A/B)	Average conversion ratio
Experiment 1												
A Whole	54	47	55	30	66	93	32			53.9 ± 21.5	7.7	8.7
B 0.05 mm^2	6	5	8	3	14	8	5			7.0 ± 3.6	–	
Experiment 2												
A Whole	73	91	90	46	98	88	69	59	71	76.1 ± 17.0	9.6	
B 0.05 mm^2	6	7	6	6	13	9	5	10	9	7.9 ± 2.6	–	

Coelomic eggs were inseminated in 0.33 De Boer's solution, and the number of sperm bound to the whole animal hemisphere (A) and to an area of 0.05 mm^2 of the animal hemisphere (B) on the same egg was recorded.
[a] ±SD.

Table 5 Inhibition of sperm binding to VE with 6964M

Treatment	No. of eggs examined sperm no.[a]	Average no. converted	Average sperm sperm	% bound
IgM (20 µg/ml)	7	164.1 ± 28.3	1427.7	100
6964M (20 µg/ml)	8	15.3 ± 6.5	133.1	9.3
6964M (2 µg/ml)	10	16.0 ± 4.5	139.2	9.8
6964M (0.2 µg/ml)	7	132.6 ± 63.3	1153.6	80.8
None (control)	6	145.2 ± 20.2	1263.2	100
Activated[b]	10	1.8 ± 0.9		0.1
MR (pH 6.5)	10	176.0 ± 61.4	1531.2	100
Type I collagenase	10	9.3 ± 1.6		0.6

Dejellied uterine eggs were treated with a monoclonal antibody (6964M) or other reagents indicated, washed with 0.33 De Boer's solution, and inseminated for counting the number of bound sperm.
[a] ± SD.
[b]Eggs were electrically activated and dejellied to remove the F-layer (see Materials and methods).

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pretreated CE (the 'CE level'). Sperm binding was inhibited by pretreatment of the eggs with 6964M in a dose-dependent manner. In addition, sperm binding was competitively inhibited by the presence of heat-solubilised VE or purified gp69/64 on insemination (Table 3). These results indicate that gp69/64 exposed on the CE surface is indeed recognisable by sperm.

In an attempt to estimate the actual total number of sperm bound to CE, the number of sperm bound to the animal hemisphere of CE was counted by two different methods: (A) the total number of sperm bound to the entire animal hemisphere of CE, and (B) the highest number of sperm bound within 0.05 mm^2 on the animal hemisphere of CE (Table 4). The ratio (A/B) based on two different experiments was calculated to be 8.7. Thus, in cases where hundreds of sperm bind to egg envelopes (especially VE), the actual number of sperm bound to the whole animal hemisphere could be estimated by counting the number of sperm bound within 0.05 mm^2 and multiplying this number by 8.7. This conversion enabled us to compare directly the number of sperm bound to CE and VE.

The number of sperm bound on the animal hemisphere of VE was about 1200–1600 (the 'maximum level') (Table 5), based on counting the number of bound sperm within 0.05 mm^2 on the animal hemisphere followed by multiplication by 8.7. The number of bound sperm was dose-dependently decreased by pretreatment of uterine eggs with various concentrations of 6964M (Table 5). When activated eggs with VE* were inseminated, the number of the bound sperm was about 2 (Table 5), which was regarded as a 'background level' of sperm binding. In the uterine eggs pretreated with type I collagenase, however, the number of bound sperm (about 10; Table 5) was significantly larger than the background level (~2) and definitely smaller than the CE level (50–80) and maximum level (1200–1600). Since the N-terminal portion of gp69/64 must have been deleted at Pro160 by type I collagenase (Tian *et al.*, 1999), these results strongly suggest that envelope components and/or portions other than the N-terminal glycopeptide of gp69/64 (Tian *et al.*, 1999) are involved at least in part in sperm binding to VE.

Discussion

Our finding that the molecules reactive with our monoclonal antibody 6964M are exposed on the CE surface (Fig. 2*A*) is inconsistent with the immunological observation by Tian *et al.* (1997*b*) showing that the molecules recognised by polyclonal antibodies against gp69/64 were hidden from the CE surface. The inconsistency can be attributed to the difference in the epitopes recognised by the antibodies used. The epitope recognised by our 6964M evidently resides within the N-terminal peptide portion of gp69/64 comprising 27 amino acids (Fig. 1), which is closely adjacent to the putative sperm binding site proposed by Tian *et al.* (1999). In the previous studies using coelomic eggs of *Xenopus* (Katagiri *et al.*, 1999) and *B. japonicus* (Omata & Katagiri, 1996), levels of sperm binding similar to those shown in Table 4 in our experiments were regarded as the background level, typically low in CE. However, sperm binding to CE is undoubtedly mediated by epitopes recognised by 6964M, as demonstrated by its specific blockade by the pretreatment of coelomic eggs with 6964M (Table 2) as well as its competitive inhibition by purified gp69/64 on insemination (Table 3). Thus, we conclude that the positive immunohistochemical reaction on the CE surface with 6964M, although to a lesser extent than the VE surface, represents the exposure on CE of the sperm binding site contained in gp69/64.

The higher amount of gp69/64 in the animal than the vegetal hemisphere revealed by immunoelectron microscopy (Table 1) is consistent with an apparently higher sperm binding rate observed at the animal hemisphere of eggs (cf. Katagiri *et al.*, 1999), thus again supporting the notion that the observed immunoreactivity is relevant to the sperm binding observed on CE. Our electron microscopic studies (Figs. 3, 4) reveal that the gross ultrastructural changes accompanying the CE-to-VE conversion do not alter the distribution pattern but increase the exposure rate of the pertinent gp69/64 epitope. The distribution of gp69/64 shown in Fig. 4 is apparently reminiscent of the swirling pattern and the bundles among the large cable-like fibres described by Larabell & Chandler (1988) on the basis of a quick-freeze, deep-etch analysis. The oviductin supposedly selectively affects gp43 on large cable-like fibres and/or bundles, so that more gp69/64 epitopes are exposed upon ultrastructural conversion from CE to VE.

Apparently, however, the about 2-fold increase in the exposure level of gp69/64 epitope upon CE-to-VE conversion (Table 2) does not directly correlate with the 20-fold increase in the number of bound sperm on the envelope. A possible explanation is that gp69/64 may be more effectively exposed upon the CE-to-VE conversion, sufficient to be fully accessible to sperm. The difference between gp69/64 on CE and VE cannot be detected by this monoclonal antibody. A similar situation may also hold true for the observation by Tian *et al.* (1997*b*) who were able to stain the surface of the VE but not CE surface with their polyclonal antibodies against gp69/64. The maximum level of sperm binding to VE is reportedly mediated by gp69/64 but not by other envelope components including gp41 (Tian *et al.*, 1997a), consistent with the inhibition experiments of sperm binding to VE with 6964M (Table 5). Therefore, it is quite reasonable to speculate that the

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elevation of sperm binding is exclusively due to the conformational change of gp69/64 on VE. Another possibility is that envelope components other than gp69/64 are involved in the maximum level of sperm binding observed on VE. This is exemplified by the observation that the VE digested with Type I collagenase still showed a low but a significant level (~10) of sperm binding (Table 5) beyond the background level (below 2), despite the fact that collagenase digestion was expected to have truncated gp69/64 into gp65/60 by deleting the sperm-accessible N-terminal peptide (Fig. 1) (Tian *et al*., 1999). In this respect, gp41 produced from CE gp43 accompanying the CE-to-VE conversion will be one of the most plausible candidates as an additional sperm receptor for the following reasons: Firstly, contrary to previous reports showing that gp41 is hidden from the VE surface as evidenced by radioactive iodine labelling experiments (Nishihara *et al*., 1983; Tian *et al*., 1997*b*), our recent immunofluorescence study with a monoclonal antibody specific to the N-terminus of gp41 (anti-N41; Kubo *et al*., 1999) indicated the surface exposure of gp41 on VE (Kubo, unpublished data). Secondly, gp41 covalently coupled to silanised glass slides has recently been reported to be accessible to sperm (Vo & Hedrick, 2000). Thirdly, the pretreatment of dejellied uterine eggs with anti-N41 significantly inhibited sperm binding but to a lesser extent than did 6964M (Kubo, unpublished data). Fourthly, insofar as they have been analysed by Western blotting of VE with monoclonal antibodies anti-N41 and anti-gp43/gp41 (Katagiri *et al*., 1999), the mobility and immunoreactivity of gp41 were not altered by type I collagenase treatment of uterine eggs (Kubo, unpublished data). Finally, the 36–39 kDa component in the egg envelope of *B. japonicus*, a putative homologue of *Xenopus* gp41, has been shown to be involved in homologous sperm binding to VE (Omata & Katagiri, 1996). Nevertheless, there is a critical problem that sperm binding to intact VE is not competitively inhibited by the presence of purified gp41 (Tian *et al*., 1997*a*), which was also repeated in our experiments (Kubo, unpublished data). Therefore, even if gp41 is involved in sperm binding to intact VE, its involvement is supposed to be far more limited than that of gp69/64. To understand more exactly how fertilising sperm recognise quite a subtle structure of the VE surface of *Xenopus*, it will be important to localise the gp41 molecules on an intact envelope surface and analyse whether they function as an intrinsic and natural binding site for sperm.

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