BIOLOGY OF REPRODUCTION **64,** 822–830 (2001)

Morphological and Biochemical Changes of Isolated Chicken Egg-Envelope During Sperm Penetration: Degradation of the 97-Kilodalton Glycoprotein Is Involved in Sperm-Driven Hole Formation on the Egg-Envelope[1]

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ABSTRACT

The chicken egg-envelope is made of two major glycoprotein components, which are designated as gp97 and gp42 (after their molecular masses). To elucidate how these two components are involved in macromolecular organization of the chicken egg-envelope, the isolated egg-envelope was characterized by immunochemical and biochemical methods. The gp97 was suggested to be a homologue of mouse ZPB based on the similarities of N-terminal and internal sequences. Immunoblotting using anti-gp97 monoclonal antibodies and two-dimensional gel electrophoresis with or without mercaptoethanol treatment revealed that gp97 formed a homodimer through disulfide bonds, whereas gp42 did not. Under indirect immunofluorescence microscopy, the anti-gp97 antibody visualized indistinct, small spots on the egg-envelope, whereas the anti-gp42 antibody showed a meshwork of blurry, fibrous structures. The hole formation on the egg-envelope by in vitro sperm penetration was completely inhibited by two anti-gp97 monoclonal antibodies. Interestingly, the anti-gp97 monoclonal antibodies blocked the proteolysis not only of gp97 but also of gp42 during incubation of the egg-envelope with either sperm or the crude chicken acrosin. Taken together, these results indicate that gp97 may play pivotal roles not only in constitution of the macromolecular organization of the egg-envelope but also in triggering hydrolysis of the egg-envelope during sperm penetration.

fertilization, ovum, sperm

INTRODUCTION

The egg-envelope is an extracellular matrix that surrounds oocytes and plays important roles in sperm-egg interaction during the initial step of fertilization. In mammals, the major components of the egg-envelope, or zona pellucida, are three kinds of glycoproteins, which are termed ZPA, ZPB, and ZPC (according to their genes) [1, 2]. Another nomenclature system, with the terms ZP1, ZP2, and ZP3 (according to their electrophoretic mobility/molecular masses), has also been used [3–6], although ZP1, ZP2, and ZP3 are not necessarily related to ZPA, ZPB, and ZPC, respectively. Glycoproteins homologous to these zona proteins have been identified in egg-envelopes of amphibian [7–10] and fish species [11–13]. Although significant sequence similarity among species is found in these egg-envelope glycoproteins, the egg-envelope component that is responsible for sperm binding has been reported to differ among several species, including ZPC (ZP3) in mouse [3], ZPB (ZP3a) in pig [14, 15], and ZPC (gp41) [16] and ZPA (gp69/64) [7, 8] in *Xenopus.* Such differences can be lessened, in part, by the critical role of glycan chains of the glycoproteins in sperm binding [7, 17–19].

Avian egg-envelope, which is termed the perivitelline layer, has been reported to be morphologically homologous to mammalian zona pellucida [20, 21]. Although the egg-envelopes of chicken [22] and Japanese quail [23] are composed of several major components, to our knowledge, the structure and function of these components remain to be elucidated. We have cloned a cDNA encoding the 42-kDa glycoprotein (gp42) of chicken egg-envelope and have identified this glycoprotein as the chicken homologue of vertebrate ZPC-family proteins [24, 25]. Chicken ZPC differs from those of other species, in that it is expressed and secreted by granulosa cells surrounding an oocyte during rapidly growing stages [25, 26], which contrasts with the oocyte-specific expression of mouse zona proteins [27]. In some other mammals, not only oocyte but also the follicle cells contribute to ZPC synthesis [28, 29]. In the chicken, another major component, the 97-kDa glycoprotein (gp97), has not yet been characterized.

The aims of this study were to characterize gp97 and to reveal its roles in formation of the macroscopic structure of the egg-envelope and in sperm-egg interaction during fertilization. We produced monoclonal antibodies against the egg-envelope to utilize as molecular probes for the morphological and biochemical analyses. Effects of some monoclonal and polyclonal antibodies specific for gp97 or gp42 were also investigated on the sperm-driven hole formation on the egg-envelope and the proteolytic degradation of the egg-envelope component glycoproteins.

MATERIALS AND METHODS

Isolation of Chicken Egg-Envelopes

Egg-envelopes were isolated from large follicles of laying White leghorn hens as described previously [25]. Briefly, the granulosa cell layer composed of the perivitelline layer (i.e., egg-envelope), the monolayer of granulosa cells, and the basal lamina (i.e., basement membrane) was mechanically separated from oocytes, and the egg-envelope was further separated by incubating the granulosa cell layer in saline containing 50 mM EDTA. The pieces of egg-envelope free from the cells and basement membrane were

[1]Supported in part by Grant-in-Aid for Scientific Research (10660121 and 12556060 to T.M.) from the Ministry of Education, Science, Sports and Culture of Japan.
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Received: 17 July 2000.
First decision: 21 August 2000.
Accepted: 16 October 2000.

ISSN: 0006-3363. http://www.biolreprod.org

isolated by picking up with forceps under a stereoscopic microscope.

Production of Monoclonal Antibodies

Female BALB/c mice (Japan SLC, Inc., Hamamatsu, Japan) were immunized with the isolated egg-envelope that had been homogenized and suspended by sonication. Several pieces of the egg-envelope were placed in a test tube containing 0.5 ml of saline (0.15 M NaCl) and sonicated on ice using sonication equipment (Sonifier model 250; Branson, Danbury, CT) at 40% of its maximum output for 30 sec three times. The egg-envelope suspension in saline was mixed and emulsified with Freund's complete adjuvant (Difco, Sparks, MD) and injected i.p. into mice (40 μg dry mass of the envelope per mouse). Two weeks later, the mice were given the booster injection of the egg-envelope (20 μg dry mass per mouse) with Freund's incomplete adjuvant (Difco). The final i.v. injection was done with the egg-envelope suspension (50 μg dry mass per mouse) 4 days before the fusion. The spleen cells were fused with a myeloma cell line (P3Ag8.653) by the polyethylene glycol method, and hybridoma cells were selected with the hypoxanthine/aminopterin/thymidine medium as described previously [30, 31]. The hybridomas secreting specific antibodies were screened by ELISA using the egg-envelope suspension as the antigen for the ELISA plate coating and peroxidase-labeled anti-mouse immunoglobulin (Ig) G (E.Y. Laboratories, Inc., San Mateo, CA) as the secondary antibody. The positive hybridoma cells were cloned by limiting dilution three times. Ascitic fluids containing monoclonal antibodies were prepared by injecting the hybridoma in pristane-primed CDF1 mice.

Sperm Preparation and Reaction with Egg-Envelopes

Semen were collected from three cocks after ejaculation induced by lumbar massage. Pooled sperm were washed with the minimum essential medium (MEM; Gibco BRL, Rockville, MD) [32] three times by centrifugation at 1000 $\times$ *g* for 10 min and suspended with MEM (two volumes of the pooled semen). The washed sperm, which had been diluted appropriately with PBS (Gibco BRL), were kept for 15 min in a water bath at 39°C, then incubated with the isolated egg-envelope in PBS for 10–40 min at 39°C at a final concentration of 10^5–10^8 cells/ml in a humidified incubator. The sperm concentration was determined by counting sperm under a fluorescence microscope after appropriate dilution and fluorescent staining as described below.

Crude Acrosin Preparation and Assay for Its Trypsin-Like Protease Activity

To the washed sperm ($\sim 10^7$ sperm/ml in PBS) was added a calcium ionophore (A23187; Wako Pure Chemical Industries, Osaka, Japan) at a final concentration of 5 μM, and the sperm suspension was incubated at 39°C for 60 min to induce sperm acrosome reaction. After being centrifuged at 1000 $\times$ *g* for 10 min, the supernatant was used as a crude acrosin solution for the protease activity measurement and the experiments for reaction with egg-envelopes. A trypsin-like protease activity of the crude acrosin solution was assayed using a synthetic substrate, *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BAPA; Sigma, St. Louis, MO). The substrate solution (0.5 mg/ml in PBS) was added to a 96-well microtiter plate (150 μl/well) preincubated at 37°C for 15 min. The crude acrosin solution (5–20 μl) was added to each well, and the increase in absorbance of the reaction mixture at 405 nm was monitored for 60 min by a microplate spectrophotometer (SpectraMax 250; Molecular Devices Corp., Sunnyvale, CA).

Morphological Analyses

For immunofluorogenic analysis, the egg-envelope was cut into small pieces (~ 1 cm^2), placed on a slide glass, incubated at room temperature for 1 h with 100 μl of each antibody (diluted 25-fold with 2% [w/v] BSA/PBS), and washed with PBS three times. The egg-envelope was then incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (E.Y. Laboratories) in 2% w/v BSA/PBS for 20 min, washed with PBS three times, and observed under a fluorescence microscope (BX60; Olympus, Tokyo, Japan). To detect sperm interacted with egg-envelope, the egg-envelope was incubated with sperm (10^6 cells/ml) at 39°C for 15 min and rinsed gently with PBS. The sperm on the egg envelope was then stained for 10 min with 4,6-diamidinophenyl-indole (DAPI) staining solution (0.005% [w/v] DAPI, 10 mM Tris, 100 mM NaCl, 10 mM EDTA, and 0.015 mM 2-mercaptoethylamine) and observed under the fluorescence microscope. For observation of the holes formed by sperm interaction, the egg-envelope pieces were incubated without or with sperm (10^5–10^7 cells/ml) on a slide glass for 40 min, then fixed with a 20% (w/v) formaldehyde aqueous solution (formalin diluted with distilled water) for 30 sec. After being stained with Schiff reagent (Wako Pure Chemical Industries) for 30 min and subsequently washed with distilled water, the holes formed by sperm penetration into the egg-envelope were observed under a light microscope. Some other pieces of the egg-envelope were preincubated individually on a glass slide with each of the antibodies specific for the egg-envelope (50 μl of ascitic fluid or serum diluted fivefold with PBS) at 37°C for 30 min, then 50 μl of sperm (10^7 cells/ml in PBS) were added to the egg-envelope and incubated as described above.

The pieces of egg-envelope before and after incubation with sperm (10^5 cells/ml) for 10 min were also observed by scanning electron microscopy. The egg-envelope was fixed with the 1% (w/v) paraformaldehyde/2% (w/v) glutaraldehyde mixture, refixed with osmium tetraoxide/Millonig buffer (pH 7.4), dehydrated, and critical-point-dried as described previously [33]. The specimen was then ion-spatter-coated and observed with a scanning electron microscope (JSM-820; Japan Electron Optics Laboratory Company, Tokyo, Japan).

Gel Electrophoresis and Immunoblotting Analyses

The egg-envelope was dissolved with the Laemmli's sample buffer in the presence and absence of 1% 2-mercaptoethanol and applied to the SDS-PAGE system [34]. The gel sheet was stained with Coomassie brilliant blue (CBB) or subjected to the electric transfer onto a polyvinylidene difluoride (PVDF) membrane [35]. The membrane was incubated first with the indicated antibodies and subsequently with the peroxidase-labeled anti-mouse IgG. The activity staining for the peroxidase was done with 4-chloro-1-naphtol as a substrate [31]. For a modified two-dimensional gel electrophoresis, the first-dimensional electrophoresis was done with 5% acrylamide gel under a nonreducing condition, then the gel strip was incubated in the Laemmli's sample buffer containing 2% mercaptoethanol for 20 min with gentle shaking. The gel strip was applied

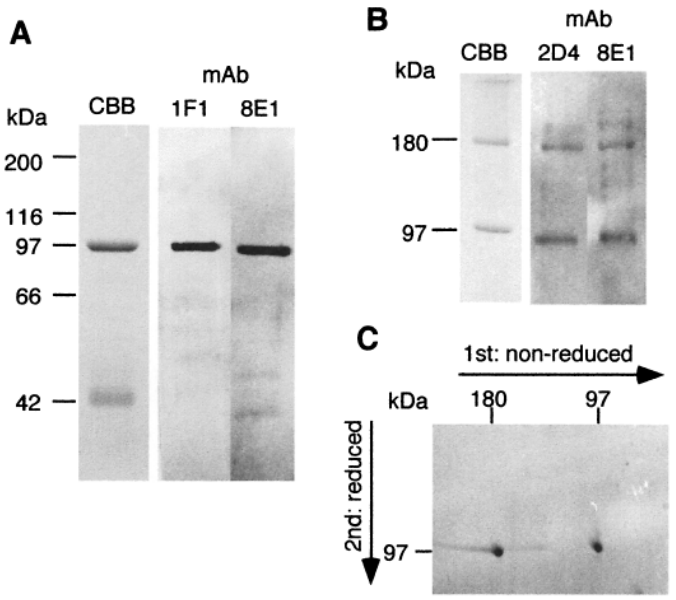

FIG. 1. SDS-PAGE and immunoblotting analyses for the 97-kDa antigen recognized by the monoclonal antibodies raised against chicken egg-envelope. The proteins in isolated egg-envelope were separated by SDS-PAGE (10% gel) under reducing (**A**) and nonreducing (**B**) conditions and stained with CBB. Those separated in another gel were transferred onto a PVDF membrane and immunologically stained with the monoclonal antibodies (mAb) 1F1, 2D4, and 8E1. The proteins in the egg-envelope were also analyzed by two-dimensional SDS-PAGE (5% acrylamide) under the first nonreducing and the second reducing conditions and then stained with CBB (**C**).

to the second-dimensional electrophoresis using 5% acrylamide gel.

For electrophoretic analyses of egg-envelope degradation and solubilization during the incubation with sperm, small pieces of the egg-envelope (~10 μg dry mass in 20 μl of PBS) were put in test tubes, and sperm (10^2–10^4 cells in 20 μl of MEM) were added. After incubation at 39°C for 15 min, the test tubes were centrifuged at 10 000 × *g* for 15 min and the supernatant subjected to SDS-PAGE and immunoblotting analyses. To examine the effect of the specific antibodies on the sperm-driven degradation of the egg-envelope, the egg-envelopes (~10 μg dry mass) in the test tubes were incubated with 2 μl of the ascitic fluid, antiserum, or PBS at 37°C for 60 min, then sperm (10^4 cells in 20 μl of MEM) were added to the test tubes and incubated for 30 min. The SDS-PAGE sample buffer containing 2-mercaptoethenol was added to the test tubes to dissolve the whole of the egg-envelope/sperm mixture, and the proteins were subjected to SDS-PAGE, followed by immunoblotting. For analysis of the effect of the antibodies on proteolysis of the egg-envelope by the crude acrosin solution, to the egg-envelopes pretreated with each antibody as described above were added 50 μl of the crude acrosin or *N*-α-tosyl-L-phenylalanylchloromethyl ketone (TPCK)-treated trypsin (Sigma) in 50 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ and 0.15 M NaCl, which had been adjusted to be as active as the crude acrosin for the synthetic substrate. The proteolytic activity of these enzyme preparations added to the reaction mixture was ΔA405 of 0.052 (absorbance units/min·ml·cm) at 37°C for BAPA as a substrate. The reaction mixtures were then incubated at 37°C for 3, 8, and 24 h. The enzyme reaction was stopped by adding the SDS-PAGE sample buffer containing 2-mercaptoethenol and boiling for 3 min, and proteins were then analyzed by SDS-PAGE.

Protein Purification and Antiserum Preparation

To purify individual egg-envelope glycoproteins (i.e., gp97 and gp42), the isolated egg-envelopes were solubilized with the Laemmli's SDS-PAGE sample buffer [34] and subjected to SDS-PAGE (10% acrylamide gel). The protein bands on the gel were visualized by staining the gel with 0.3 M $CuCl_2$ for 5 min and washing with distilled water three times. Each protein band was excised from the gel and destained in an EDTA solution (0.25 M EDTA and 0.25 M Tris [pH 9.0]). For extraction of proteins, the gel pieces were ground in a small mortar and incubated with shaking in the SDS-electrophoresis buffer for 18 h at room temperature. After being separated from the gel pieces by centrifugation at 10 000 × *g* for 15 min, the supernatant was concentrated fivefold by a vacuum concentrator. One volume of the concentrated supernatant was mixed with five volumes of cold acetone and kept at −80°C for 2 h to make the proteins precipitate. The protein precipitates were then recovered by centrifugation (10 000 × *g* for 15 min at 4°C), dissolved in distilled water, and used for immunization and peptide preparation as described below. Mice (10-wk-old female ddY; Japan SLC) were immunized by i.p. injection of the purified protein (gp97 or gp42, 30 μg/mouse) emulsified with Freund's complete adjuvant (Difco), then boosted twice by the injection with each protein (10 μg/mouse) emulsified with Freund's incomplete adjuvant every 2 wk. Seven days after the last injection, blood was collected from individual mice and centrifuged at 10 000 × *g* for 15 min to separate serum. The antisera were kept at −20°C before use.

Amino Acid Sequence Analysis

The N-terminal amino acid sequences were determined using a protein sequencer (Model 476A; Applied Biosystems Inc., Foster City, CA) for the proteins and peptides separated by SDS-PAGE and electroblotted onto PVDF membranes. For the peptide preparation, the isolated egg-envelope was digested with TPCK-treated trypsin (Sigma) at the enzyme:protein mass ratio of 1:50 in 10 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, and 150 mM NaCl at 37°C for 16 h, whereas the purified gp97 was digested with the endoproteinase Lys-C (Sigma) at the enzyme:protein mass ratio of 1:100 in 0.1 M Tris-HCl (pH 8.0) and 0.1% (w/v) SDS at 37°C for 16 h. These proteolytic digests were subjected to SDS-PAGE (12.5% or 15% acrylamide gel), followed by electroblotting. The membrane was stained with 0.025% (w/v) CBB in 40% (v/v) methanol for 10 min, destained with 50% (v/v) methanol, and subjected to the protein sequencer.

RESULTS

gp97 Forms Homodimer Through Disulfide Bonds and Resembles Mouse ZPB

By immunoblotting analysis after SDS-PAGE separation of egg-envelope proteins under reducing condition, a single 97-kDa band of gp97 was specifically immunostained with two monoclonal antibodies, 1F1 and 8E1 (see below). On the other hand, a 180-kDa band in addition to the 97-kDa band was also detected by the analysis under nonreducing condition with the four monoclonal antibodies. The typical results of immunoblotting analyses are shown in Figure 1,

A and B. The additional 180-kDa protein band was also detected for the nonreduced sample in the gel stained with CBB (Fig. 1B). The molecular mass of 180 kDa, which is approximately twice the 97-kDa mass, and the immunological similarity between the 180- and 97-kDa proteins prompted us to analyze these proteins by the nonreduced/reduced two-dimensional gel electrophoresis. As shown in Figure 1C, the protein that migrated to the 180-kDa position by the first electrophoresis under the nonreducing condition moved to the 97-kDa position by the second electrophoresis after the reducing treatment. The ratio of the 180- to the 97-kDa band was higher in the egg-envelope preparations from laid eggs than in those from ovarian follicles (data not shown). These results suggest that the 180-kDa band was the dimer of gp97 formed through intermolecular disulfide bond(s), and that a part of the gp97 molecules existed as a dimer form in the egg-envelope. No band corresponding to gp42 appeared after the reducing treatment either from the 180- or the 97-kDa band (data not shown), indicating no intermolecular disulfide bonding between gp97 and gp42.

To identify the 97-kDa glycoprotein antigen of chicken egg-envelope, the N-terminal amino acid sequence was determined to be LLQYHYDXGDFGMQLLAYPTR, and its sequence similarity to known proteins was analyzed by the BLAST program at the National Center for Biotechnology Information. Significant similarity (10 of the 21 residues were identical) of the gp97 N-terminal sequence was obtained against the mouse zona pellucida glycoprotein ZPB. The N-terminus of gp97 corresponded to the Gly^{28} of the mouse ZPB sequence, which is the eighth residue downstream from the possible signal-peptide cleavage site [36]. The isolated chicken envelope was digested with trypsin, resulting in the production of 34-, 31-, and 22-kDa fragments. By immunoblotting analysis, only the 31-kDa fragment was detected with the monoclonal antibody 1F1 among the tryptic fragments of the egg-envelope, and its N-terminal amino acid sequence was determined to be LASTQPGXQPTQXTDAFVLF. This internal amino acid sequence of gp97 also was similar to the sequence stretch (Leu^{299}–Phe^{318}) of mouse ZPB (11 of the 20 residues were identical). The 34-kDa fragment was not stained with the anti-gp97 antibodies but was with the anti-gp42 antibody, and its N-terminal amino acid sequence was determined to be AMAGSHPVAVQ. This sequence was identical to an internal sequence (Ala^{47}–Gln^{57}) of chicken ZPC [25]. The endoproteinase Lys-C digestion of purified gp97 produced 19-, 13-, and 11-kDa fragments by SDS-PAGE (15% acrylamide gel), and the N-terminal amino acid sequences of these fragments were VLRDPIYVEVRLL and TDPNLVLVLHQXWAAP, respectively. Both sequences were also similar to the internal sequence stretches (Leu^{425}–Leu^{437} and Leu^{440}–Pro^{455}) of mouse ZPB. The sequence of the 19-kDa fragment was identical to the N-terminal sequence of gp97.

Four Monoclonal Antibodies Raised Against Egg-Envelope Were Specific for gp97

Five hybridomas secreting monoclonal antibodies specific for the egg-envelope were obtained from three fusions of spleen cells from the BALB/c mice. The antigen-binding properties of these monoclonal antibodies are summarized in Table 1. The monoclonal antibodies were classified into three groups based on their antigen-binding properties: 1) 8E1 and 1F1, which were reactive to the antigen treated with SDS under reducing condition; 2) 2D4 and 5G9, which were reactive to the antigen only under nonreducing condition; and 3) 3A6, which showed reactivity only for the native antigen on ELISA. The two monoclonal antibodies of the first group also showed reactivity to the egg-envelope that had been heat-denatured in boiling water, indicating that these two antibodies recognize conformationally independent and/or structurally stable epitopes of the egg-envelope antigen. The antigenic protein recognized by the monoclonal antibodies of groups 1 and 2 was identified as gp97 (Fig. 1). The antigen recognized by the monoclonal antibody 3A6 has not yet been determined.

TABLE 1. Properties of monoclonal antibodies raised against chicken egg-envelope.

		Reactivity to egg-envelope ELISA value (A492)[a]			Reactivity to gp97 on immunoblotting[b]	
Clone	Class	Native	Acid treated	Boiled	Reduced	Nonreduced
8E1	IgG_1	0.45	1.02	0.35	Yes	Yes
1F1	IgG_1	0.57	0.87	0.48	Yes	Yes
2D4	IgG_1	0.43	0.29	0.09	No	Yes
5G9	IgG_1	0.39	ND[c]	0.06	No	Yes
3A6	IgG_1	0.31	0.01	0.05	No	No

[a] Isolated egg-envelope was suspended at 10 μg protein/ml in 20 mM sodium phosphate buffer (pH 7.0, native) and 20 mM glycine/HCl buffer (pH 2.0; acid treated). A portion of the egg-envelope suspended in the phosphate buffer was heated in boiling water for 10 min (boiled). Wells of ELISA plates were coated with 10 μl of each egg-envelope suspension and then incubated with the supernatant of each hybridoma culture.
[b] Reactivity to gp97 was assessed by immunoblotting after SDS-PAGE. The egg-envelope proteins separated by SDS-PAGE were blotted onto a PVDF membrane and incubated with the hybridoma culture supernatant.
[c] ND, Not determined.

gp97 Is Present on the Surface of Egg-Envelope

The egg-envelope isolated from the first- or second-largest oocyte in the ovary was stained with the monoclonal antibodies specific for gp97 or the polyclonal anti-gp42 antiserum by an indirect immunofluorescence method. Typical immunofluorescence micrographs are shown in Figure 2. The egg-envelope was strongly stained with these antibodies, but those images visualized by 8E1 and 5G9 were clearly different from those obtained by the polyclonal antiserum to gp42. The characteristic meshwork fibrous structure of the chicken egg-envelope, as revealed by the scanning electron micrograph (see Fig. 4), was visualized by staining with the anti-gp42 antiserum but not with the anti-gp97 antibodies (i.e., 8E1 and 5G9). Indistinct, small spots were observed with the anti-gp97 antibodies.

Hole Formation on the Egg-Envelope During Sperm Penetration and Its Inhibition by Monoclonal Antibodies to gp97

The egg-envelope was incubated with sperm and stained with DAPI to see if sperm bind to the isolated egg-envelope. As shown in Figure 3A, the heads of sperm bound to the isolated egg-envelope were stained with DAPI and visualized under a fluorescent microscope. Such bent rod-shaped structures stained with DAPI were not observed for egg-envelope incubated without sperm (data not shown). Sperm binding was detected on any piece of the isolated egg-envelope, suggesting that binding sites for sperm are distributed on the entire surface of the egg-envelope. Under a light-microscope after staining with the Schiff reagent, holes were found on a wide area of the egg-envelope sheet

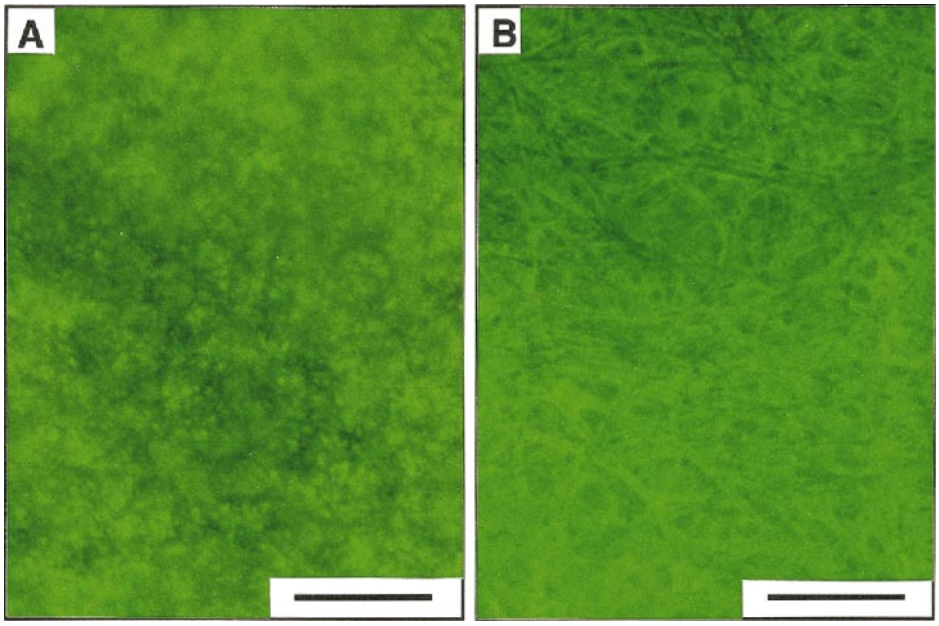

FIG. 2. Differential localization of gp97 and gp42 on the egg-envelope. The pieces of egg-envelope were stained by the indirect fluorescence-antibody method with the anti-gp97 monoclonal antibody 8E1 (**A**) and the polyclonal antiserum to gp42 (**B**), respectively. Bars = 10 μm.

(Fig. 3B). The holes were presumably made by sperm penetration in the egg-envelope and were visualized as unstained, circular areas on the Schiff's reagent-positive egg-envelope matrix. The holes were approximately 20–40 μm in diameter. A faintly bright, circular area (~50–80 μm in diameter) surrounding each hole was also observed (Fig. 3C).

The effect of monoclonal antibody 8E1 on hole formation by sperm penetration into the egg-envelope was also examined. No holes were observed on the egg-envelope after in vitro incubation with sperm when the egg-envelope had been preincubated with the anti-gp97 monoclonal antibody 8E1 (Fig. 3D). Thus, the sperm-driven hole formation on the egg-envelope was completely blocked by the monoclonal antibody to gp97. The same results were obtained with another anti-gp97 monoclonal antibody, 2D4. In contrast, holes were still observed when the egg-envelope had been preincubated with the polyclonal antiserum to gp42 and an unrelated monoclonal antibody, 1C10 (data not shown).

To observe the fine structure of holes on the egg-envelope, the egg-envelope incubated with sperm was observed under a scanning electron microscope. As shown in Figure 4, the meshwork structure disappeared at the circular area (20–40 μm in diameter), and instead, several thinner strings (~10 nm in thickness) were seen in that area. A sperm was

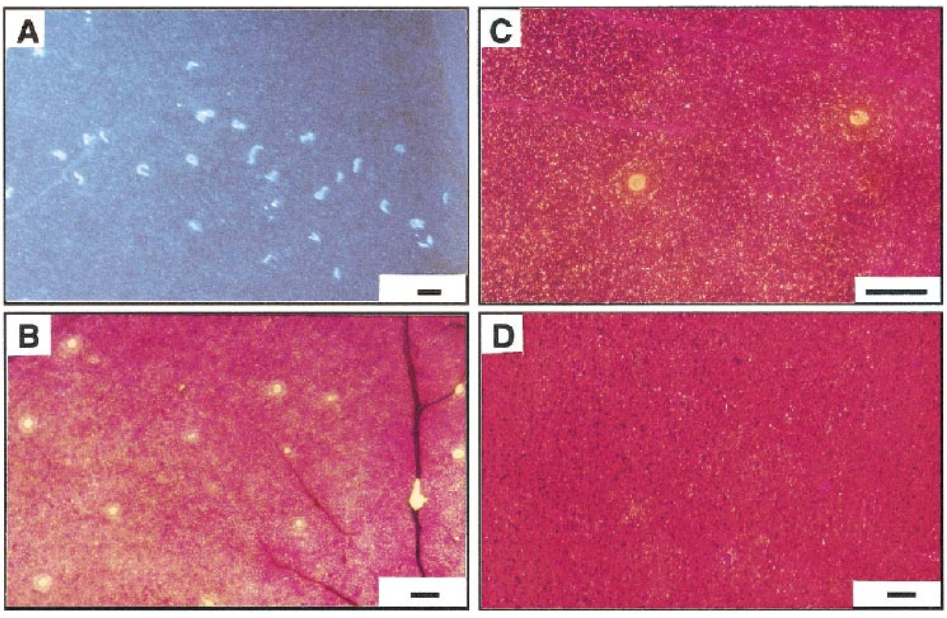

FIG. 3. Sperm-driven hole formation and its inhibition by the monoclonal antibodies to gp97. Small pieces of the egg-envelope were incubated with sperm (10^9 cells/ml) for 10 min and observed under a fluorescent microscope after DAPI staining (**A**). The egg-envelope pieces were incubated with sperm (10^7 cells/ml) for 40 min, fixed with formaldehyde, stained with the Schiff reagent, and observed under a light microscope (**B–D**). An egg-envelope piece was preincubated with the anti-gp97 monoclonal antibody 8E1 before the incubation with sperm (**D**). Bars = 100 μm.

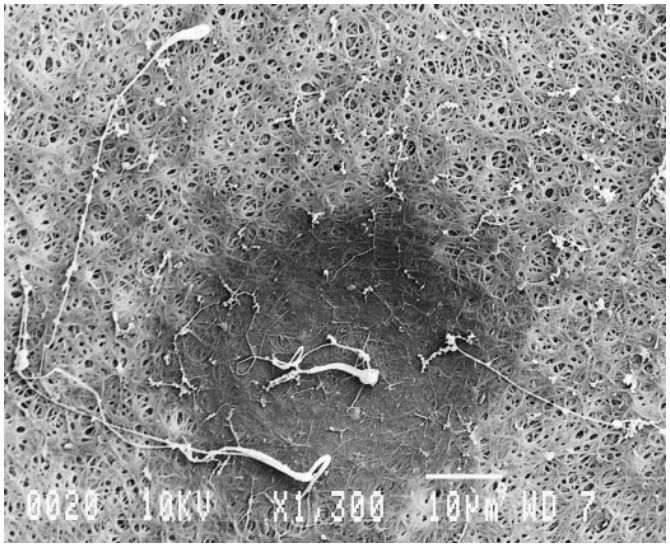

FIG. 4. Scanning electron microscopic observation of the holes on the egg-envelope incubated with sperm. The small pieces of egg-envelope incubated with sperm (10^5 cells/ml) for 10 min were observed under a scanning electron microscope after fixing, drying, and ionspattering treatments.

observed at the central part of the circular area. Compared with the size of a sperm, the holes formed by sperm penetration on the chicken egg-envelope appeared to be large (20–40 μm in diameter, or approximately half in diameter as large as mammalian eggs or oocytes [37, 38]).

Degradation of Egg-Envelope Glycoproteins During Sperm/Egg-Envelope Interaction and Its Inhibition by Monoclonal Antibodies to gp97

A small piece of egg-envelope was incubated with sperm (10^2, 10^3, and 10^4 cells), and peptide fragments that were degraded and released from the egg-envelope during sperm interaction were analyzed by SDS-PAGE and immunoblotting using the anti-gp97 and anti-gp42 antisera (Fig. 5). The sheets of egg-envelope were partially fragmented to be dispersed in the medium during incubation with sperm as previously reported [39]. In the CBB-stained gel for supernatant of the egg-envelope incubated with sperm, no band for gp97 but several low molecular bands, most of which appeared to be sperm derived, were detected. The immunoblotting analyses of the supernatant demonstrated that 43- and 34-kDa fragments were produced and released, respectively, from the major 97- and 42-kDa components (i.e., gp97 and gp42) of the egg-envelope during incubation with 10^4 cells of sperm. Neither apparent morphological changes in the egg-envelope nor peptide fragments released from the egg-envelope were observed during the incubation without sperm. When the egg-envelope was incubated with 10^3 cells of sperm, several gp97 fragments of 35–50 kDa were clearly detected in the supernatant of the egg-envelope/sperm reaction mixture. On the other hand, no gp42 fragment was detected in that supernatant, suggesting that gp42 had not yet been degraded under these conditions. No released peptide fragments were detected for the egg-envelope incubated with 10^2 cells of sperm or without sperm.

The effect of monoclonal antibodies on protein degradation during the sperm interaction was examined by preincubating the egg-envelope with the two monoclonal antibodies, 2D4 and 5G9. The whole of each egg-envelope/sperm mixture was dissolved with the SDS-PAGE sample

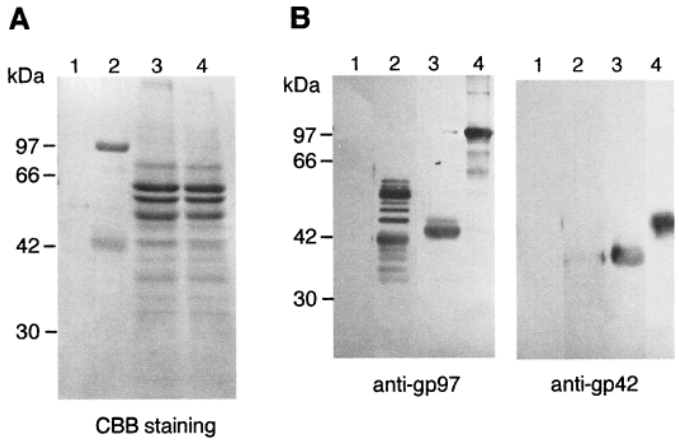

FIG. 5. The sperm-driven degradation of the egg-envelope glycoproteins. **A**) After incubation of the egg-envelope without or with sperm (10^4 cells in 20 μl of MEM) for 15 min (lanes 1 and 3), the supernatant was separated by centrifugation at 10 000 × *g* for 15 min, treated with the SDS-PAGE sample buffer, and subjected to SDS-PAGE (10% acrylamide gel). A piece of egg-envelope was directly dissolved with the SDS-PAGE sample buffer and analyzed as intact glycoproteins (lane 2). The sperm (10^4 cells in 20 μl of MEM) was incubated alone and centrifuged under the same conditions, then subjected to SDS-PAGE (lane 4). The gel was stained with CBB. **B**) After incubation of the egg-envelope with sperm (10^2, 10^3, and 10^4 cells in 20 μl of MEM) for 15 min (lanes 1, 2, and 3, respectively, of each panel), the supernatant was separated by centrifugation at 10 000 × *g* for 15 min, treated with the SDS-PAGE sample buffer, and subjected to SDS-PAGE (10% gel) and immunoblotting. A piece of egg-envelope was directly dissolved with the SDS-PAGE sample buffer and analyzed as intact glycoproteins (lane 4 of each panel). The blotted membranes were immunostained with anti-gp97 (left) and anti-gp42 (right) antisera, respectively.

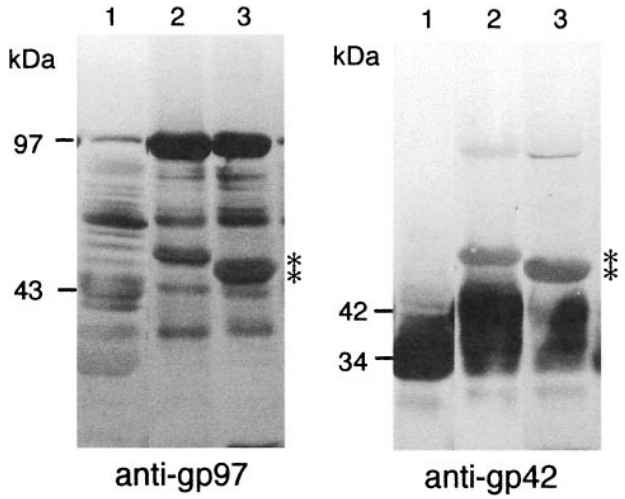

FIG. 6. Inhibition of the sperm-driven degradation of gp97 and gp42 by the anti-gp97 monoclonal antibodies. Small pieces of the egg-envelope were preincubated with the anti-gp97 monoclonal antibodies 2D4 (lane 2) and 5G9 (lane 3) and without antibody (lane 1). Sperm (10^4 cells in 20 μl of MEM) were added to the egg-envelope and then incubated for 30 min. The whole of the incubation mixture was dissolved with the SDS-PAGE sample buffer and analyzed by the SDS-PAGE (10% gel)/immunoblotting analysis using the antisera specific for gp97 (left panel) and gp42 (right panel), respectively. Asterisks indicate IgG heavy-chains of the monoclonal antibodies 2D4 and 5G9 as detected by the secondary antibody.

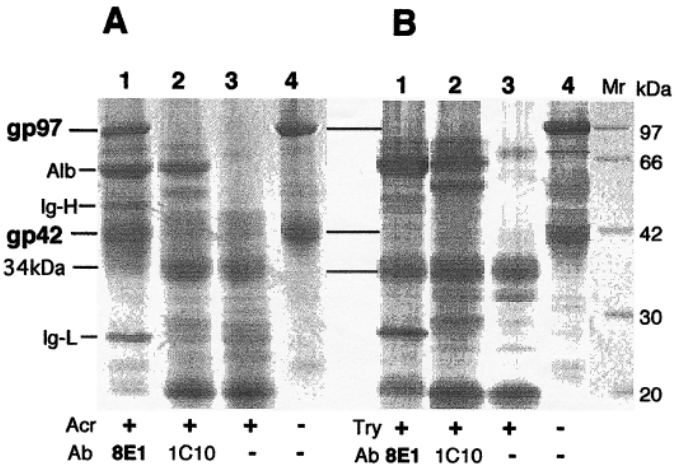

FIG. 7. Specific inhibitory effect of the anti-gp97 monoclonal antibody on the degradation of gp97 and gp42 by the crude acrosin. Small pieces of the egg-envelope were preincubated with the anti-gp97 monoclonal antibody 8E1 (lane 1) and a nonspecific antibody, 1C10 (lane 2), and without antibody (lanes 3 and 4), respectively. The crude acrosin (**A**) and the trypsin with equivalent activity (**B**) were added to the egg-envelopes (lanes 1–3) and not to another one (lane 4). The reaction mixtures were then incubated for 8 h. The whole of the incubation mixture was dissolved with the SDS-PAGE sample buffer and analyzed by SDS-PAGE (12% gel). A molecular mass standard (M_r) is shown on the right, and migration positions of gp97 and gp42 as well as the ascites-derived proteins, serum albumin (Alb), and antibody heavy and light chains (Ig-H and Ig-L) are shown on the left.

buffer and analyzed to characterize both the soluble fragments and the insoluble egg-envelope matrix. Figure 6 shows the results of immunoblotting analyses of the egg-envelope with or without the antibody preincubation. Several bands corresponding to gp97-derived fragments, including the 43-kDa band as well as a faint 97-kDa band, were detected by the anti-gp97 antibodies for the egg-envelope that had been preincubated without antibody and then incubated with sperm. In contrast, the major part of gp97 remained intact even after incubation with sperm when the egg-envelope had been preincubated with the monoclonal antibodies 2D4 and 5G9. Degradation of gp42 into the 34-kDa fragment by incubation with sperm was also inhibited by the anti-gp97 monoclonal antibodies. Although the 42-kDa band of the egg-envelope without the antibody preincubation was shifted almost completely to 34 kDa by incubation with sperm, it was still detected as a major component for the egg-envelope pretreated with the anti-gp97 monoclonal antibodies. These results indicate that the monoclonal antibodies to gp97 suppress the sperm-driven degradation not only of gp97 itself but also of another component, gp42, in the egg-envelope.

To determine whether the monoclonal antibodies to gp97 sterically inhibit acrosin from affecting gp97 and gp42, the egg-envelopes were preincubated with a specific antibody (i.e., 8E1), a nonspecific antibody (i.e., 1C10), or without antibody and then reacted with the crude acrosin or trypsin (Fig. 7). The band of gp97 completely disappeared with incubation for 8 h using the crude acrosin when the egg-envelope was preincubated with nonspecific antibody or without antibody. However, gp97 remained almost intact after the reaction for 8 h, and even for 24 h, with the crude acrosin when the egg-envelope was preincubated with the specific antibody, 8E1. The 34-kDa band produced by the reaction with the crude acrosin, which was identified as a fragment of gp42 by immunoblotting (data not shown), was not detected for the egg-envelope preincubated with 8E1. Similar degradation profiles were also observed for the egg-envelope without the 8E1 treatment by incubation with trypsin having an equivalent proteolytic activity (Fig. 7B). The band of gp97 disappeared, and that of gp42 shifted to 34 kDa, even after incubation with trypsin for 8 h. Interestingly, pretreatment of the egg-envelope with 8E1 was not at all effective on the tryptic degradation of gp97 and gp42. These results suggest that the monoclonal antibody 8E1 binds to gp97 on the surface of the egg-envelope and inhibits degradation of gp97 by blocking access of soluble acrosin to its target substrate(s) on the egg-envelope.

DISCUSSION

Using specific monoclonal antibodies, we have demonstrated, to our knowledge for the first time, that the ZPB-like, 97-kDa glycoprotein is exposed on the surface of isolated egg-envelopes. These monoclonal antibodies were shown to inhibit hole formation and protein degradation of isolated egg-envelopes during in vitro incubation with sperm and the crude acrosin, suggesting important roles for gp97 in sperm binding and penetration. Indeed, during preliminary experiments, we have found that gp97 enhances the release of an acrosin-like protease from chicken sperm in vitro. Further studies using Fab′ fragments of these antibodies and purified gp97 would enable more precise identification of sperm receptor(s) and the primary target of acrosin on the chicken egg-envelope.

Four of the five antibody clones raised against the native egg-envelope were specific for gp97. The reasons for the high frequency in obtaining the gp97-specific hybridoma clones are uncertain, but the plausible explanation refers to macromolecular organization of the egg-envelope rather than to differences in immunogenicity between gp97 and gp42. Immunogenicity of gp97 would not differ largely from that of gp42, because a high titer of antisera could be obtained through immunizing mice with both glycoproteins isolated by recovery from the gel of SDS-PAGE [25]. The gp97 in intact egg-envelope might localize at a position easily accessible to cell-surface antigen receptors of B lymphocytes. The staining images of egg-envelope with the anti-gp97 monoclonal antibodies, which markedly differ from those with the anti-gp42, would also suggest differences in localization on the egg-envelope surface between the two glycoproteins.

The identification of gp97 as mouse ZPB-like glycoprotein in the present study and the results of our previous work showing gp42 as a member of ZPC family [25] indicate that the chicken egg-envelope is composed of at least two abundant glycoproteins belonging to the ZPB and ZPC families. Further studies are needed to determine whether a ZPA-like component is present in avian egg-envelope. A part of the gp97 molecule is present as a dimer in the egg-envelope, as is the case with mouse ZPB [4, 40], suggesting a functional similarity of gp97 to mouse ZPB. The mouse zona pellucida is suggested to consist of long, interconnected filaments, each approximately 7 nm in width, in which the fundamental building block is a repeating unit composed of one ZPA and one ZPC and each filament is interconnected with intermolecular disulfide bonds between homodimers of the least abundant component, ZPB [4]. In contrast, chicken egg-envelope is found to be built of a much wider fibrous structure, approximately 500 nm in width, by immunofluorogenic and electron microscopic observations (Figs. 2 and 4). Considering these results and the fact that gp97 is an abundant component of chicken egg-

envelope, we propose that gp97 is a key component for the matrix architecture of chicken egg-envelope.

When the isolated egg-envelope was incubated with sperm for 40 min, holes through the egg-envelope were made, presumably by local degradation of the egg-envelope matrix with a sperm-derived protease (Fig. 3). On the other hand, electron microscopic observations after incubation for 10 min (Fig. 4) showed that the holes were made on, but not through, the egg-envelope. These results suggest that sperm did not pass through these holes of the egg-envelope during the 10-min incubation but did pass through after the 40-min incubation in vitro. Further studies with varied incubation periods are needed to determine the time required for sperm to penetrate the egg-envelope.

The number of holes formed on the isolated egg-envelope by sperm varied depending on the egg-envelope preparation. Such variations in sperm binding might result from preferential sperm binding to the egg-envelope over the ovum germinal disc, as previously reported [41, 42]. Nevertheless, the complete inhibition of hole formation by the monoclonal antibodies was reproducible for several egg-envelope preparations. When the egg-envelope was incubated with sperm in the presence of soybean trypsin inhibitor or with the sperm pretreated using a calcium ionophore (i.e., A23187), no hole was detected on the egg-envelope (unpublished results), suggesting that the holes could have been formed by the action of the penetrating sperm. The inhibitory effect of the anti-gp97 monoclonal antibodies raises a question of whether these antibodies inhibit the sperm binding and subsequent acrosome reaction or protect the egg-envelope from attack by acrosomal proteases. The latter possibility (i.e., protection against proteolytic degradation) is strongly supported by the results of reaction with the crude acrosin prepared from sperm (Fig. 7). However, these results would not necessarily rule out the possibility that the antibodies inhibited sperm binding. Further studies using a more precise assay for sperm binding are needed to clarify the role(s) of gp97 in the sperm/egg-envelope interaction.

It is interesting to note that the anti-gp97 antibodies inhibited proteolytic degradation not only of gp97 but also of gp42, to which these antibodies did not bind (Figs. 6 and 7), and that such protection by the antibodies was ineffective against tryptic hydrolysis (Fig. 7). These results also suggest important role(s) of gp97, and of the region around the antibody-binding site of a gp97 molecule, in egg-envelope degradation by acrosin. The acrosome reaction of cock sperm was reported to be initiated on contact with the egg-envelope, resulting in release of a trypsin-like enzyme that digests the envelope to make a pathway for sperm penetration [39, 43, 44]. In the present study, gp97 was degraded and its fragments released from the egg-envelope more easily than gp42 and its fragments during the sperm/egg-envelope interaction (Fig. 5). Such proteolytic degradation of each component of the chicken egg-envelope is in marked contrast to that previously reported in the pig [45, 46]. In the pig, ZPA is susceptible to acrosin hydrolysis, whereas ZPB and ZPC, which are equivalent to the chicken gp97 and gp42, are resistant to acrosin hydrolysis. In the chicken, ZPB (i.e., gp97) would be presumed to interact with acrosome-intact sperm and to be degraded subsequently by protease(s) released from acrosome-reacted sperm. The proteolysis of gp97 and/or release of degraded gp97 from the egg-envelope might result in exposure of gp42 to the sperm proteases, leading to hydrolysis of gp42 and final degradation of the egg-envelope meshwork structure. Current efforts are being made to determine the complete amino acid sequence of gp97 and to map the regions recognized by the anti-gp97 monoclonal antibodies and the soluble acrosin released from sperm as well as possible receptor sites for sperm on the gp97 molecule.

Fertilization and development in avian species is unique, in that fertilization occurs in the infundibulum at the entrance of oviduct and embryonic development already starts during albumen and egg shell formation in the oviduct and continues in laid eggs [47]. Especially during the egg-sperm interaction, avian eggs exhibit physiological polyspermy, in which several spermatozoa penetrate the egg-envelope [48, 49]. Such polyspermy would be in contrast to the polyspermy block of the other viviparous and oviparous vertebrates, in which the dynamic changes in egg cortical granules and egg-envelope are induced in fertilized eggs [4, 50]. A set of the monoclonal antibodies to gp97 with different binding specificities would be a powerful tool to investigate details of the structural changes of the egg-envelope and the biological roles of gp97 during fertilization and early development in avian species.

ACKNOWLEDGMENTS

We thank Prof. William J. Lennarz for critical reading of the manuscript. We also thank Dr. Katsutoshi Kino for kind help in the sperm preparation and Dr. Makoto Mori for helpful advice on histochemical analyses.

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