Title: Molecular characterization of egg envelope glycoprotein ZPD in the ovary of Japanese quail (*Coturnix japonica*)

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Cloning and expression of quail ZPD (35 characters including spaces)

Abstract

The egg envelope surrounding avian oocytes exhibits a three-dimensional network of coarse fiber between the granulosa cells and the oocyte. Our previous studies have demonstrated that one of the matrix's components, ZP3, is synthesized in the ovarian granulosa cells. Another component, ZP1, which is critically involved in triggering the sperm acrosome reaction, is synthesized in the liver. We have previously isolated cDNAs encoding quail *ZP3* and *ZP1*, and we now report the isolation of cDNA encoding quail *ZPD*. By RNase protection assay and *in situ* hybridization, we have demonstrated that ZPD transcripts are restricted to the granulosa cells of preovulatory follicles. The expression level of *ZPD* increased progressively during follicular development, and the highest expression was observed in the largest follicles. Western blot analyses using the specific antibody against ZPD indicate that the 40-kDa protein is the authentic ZPD, and the contents of ZPD protein also increased during follicular development. Moreover, we found that the addition of follicle stimulating hormone to the culture media enhances the ZPD secretion in the cultured granulosa cells. Two-dimensional gel electrophoresis revealed the presence of several ZPD isoforms with different pI values ranging from 5.5 to 7. Immunohistochemical analyses indicate that the materials recognized with anti-quail ZPD antibody were accumulated in the egg envelope of large yellow follicles. These results demonstrate the presence of ZPD protein in the egg envelope, and that the amount of ZPD in the egg envelope as well as the mRNA in the cells increases at the latter stages of folliculogenesis.

Introduction

The avian egg is surrounded by multiple layers of different extracellular matrices, including an inner layer of vitelline membrane, continuous membrane, and an outer layer of vitelline membrane (Bellairs *et al.*, 1963). At the time of ovulation, the avian oocyte is only surrounded by the inner layer of vitelline membrane, also referred to as the perivitelline layer (PL), which is a rough meshwork of thick filaments mainly composed of glycoproteins (Wyburn *et al.*, 1965). PL is a homologue of the egg envelope in other vertebrates, the zona pellucida in mammals, the vitelline membrane in amphibians and the chorion in teleosts. The vertebrate's egg envelope serves several functions in the process of fertilization, including species-specific sperm-egg binding, induction of the acrosome reaction, and prevention of polyspermy (McLeskey *et al.*, 1998). Although the penetration of the PL by supernumerary spermatozoa is found regularly in birds, the PL behaves in a manner analogous to the zona pellucida in mammalian species (Howarth, 1990).

These egg envelopes are mainly constructed of glycoproteins belonging to different subclasses of the zona pellucida (ZP) gene family (Spargo & Hope, 2003; Conner *et al.*, 2005; Smith *et al.*, 2005). The components of this matrix include three glycoproteins (*i.e.*, ZP1, ZP2, and ZP3, which are also known as ZPB1, ZPA, and ZPC, respectively, according to Spargo & Hope (2003) and Smith *et al.* (2005)) in most mammalian species (Harris *et al.* 1994) and four glycoproteins in several organisms including humans, bonnet monkeys and rats (ZP1, ZP2, ZP3, and ZP4 (ZPB2); Lefievre *et al.* 2004; Ganguly et al., 2007; Hoodbhoy *et al.*, 2005), while non-mammalian

vertebrates such as amphibians (i.e., *Xenopus laevis* and *Xenopus tropicalis*), 5 ZP glycoproteins (ZP2, ZP3, ZP4, ZPD and ZPAX) have been identified (Lindsay *et al.*, 2003; Goudet *et al.*, 2008). ZP gene family proteins are characterized by a highly conserved amino acid sequence called the ZP domain, consisting of about 260 amino acid residues with 8 or 10 conserved cys residues (Bork & Sander, 1992).

In avian species, at least two glycoproteins have been identified as the component of PL, ZP1 and ZP3 in quail (Pan *et al.*, 2001; Sasanami *et al.*, 2003b) and in chickens (Waclawek *et al.*, 1998; Takeuchi *et al.*, 1999; Bausek *et al.*, 2000). In addition, a recent study revealed that a new member of the ZP protein family, ZPD, is a structural component in chicken PL (Okumura *et al.*, 2004). These glycoproteins coordinately assemble into the fibrous matrix on the surface of the oocyte (Sasanami *et al.*, 2004), and the one of its major components, ZP1, has the ability to induce the sperm acrosome reaction in Japanese quail (Sasanami *et al.*, 2007).

We have previously cloned the cDNA encoding ZP3 (GenBank Accession no. AB012606) and ZP1 (GenBank Accession no. AB061520) of the quail PL. Although the hormonal control as well as the machinery of post-translational modification of the major components has been investigated in detail (Pan *et al.*, 2001; Sasanami *et al.*, 2002; Sasanami *et al.*, 2003b; Sasanami *et al.*, 2003c), knowledge of the remaining components is still lacking. In the present study, we have cloned a novel constituent of quail PL protein, ZPD, which was first discovered in the egg envelope of *Xenopus laevis* (Lindsay *et al.*, 2002), and show that the *ZPD* gene is expressed in the granulosa cells of the preovulatory follicles. Moreover,

we also provide details about the biochemical properties of the ZPD protein as well as the expression pattern of the gene.

Materials and Methods

Animals and tissue preparation

Female Japanese quail, *Coturnix japonica*, 15-30 weeks of age (Tokai-Yuki, Toyohashi, Japan), were maintained individually under a photoperiod of 14L: 10D (with the light on at 0500) and were provided with water and a commercial diet (Tokai-Hokuriku Nosan, Chita, Japan) *ad libitum*. Animals were decapitated, and the preovulatory follicles were dissected and placed in physiological saline. The granulosa layer was isolated as a sheet of granulosa cells sandwiched between the PL and the basal laminae, as previously described (Gilbert *et al*. 1977). PL was isolated by the procedure described by Sasanami *et al*. (2002), and the isolated PL was confirmed to be free from the granulosa cells by phase contrast microscopy. All experimental procedures for the use and the care of animals in the present study were approved by the Animal Care Committee of Shizuoka University (approval number, 19-13).

cDNA cloning of quail ZPD

Total RNA was extracted from the granulosa cells of the largest follicles with a commercial kit, RNAiso (Takara Biomedicals, Otsu, Japan), according to the manufacturer's instructions. Messenger RNA was isolated using an oligotex-dT30 mRNA purification kit (Takara Biomedicals, Otsu, Japan), according to the manufacturer's instructions, and was reverse transcribed using a Super Script II First-Strand Synthesis System for RT-

PCR kit with oligo (dT) primer (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) was performed using degenerate primers (sense primer; 5′-TCTMACASRKGAAAACCACACT-3′, antisense primer; 5′-ATKGGTCCATARGASACAATT-3′) designed from the alignment of available sequences of frog *ZPD* (GenBank accession number: U44949) and chicken *ZPD* (GenBank accession number: AB114441). PCR was performed in a 50 μl volume with PCR buffer, 5 mmol l^{-1} $MgCl_2$, 0.4 mmol l^{-1} dNTP, and 2.5 U of Taq DNA polymerase (Takara EX Taq, Takara Biomedicals, Otsu, Japan). Following the addition of cDNA as the template, the reaction mixtures were incubated at 94 ℃ for 1 min and amplified for 30 cycles. Each cycle consisted of 30 sec denaturation at 94 ℃, 30 sec annealing at 51.7 ℃, and 1 min elongation at 72 ℃. The amplicon was separated on 1.0% (w/v) agarose gel electrophoresis, and a band of the expected size was eluted from the gel with a Rapid gel extraction system (Marligen Bioscience, Ijamsville, MD, USA) according to the manufacturer's instructions. Purified DNA was subcloned into pT7 Blue T-Vector (Novagen, Madison, WI, USA) and sequenced by dye-terminator chemistry on an Applied Biosystem Model 310 sequencer by the dideoxy-mediated chain-termination method (Sanger *et al*., 1977). Based on the sequence information of the presumptive *ZPD* cDNA amplicon, 3′-RACE (rapid amplification of 3′ cDNA end) was performed using the 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA, USA). A gene-specific primer (5′-AGWRTRGAGGACTGCTGGGC-3′) in combination with adaptor primers was used for 3′-RACE. For 5′-RACE (rapid amplification of 5′ cDNA end), cDNA was synthesized with a gene-specific primer (5′-GCCCAGCAGCCCTCCACACT-3′), and RACE reaction was performed using the 5′ RACE System for Rapid

Amplification of cDNA Ends, Version 2.0 (Invitrogen, Carlsbad, CA, USA). The gene-specific primer in combination with adaptor primers used was 5'-CACCTCCAGCTGAAAGCTCCT-3'. RACE amplicons were subcloned and sequenced as described above.

Sequence computations

Homology search by the position-specific iterated, basic local alignment search tool (PSI-BLAST) method (Altschul *et al*., 1997) and alignment of multiple protein sequences by the CLUSTAL W method (Thompson *et al*., 1994) were performed using the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp). The signal peptide and its putative cleavage site were predicted according to Nielsen *et al*. (1997) using SignalP v2 (http://www.cbs.dtu.dk/services/SignalP/).

RNase protection assay

PCR-amplified fragments derived from quail *ZPD* cDNA (345 nt; 172-516 of GenBank accession number AB301422) or quail S17 ribosomal protein cDNA (Yamamoto *et al*., 2003) were subcloned into pGEM-3Z vector (Promega Corporation, Madison, WI, USA). After linearization with appropriate restriction enzymes, radioactive antisense or sense riboprobes were synthesized using Riboprobe in vitro Transcription System (Promega Corporation, Madison, WI, USA) and [α-^{32}P] CTP (NEN Life Science Products, Boston, MA, USA). The RNA probes were hybridized with total RNA overnight and digested with RNase A and T1 (Tanaka *et al*., 1999). The protected RNAs were separated on 6% (w/v) polyacrylamide/urea gels. The radioactive bands on the dried gels were detected with autoradiography

with the aid of an intensifying screen (Kodak, Rochester, NY, USA).

In situ hybridization

Birds were decapitated, follicles were immediately removed, and frozen sections were prepared. *In situ* hybridization was carried out as described previously (Yoshimura *et al.*, 2000). The antisense 45 mer oligonucleotide probe (nucleotides 162-206 of GenBank accession number AB301422) was labeled with [^{33}P] dATP (NEN Life Science Products, Boston, MA, USA) using terminal deoxyribonucleotidyl transferase (Gibco, Frederic, MD, USA). The sense probe was also labeled as described above. Hybridization was carried out overnight at 42℃. Washing was performed at room temperature for 30 min and at 55℃ for 40 min twice. After washing, slides were exposed to Biomax-MR film (Kodak, Rochester, NY, USA) for 2 weeks.

Culture of granulosa cells

Granulosa layers were incubated for 10 min at 37 ℃ with 500 U ml^{-1} collagenase (Type I, Sigma Chemical Company, St. Louis, MO) in Hanks' balanced salt solution (Hanks' BSS). Dispersed cells were washed three times with RPMI 1640 medium (Gibco/Life Sciences, Gaithersburg, MD, USA) containing 25 m mol ml^{-1} Hepes and antibiotics (100 U ml^{-1} penicillin and 75 U ml^{-1} streptomycin) by repeated centrifugation at 80 x g for 3 min and finally suspended in RPMI 1640 medium containing 10% (v/v) foetal calf serum, 25 m mol ml^{-1} Hepes and antibiotics. After counting the cell numbers by hematocytometer, the granulosa cells were inoculated in 24-

well culture plates at a concentration of 1 x 10^5 viable cells per well and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 41 ℃. The viability of the cell preparations was determined using the trypan blue exclusion technique (Roberts *et al*., 1994), and was greater than 95%. After the initial plating period of 6 h, the medium was changed to RPMI 1640 medium supplemented with or without ovine follicle stimulating hormone (FSH, 100 ng ml^{-1}). Ovine FSH (NIDDK-oFSH-17) was generous gift from National Hormone and Pituitary Program (Baltimore, MD) and the concentration of FSH was adopted from the studies of Pan *et al*. (2003) and Sasanami *et al*. (2003a). The cells were further cultured for 48 h. Total volume of culture medium was adjusted to 0.8 ml per well, and the granulosa cells used in all experiments were obtained from at least two birds. After the culture, the medium was collected and the debris was precipitated by centrifugation at 10,000 x g for 10 min. The supernatants after centrifugation were served as conditioned medium of the granulosa cells. The conditioned medium was stored at –80C until use.

Production of antiserum against quail ZPD

Rabbit polyclonal anti-quail ZPD antibody was raised against bacterially expressed His-tag fused with partial ZPD protein (from Ser^{43} to Glu^{262}). Quail *ZPD* cDNA was amplified by PCR (cycling conditions: 94 °C for 1 min, 63 °C for 1 min, and 72 °C for 1 min for 30 cycles) in order to introduce *HindIII* and *XbaI* sites upstream of the Ser^{43} and downstream of the Glu^{262}, respectively. The sense and antisense primers used were 5′-AAAAAAGCTTAGCTCAGATGCCTGCATGCCCAA-3′ and 5′-

AAAATCTAGACTCCACACTCAGCAGGAAGT-3', respectively. The PCR product containing the partial quail *ZPD* cDNA was digested with *HindIII* and *XbaI*, and ligated into pCold TF DNA vector (Takara Biomedical, Otsu, Japan) treated with the same restriction enzymes. The resulting construct was transformed into competent *Escherichia coli*, strain BL21 (Takara Biomedical, Otsu, Japan), and the ampicillin-resitant clone was selected after the nucleotide sequence analysis was performed. Recombinant quail ZPD was expressed in the presence of 1 mmol l^{-1} Isopropyl-β-thiogalactopyranoside at 15℃ for 24h, and the protein was purified from the cell lysate using nickel resin (Novagen, Madison, WI, USA) according to the manufacturer's instructions.

A single female New Zealand White rabbit (SLC, Hamamatsu, Japan) was immunized with the recombinant quail ZPD (300 μg of protein) as described previously (Kuroki & Mori 1995).

Gel electrophoresis and Western blot analysis

The PL was extracted in SDS-Tris (1% (w/v) SDS buffered at pH 6.8 with 70 mmol l^{-1} Tris-HCl). Insoluble materials were removed by centrifugation at 14,500 x *g* for 15 min, and clear supernatants served as PL extracts. Although we did not confirm whether the total protein of the PL was completely dissolved, using this method, most of the PL disappeared during the extraction step, and the visible precipitates after the centrifugation was not found in our experimental conditions. The protein concentration in each sample was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).

SDS-PAGE under non-reducing or reducing conditions was carried out as

described previously (Laemmli 1970), using 12 and 5% (w/v) polyacrylamide for resolving and stacking gels, respectively. For Western blotting, proteins separated on SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Bedford, MA, USA) (Matsudaira 1987). The membrane reacted with anti-quail ZPD antibody (1:10,000) was visualized by means of a chemiluminescent technique (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using horseradish peroxidase-conjugated anti-rabbit IgG (Cappel, Durham, NC, USA) as a secondary antibody. Visualized blots were digitized using a Macintosh-based image analyzing system (NIH Image v.1.61).

Two-dimensional gel electrophoresis was performed using a Protean IEF Cell apparatus with IPG ReadyStrip (pH range: 4-7, length: 7cm, Bio-Rad, Hercules, CA, USA). The PL isolated from the largest follicles was dissolved in sample preparation buffer (8 mol l^{-1} urea, 0.2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxypropanesulfonic acid, 0.2% (w/v) ampholine, and 0.001% (w/v) bromophenol blue). Aliquots (50μg) were then applied to the IPG ReadyStrip, and were separated by isoelectric focusing under the standard procedure suggested by the manufacturer. They were then subjected to SDS-PAGE under non-reducing conditions as described above. Separated proteins were detected with Coomassie Brilliant Blue R 250 staining or Western blotting with anti-quail ZPD antibody as described above.

PNGase F digestion

The PL extract was digested with PNGase F, an amidase that cleaves between the innermost *N*-acetylglucosamine and asparagines residues of *N*-

linked glycoproteins (E.C.3.5.1.52; New England Biolabs, Beverly, MA, USA), according to the manufacturer's instructions. Briefly, the PL extract was mixed with 1/10 volume of 10 x G7 buffer (0.5 mol l^{-1} sodium phosphate, pH 7.5) and 10% (v/v) NP-40. The mixture was incubated in the presence or absence of PNGase F at 37℃ for 20 h. It was then separated on one-dimensional SDS-PAGE or two-dimensional gel electrophoresis, and the ZPD band was detected with Western blotting as described above.

N-terminal sequence analysis of ZPD

Aliquots (1 mg of protein) of the PL extract separated on SDS-PAGE were transferred to PVDF membranes as described above. After visualization of the bands by CBB staining, the band of ZPD was directly applied to an automated gas-phase protein sequencer (Model PPSQ-21A, Shimadzu Corporation, Kyoto, Japan).

Immunohistochemical observation

For localization of ZPD in the follicle, the preovulatory follicles were dissected, fixed in Bouin's fixative, and embedded in Paraplast (Oxford Labware, St. Louis, MO, USA). Immunohistochemical techniques using anti-quail ZPD antibody (1:300) or normal rabbit serum (1:300) were described previously (Sasanami *et al.*, 2002). The immunolabeled sections were examined under an interference-contrast photomicroscope (BX 50, Olympus Optics, Tokyo, Japan).

Statistical Analysis

All experiments were repeated at least three times. Data were

analyzed for significant differences using ANOVA, and means were compared using Duncan's multiple range test. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

Results

Cloning of quail ZPD

The degenerate PCR amplified a product of 684 bp from quail granulosa cells, and RACE procedures led to the isolation of 5′ and 3′ ends of the cDNA covering the open reading frame and untranslated regions (Fig. 1A). The sequences have been deposited in the GenBank database (accession number: AB301422).

The isolated cDNA encoded 420 amino acids, including a predicted 21-amino acid signal peptide (Fig. 1A). As other ZP proteins, ZPD contains a ZP domain (Ala^{86}-Leu^{342}) and a consensus furin-cleavage site (Arg^{344}-Ser^{345}-Lys^{346}-Arg^{347}) near the C terminus. Interestingly, unlike other ZP proteins, quail ZPD possesses an additional consensus furin-cleavage site in the upstream of the ZP domain (Arg^{39}-Ala^{40}-Lys^{41}-Arg^{42}), indicate the possibility that the N terminus as well as the C-terminal region of the newly synthesized precursor protein could be proteolyticaly processed by furin-like protease. From the N-terminal amino acid sequence analysis, we found that the first 7 residues are Ser-X-Asp-Ala-X-Met-Pro, which corresponded to the sequence from Ser^{43} to Pro^{49} of the deduced amino acid sequence from the cDNA. This result also supports the hypothesis that the N-terminal proteolytic cleavage of the precursor for ZPD occurs at the consensus furin cleavage site, Arg^{39}-Ala^{40}-Lys^{41}-Arg^{42}. There are 4 putative *N*-glycosylation sites in the translated protein that are

completely conserved with chicken homologue and the 3 of them are common with the frog counterpart (Fig. 1B). A putative transmembrane domain could be identified in the C-terminal region of the protein (Ile^{394}-Ala^{410}) with a short cytoplasmic tail. We identified 10 Cys residues in the ZP domain of the quail ZPD (Fig. 1B). Among these, 10 Cys are conserved between quail and chicken, and 8 between quail and *Xenopus laevis* (Fig.1B), suggested that their 3-dimensional structures are similar to each other. Although the overall similarity of the amino acid sequence of quail ZPD with its frog counterpart was limited, these common features suggest that our clone is an authentic member of ZPD.

Pattern of ZPD mRNA expression in vivo

To determine the localization of *ZPD* transcripts, we analyzed the total RNA isolated from various tissues of laying quail using a gene-specific RNase protection assay (Fig. 2). As shown in the figure, a single protected probe fragment was detected only in the granulosa cells of the largest follicles (lane Granulosa (AS)). No such signal above the background was observed when the same sample was hybridized with sense probe (lane Granulosa (S)), confirming the specificity of the hybridization. These results demonstrated that the expression of ZPD gene is restricted to the granulosa cells in the quail ovary.

The temporal accumulation of the *ZPD* transcripts during follicular development was observed by an RNase protection assay. As shown in Fig. 3A, the band of the protected fragment in the F3 as well as F2 was faint (lanes 1 and 2), whereas the intensity of the band dramatically increased when the RNA derived from the F1 was detected (lane 3). The protected

fragment was not detected when the sample was prepared from small yellow follicles (data not shown). These results are in accord with the *in situ* hybridization data (Fig. 3B), where the intense signal was detected in the outer peripheral region of the follicular wall of F1. In comparison with that of the F1 specimens, the intensity of the signals in the F2 or the F3 was low. Again, no such intense signal was detected in the small follicles (less than 2 mm) or in any other specimens hybridized with sense probe (Fig. 3C). These results suggest that the expression of *ZPD* gene in the granulosa cells is progressively increased during follicular development, especially at the final stages of follicular maturation.

Biochemical properties of quail ZPD protein

To confirm the presence of ZPD protein in the PL, we analyzed the PL proteins by means of gel electrophoresis. Under non-reducing conditions, three major bands migrating around 175 kDa, 97 kDa and, 35 kDa, which corresponds to dimmeric and monomeric ZP1s and ZP3, respectively, were visualized in the PL extract after CBB staining (Fig. 4A lane 1). These results are consistent with our previous reports (Sasanami *et al*., 2002). No other prominent bands were detected, suggesting that the ZPD protein could be a minor component of the PL. To visualize the ZPD protein in the PL extract, we produced a specific antibody to quail ZPD and performed Western blot analysis. As shown in the figure, our anti-ZPD antibody reacted well with the band with 40 kDa in the PL extract under non-reducing conditions (Fig. 4A, lane 2). When the sample was separated under reducing conditions, a retarded mobility shift of the immunoreactive band was observed (43 kDa, Fig. 4A, lane 3). Although we

were not able to determine the reasons for this result, the intensity of the immunoreactive band under reducing conditions became very weak in comparison with that of the band under non-reducing conditions. No band was detected when the sample was reacted with normal rabbit serum (data not shown), which suggests that our anti-ZPD antibody is specific to 40-kDa ZPD protein. These results suggest that the immunoreactive materials that reacted with anti-ZPD antibody exist in the quail PL extract, and it might possess the intra-molecular disulfide bond(s) in the molecule, since the retarded mobility shift was observed when the proteins were denatured to reduce the proteins in the presence of mercaptoethanol.

The carbohydrate moieties of the ZPD protein were analyzed by digestion with PNGase F (Fig. 4B). The mobility of the immunoreactive band on SDS-PAGE increased in correspondence with the amount of the enzymes added, and five bands with higher mobilities were detected, indicating that the quail ZPD might carry multiple *N*-linked oligosaccharides. The change in the apparent molecular weight of ZPD after PNGase F digestion was estimated at 8 kDa. This value corresponds appropriately with the calculated molecular mass (34.7 kDa) of the putative mature protein without the N-terminal and C-terminal fragments from the nascent precursor protein (Ser^{43}-Arg^{347}), removed by furin-like protease.

To investigate the biochemical properties of ZPD protein further, we performed two-dimensional gel electrophoresis (Figs. 4C and D). The 40-kDa band that appeared in the one-dimensional SDS-PAGE under non-reducing conditions was separated further into 4 major spots that ranged from pI values of 6 to 7 by isoelectric focusing (Fig. 4C). A faint

immunoreactive spot was also seen in the position of pI around 5.5. To confirm the effect of *N*-glycosylation of the protein on the pI value, we digested the PL extract with PNGase F and performed two-dimensional gel electrophoresis followed by Western blot analysis. As shown in Fig. 4D, multiple spots detected in the absence of PNGase F in panel C are mainly converged into a single spot with pI around 6.5. Although the nature is not known, several spots in the position of pI around 5.5 were also detected. These results suggested that several ZPD isoforms detected as multiple spots with different pI value might possess different types or different numbers of *N*-linked oligosaccharides in the molecule.

Accumulation of ZPD protein during follicular growth

In the next experiments, we investigated the accumulation of the ZPD protein in the PL during follicular development by Western blot analysis. As shown in Fig. 5, F4 extract contains a small amount of immunoreactive proteins (0.43 ± 0.59, mean ± SD of arbitrary unit), but the intensity of the band increases in correspondence with the follicular growth (F3; 1.06 ± 0.7.2, F2; 2.17 ± 0.54). The intensity reached the maximum level when the PL was isolated from the largest follicle (2.95 ± 0.08). These results suggested that the ZPD protein accumulated in accordance with follicular growth.

Localization of ZPD protein in the ovary

To investigate the localization of ZPD protein in the follicles, we prepared paraffin sections of preovulatory follicles in various sizes and

analyzed them by immunohistochemistry. As shown in figure 6A, the immunoreactive material recognized by anti-quail ZPD antibody accumulated in the PL apposed to the apical surface of the granulosa cells of the largest follicle. In agreement with the data of Western blotting shown in Fig. 5, the amount of immunoreactive substances was low in the F3 follicles (Fig. 6B) and was the below the detection level in the small yellow follicles (Fig. 6C). No positive immunostaining was seen when the sections were stained with normal rabbit serum (Figs. 6D-F). These results indicate that ZPD could be selectively transported and secreted toward the apical surface of granulosa cells, which are apposed to the PL, and accumulate in the PL during follicular development.

Effect of FSH on the secretion of ZPD protein in the cultured granulosa cells

To investigate the effects of gonadotropic hormone on ZPD production, the granulosa cells isolated from the largest (F1), the second largest (F2) or the third largest (F3) follicles were cultured for 48 h with or without FSH. The culture media were collected and subjected to Western blotting to detect the effects of the hormone on ZPD protein contents. As shown in Figure 7, the immunoreactive band of ZPD was detected in the control medium (without FSH) irrespective of follicular development. The intensity of immunoreactive band of ZPD in the control medium increased when the follicle developed from F3 to F2. Although the addition of FSH caused no effect on the secretion of ZPD by F1 and F2 derived granulosa cells, the amount of ZPD in the medium was

significantly increased by FSH stimulation in the case of F3 cells.

Discussion

As reported previously in our studies, quail granulosa cells produce ZP3, which is one of the components of the PL (Pan *et al*. 2001), whereas another constituent, ZP1, is synthesized in the liver (Sasanami *et al*. 2003b). In the present study, we found that another minor constituent, ZPD, was produced and secreted from the granulosa cells in Japanese quail. Recent study in the chicken also demonstrated that the expression of *ZPD* is restricted to granulosa cells (Okumura *et al*., 2004). However, the origin of ZPD in the *Xenopus laevis* is the oocyte itself (Lindsay *et al*., 2002). Although no ZPD homologue has been identified in mammalian species, the expression of ZP glycoproteins appears to be regulated differently in different species. There is clear evidence showing that ZP3 is synthesized by the oocytes themselves in mice (Bleil and Wassarman, 1980) and in *Xenopus* (Yamaguchi *et al*., 1989), whereas the granulosa cells in the follicles also participate in the formation of ZP3 in rabbits (Lee & Dunbar, 1993) and in cynomolgus monkeys (Martinez *et al*., 1996). On the other hand, a glycoprotein homologous to ZP3 in chorion, the fish egg envelope, is produced in the liver and transported to the ovary by the blood circulation, like vitellogenin (Hamazaki *et al*., 1985; Hamazaki *et al*., 1989). Thus it is also possible that the expression of *ZPD* in birds and frogs could be regulated differently. Although the hormonal regulation of frog ZPD biosynthesis had not been investigated yet, our current study indicated that the secretion of ZPD protein from

the cultured granulosa cells isolated from the immature follicles was stimulated by FSH. This result suggests that FSH plays a role in stimulating the secretion of ZPD protein in quail granulosa cells. In analogous situation, we previously reported that the secretion of ZP3 by the cultured granulosa cells also stimulated with the supplementation of FSH in the medium (Pan *et al*., 2003).

From the deduced amino acid sequence analysis of quail ZPD, we found that there is a consensus furin cleavage site in the N-terminal region upstream of its ZP domain in addition to the C-terminal one, which is common in all known ZP glycoproteins (Rankin & Dean, 2000; Jovine *et al*., 2005). The C-terminal consensus furin cleavage site is suggested to be involved in the modification of precursor to form the mature ZP glycoprotein in various species (Litscher *et al*., 1999; Kubo *et al*., 1999; Sasanami *et al*., 2002; Yonezawa *et al*., 2003). In addition to the role of the consensus furin cleavage site in the biosynthesis of ZP glycoproteins, it is also reported that the removal of the C-terminal fragments at this site controls the secretion/release of the ZP glycoprotein from the cells in mice (Jovine *et al*., 2002; Jovine *et al*., 2004; Jovine *et al*., 2005). The results of our previous study in Japanese quail also suggest this C-terminal proteolytic processing is a prerequisite for ZP3 secretion from the cells (Sasanami *et al*., 2003c). Although the participation of the consensus furin cleavage sites for ZPD biosynthesis has not yet been elucidated, it is quite interesting to investigate the role of N-terminal proteolytic processing in the ZPD production, since the presence of two peptidase sites in the sequence is the unique feature of avian ZPD protein.

Another feature of quail ZPD is that there are 4 potential *N*-glycosylation sites in the amino acid sequence, and from the N-terminal amino acid sequence analysis, there might be 3 sites in the mature protein. In our results, more than 4 bands with faster mobility that reacted well with anti-quail ZPD antibody were seen after PNGase F digestion (Fig. 4B), suggesting that multiple Asp residues might be occupied by *N*-linked oligosaccharides, though direct evidence of this was not obtained. In two-dimensional electrophoresis, four immuoreactive spots were detected after the isoelectric focusing. The presence of these multiple spots with similar molecular mass is probably due to the differences of post-translational modifications in each ZPD isoform. (*e.g.*, glycosylation, sulfation, *etc.*). This statement is also supported by the result of the present study that the digestion of the PL extract with PNGase F results in the convergence of multiple spots mainly into a single spot with pI around 6.5. This value is similar to the calculated pI value of the mature ZPD peptide backbone (*i.e.*, pI 6.28).

The immunohistochemical study with anti-quail ZPD antibody showed that immunoreactive material is present only in the PL located in the apical side of the granulosa cells (Fig. 6). Therefore ZPD might be selectively transported from the Golgi apparatus toward the apical surface of granulosa cells, which are apposed to the PL. Similarly, we found that the majority of the secreted ZP3 from polarized Madin-Darby canine kidney (MDCK) cells cultured in a dual chamber apparatus were detected in the apical compartment, but not in the basolateral side chamber (Sasanami *et al.*, 2005). Moreover, when a ZP3 mutant without the consensus sequence for *N*-glycosylation was transfected into the cells,

the ZP3 lacking the *N*-glycans was detected in both the apical and the basolateral medium (Sasanami *et al.*, 2005). These results indicated that ZP3 expressed in MDCK cells is selectively released to the apical compartment, and that the *N*-linked carbohydrates might possess information that causes the efficient transport of ZP3 to the apical surface of the cells. Although we do not know whether the *N*-glycans attached on the ZPD possesses a functional role, efforts are currently in progress to investigate the topology of ZPD secretion in which ZPD selectively secreted to the apical surface of the granulosa cells forms the PL.

In our previous study, Western blot analysis using specific antisera indicated that ZP1 was first detected when the granulosa layer was isolated from the fourth largest follicle, and the intensity of the band was dramatically increased after the follicle matured to the third largest size. On the other hand, immunoreactive ZP3 appeared as early as in the granulosa layer obtained from the small yellow follicles, and the intensity of the immunoreactive band increased progressively during follicular development. These results demonstrated that the accumulation of ZP1 was not synchronized with that of ZP3 in the PL during follicular development (Sasanami *et al.*, 2004). In the present study, we found that the contents of ZPD protein in the PL as well as its transcripts in the cells increased during follicular development, especially at the latter stages of folliculogenesis, similar to ZP1 accumulation. Based on these observations, we suggest that the accretion of ZP3 protein on the surface of the oocyte by an unknown mechanism might trigger the ZP1 binding to the PL, and that the ZP1 accumulation leads to

the association of ZPD protein in the PL. Okumura *et al*. reported that in the chicken the ZPD protein might bind loosely to the PL because it is released by the ultra sonication treatment without urea, though the binding partner is not known. From the *in vivo* accumulation pattern of ZPD in the present study, we speculate that the binding partner of ZPD might be ZP1 but not ZP3, though the experimental evidence has not been obtained. This is very likely the case, since the pI value of ZP1 is around 8-9, whereas that of ZPD as well as ZP3 ranges from 5 to 7 (data not shown). This statement is also supported by our previous finding that the radio-labeled ZP3 protein failed to visualize the ZPD band when the total PL extract was detected by ligand blot analysis (Ohtsuki *et al*., 2004; Sasanami *et al*., 2006). The question of whether such interaction between ZPD and ZP1 actually occurs during the PL formation remains to be resolved, and the elucidation of the role of ZPD protein in the formation of the insoluble PL fiber must await future analysis.

Acknowledgments

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Figure legends

Fig. 1 (A) Nucleotide and deduced amino-acid sequences of quail ZPD cDNA. The deduced amino acid is represented as a single-letter abbreviation shown below the nucleotide sequence (GenBank Accession Number: AB301422). The signal sequence (italic letters), the zona pellucida domain (ZP domain, white box), the consensus site for *N*-glycosylation (bold letters), the consensus furin cleavage site (bold letters with underlining), a poly adenylation signal sequence (underlining), and the putative transmembrane domain (shaded box) are indicated. The asterisk indicates the termination codon. (B) Alignment of amino acid sequence of quail, chicken, and *Xenopus* ZPD. Conserved residues are shown in asterisk and the consensus site for *N*-glycosylation is in bold letters, and the consensus furin cleavage site is underlined. Conserved Cys residues were indicated with a shaded box.

Fig. 2 Tissue-specific expression of ZPD mRNA. RNase protection assays were performed to detect the mRNA expression of the *ZPD* gene. We hybridized 15 μg of total RNA isolated from brain, heart, lung, spleen, kidney, oviduct, adrenal gland, theca layers of the largest follicles, and granulosa layers of the largest follicles with riboprobes specific to *ZPD*. In case of the RNA isolated from the granulosa cells, we also hybridized it with sense probes (S) instead of antisencse probes (AS). Protected fragments were detected by autoradiography. Shown are the results representative of repeated experiments.

Fig. 3 Expression pattern of ZPD mRNA during follicular development. (A) Autoradiograms of protected fragments. The 5 μg of total RNA the granulosa layer isolated from the third (lane 1), the second (lane 2), or the largest follicles (lane 3) were hybridized with the indicated

riboprobes (ZPD or S17). Shown in upper panel are representative autoradiograms of triplicate experiments. Data shown in lower panel are represented as the mean ± SD of triplicate experiments (each value was normalized against an S17 control). (B-C) *In situ* hybridization of quail ovary. Autoradiograms of ovarian sections after hybridization with ^{33}P-labeled antisense probe specific to ZPD are shown (B). Control hybridization with ^{33}P-labeled sense probe is depicted in (C). Scale bar, 5 mm. The results are representative of repeated experiments.

Fig. 4 Western blot analysis of ZPD protein in the PL extract. (A) The PL extract (30 μg) was transblotted onto a PVDF membrane after separation on SDS-PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue R 250 (lane 1). The sample separated under non-reducing (lane 2, 1 μg/lane) or reducing (lane 3, 10 μg/lane) conditions was probed with anti-quail ZPD (1:10,000 dilution). The blots shown are representative of 3 experiments. (B) The PL extract (2 μg) was digested with 0 (lane 1), 15 (lane 2), 20 (lane 3), 25 (lane 4), 30 (lane 5), 40 (lane 6), or 50 units (lane 7) of PNGase F as described in Materials and methods. Samples were then separated on SDS-PAGE under non-reducing conditions, transblotted onto PVDF membrane, and detected with anti-quail ZPD antibody (1:10,000). The 40-kDa ZPD and 5 bands with faster mobility are indicated by arrowheads. Note that lane 6 contains a doublet bands. Shown are the results representative of repeated experiments. (C) Two-dimensional gel electrophoresis. 50 μg of the PL extract was first separated by isoelectric focusing (IEF; pH 4-7), then subjected to SDS-PAGE under non-reducing conditions, and detected by means of Western blotting. Theoretical pI values are indicated on the top of the panel.

Shown are representative of repeated experiments. (D) 50 μg of the PL extract was digested with 1250 units of PNGase F as described in Materials and methods. They were then separated on two-dimensional gel electrophoresis as described in the legend for panel C. Shown are representative of repeated experiments.

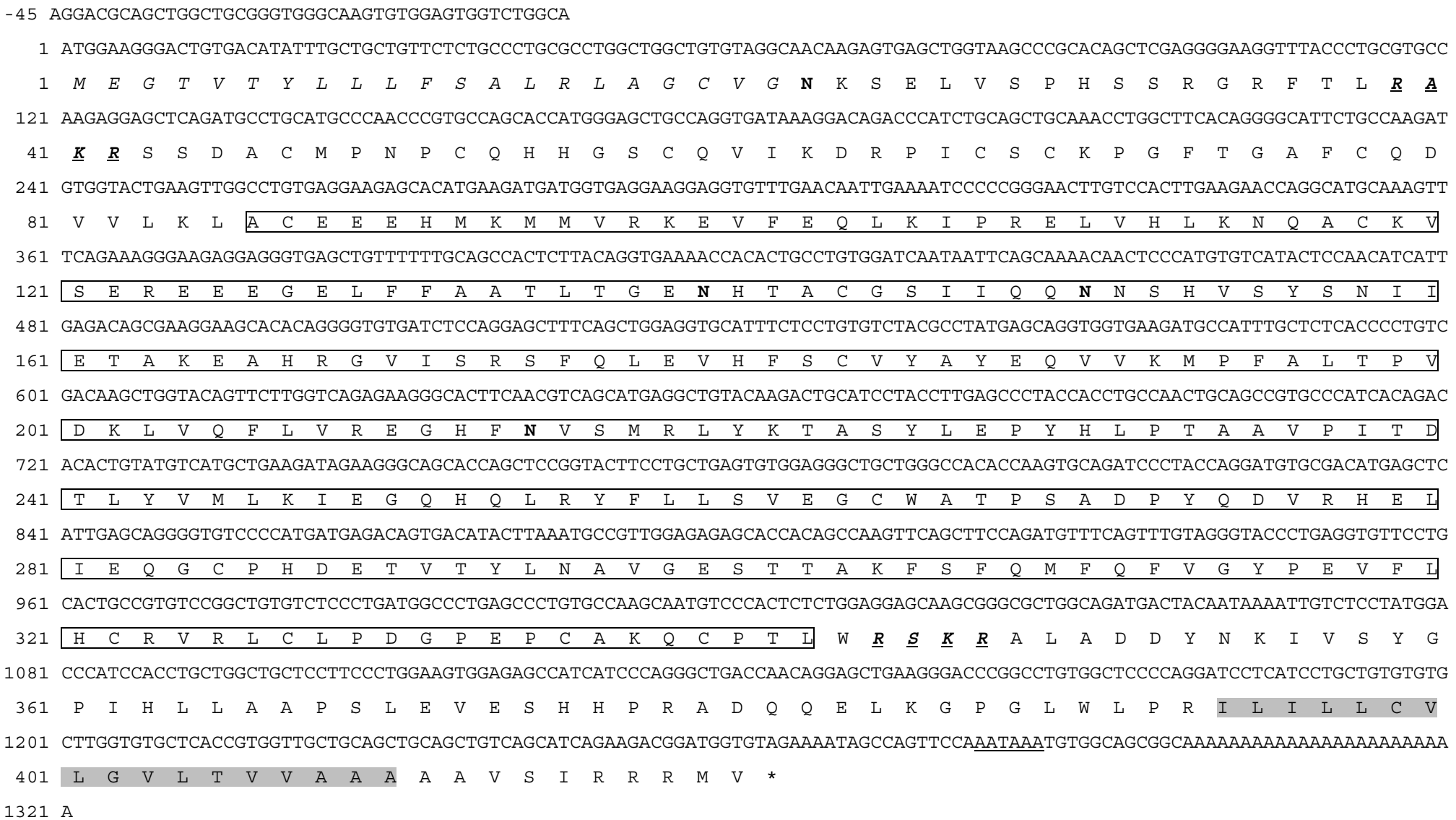
Fig. 5 Accumulation of ZPD protein during follicular development. (A) Aliquots (3 μg protein per lane) of the SDS-solubilized PL isolated from the largest (F1), the second largest (F2), the third largest (F3), and the fourth largest (F4) preovulatory follicles were subjected to Western blot analysis using anti-quail ZPD antibody (1:10,000). Representative blots are shown. (B) The signals were quantified as an arbitrary unit, and the data in the figure were expressed as means ± SD of the arbitrary unit of 3 independent experiments.

Fig.6 Immunohistochemical analysis of ZPD in follicular wall. Sections of follicular wall obtained from the largest (A and D), the third largest (B and E), and small yellow follicles (C and F) were processed for immunohistochemical observation using anti-quail ZPD antibody (A, B, C; 1:300) or normal rabbit serum (D, E, F; 1:300). Shown are the results representative of repeated experiments. Bar = 50 μm

Fig.7 Effect of FSH on ZPD protein secretion. (A) Granulosa cells isolated from the largest (F1), the second largest (F2) or the third largest (F3) follicles were cultured with (+) or without (-) 100 ng ml^{-1} FSH for 48 h. The ZPD protein in the medium was detected using anti-quail ZPD antibody. Immunoblots shown here are representative of three experiments. (B) The signals were quantified as an arbitrary unit, and

the data in the figure were expressed as means ± SD of the arbitrary unit of 3 independent experiments. Values with different superscripts are significantly different ($P < 0.01$).

Sato et al., Fig. 1A



Sato et al., Fig. 1B

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Quail          MEGTVTYLLLFSALRLAGCVGNKSELVSPHSSRGRFTLRAKRSSDACMPNPCQHHGSCQV
Chicken        MEGTVTYLLLFSALRLAGCEGN*SELVS*HNSR*RFA*RAK*S*DACVPNPCQHHGGCQV
Xenopus        ----------------------M*YCHST*WLLV*FIT*VID*I*SDLIQQ----------

Quail          IKDRPICSCKPGFTGAFCQDVVLKLACEEEHMKMMVRKEVFEQLKIPRELVHLKNQACKV
Chicken        IEDRPICSCKPGFTGAFC*DVVLK*A*EEEHMKMMVRKE*FEL*K*PRELV*LK*QA*KV
Xenopus        -------------------*NEMAD*K*DNDQ**ITLLSS*LDE*D*NASQL*MN*PL*NM

Quail          SEREEEGELFFAATLTGENHTACGSIIQQNNSHVSYSNIIETAKEAHRG------VISRS
Chicken        SEREEEGEMFFAAT**G****A**SVI*Q*S**VS***IIETGREAHRG------V*S**
Xenopus        QNTSG---LYLSIV**H****F**TAV*V*G**LI***ELTSGTSFVNQPVGPGSL*T**

Quail          FQLEVHFSCVYAYEQVVKMPFALTPVDKLVQFLVREGHFNVSMRLYKTASYLEPYHLPTA
Chicken        FQLEV******A*EQ**KM*FA*TPVDKL*Q*M*R**H***S*RL*K*ASYLE**DLLTA
Xenopus        SDIRI******K*DG**SL*YP*LTSFSS*T*V*K**I***T*TS*P*SEFKQ**EWLP-

Quail          AVPITDTLYVMLKIEGQHQLRYFLLSVEGCWATPSADPYQDVRHELIEQGCPHDETVTYL
Chicken        AV*ITDT*Y*M*KIEGQHQLR**L*SV*D*****S*D*YQDVLHEL*EQ*C*H*E**TYL
Xenopus        VI*LSQN*N*Q*QVHEHDLDN**S*RI*D*****T*N*EDKIRYPI*SS*Y*N*T**AMI

Quail          NAVGESTTAKFSFQMFQFVGYPEVFLHCRVRLCLPDGPEPCAKQCPTLWRSKRALADDYN
Chicken        NAIGESTTAK*SF***Q*VG*PK*F***R*R**L*DGPEP*AKQC*TLW*S**A*AD**N
Xenopus        QTLDN-SLTR*VM***H*IN*SE*Y***K*L**Q*NSTVF*NRPD*YVG*K**D*ES**S

Quail          KIVSYGPIHLLAAPSLEVESHHPRADQQELKGPGLWLPRILILLCVLGVLTVVAAAAAVS
Chicken        ********H*L*A*SLR**SHHPRADQQE*K*PSLWLPGILILLC*LGVLTMAAAAVSRR
Xenopus        ********T*T*T*LSG**RAESGMSDLA*L*SVSAGTMFVALFF*VIAKSLKWIRKLNG

Quail          IRRRMV----
Chicken        RRMV------
Xenopus        PTTYKVQATP
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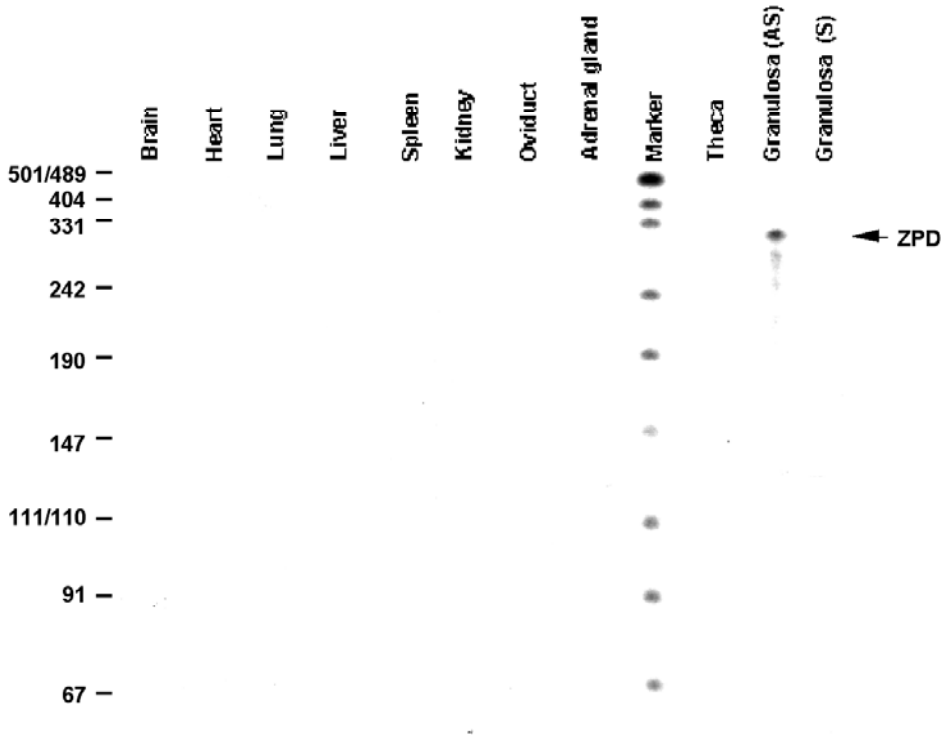
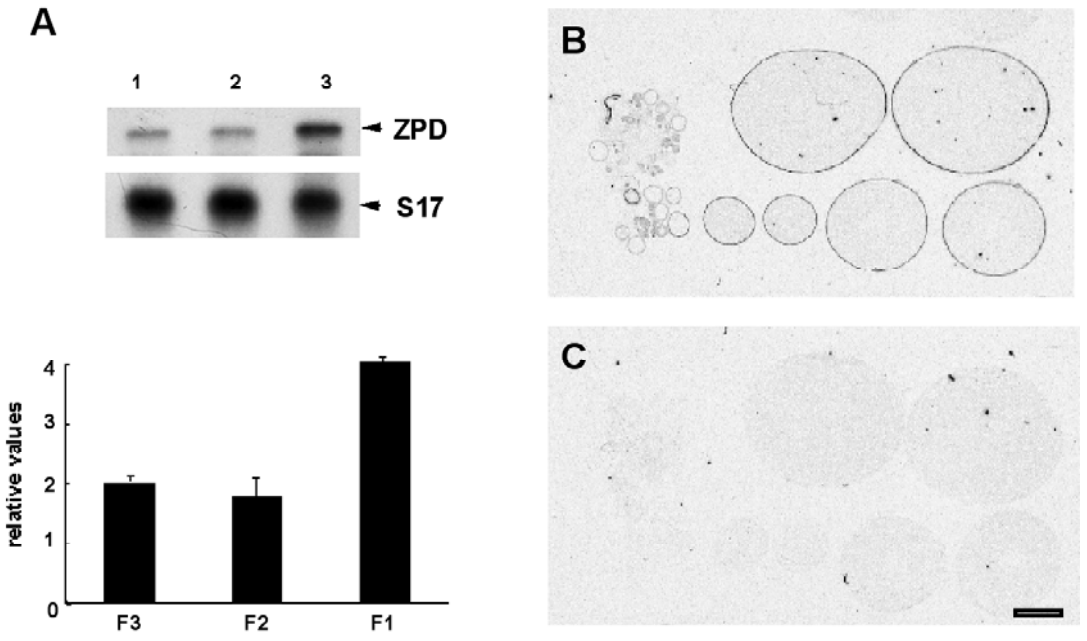
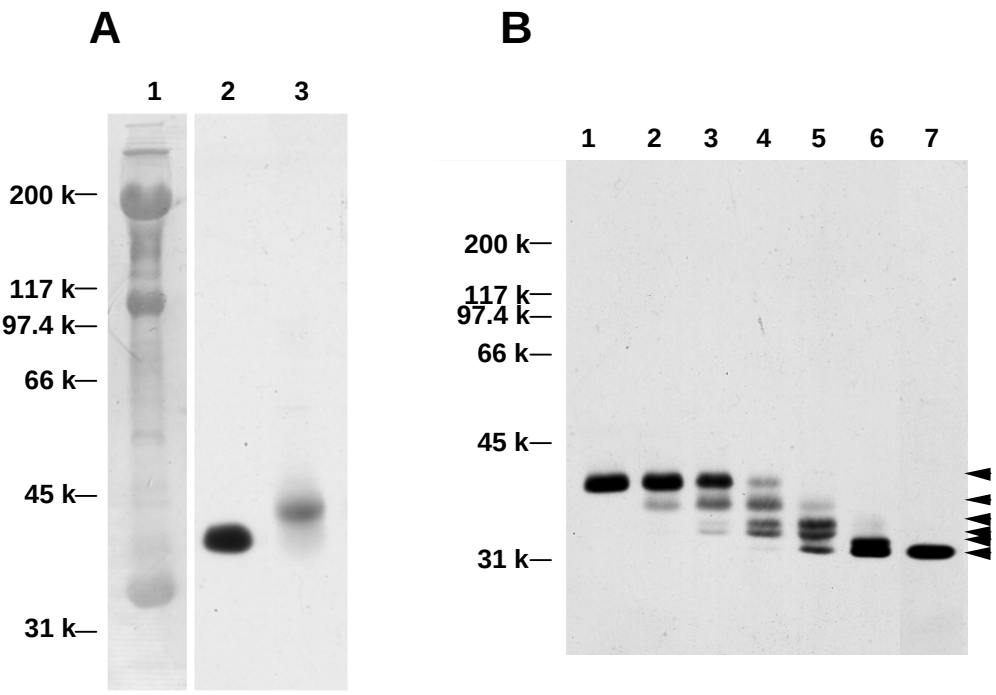
Sato et al., Fig. 2



Sato et al., Fig.3



Sato et al., Fig 4



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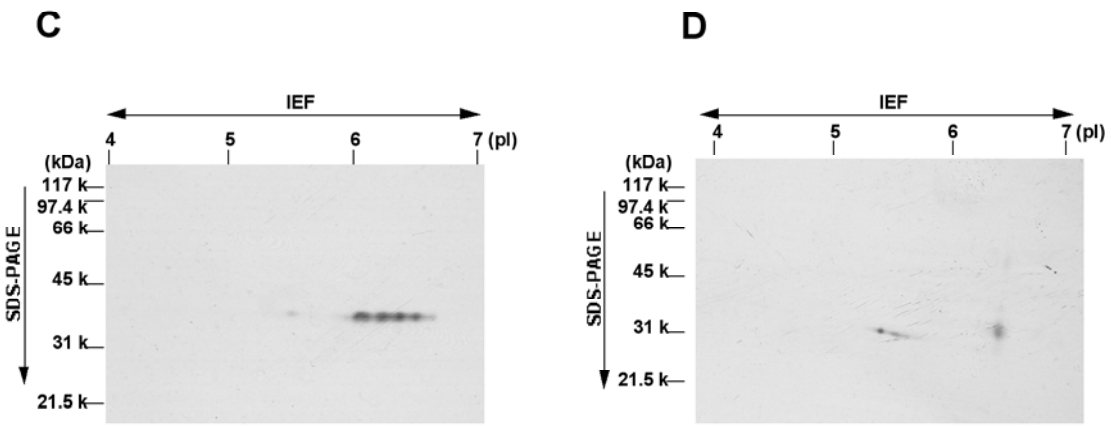
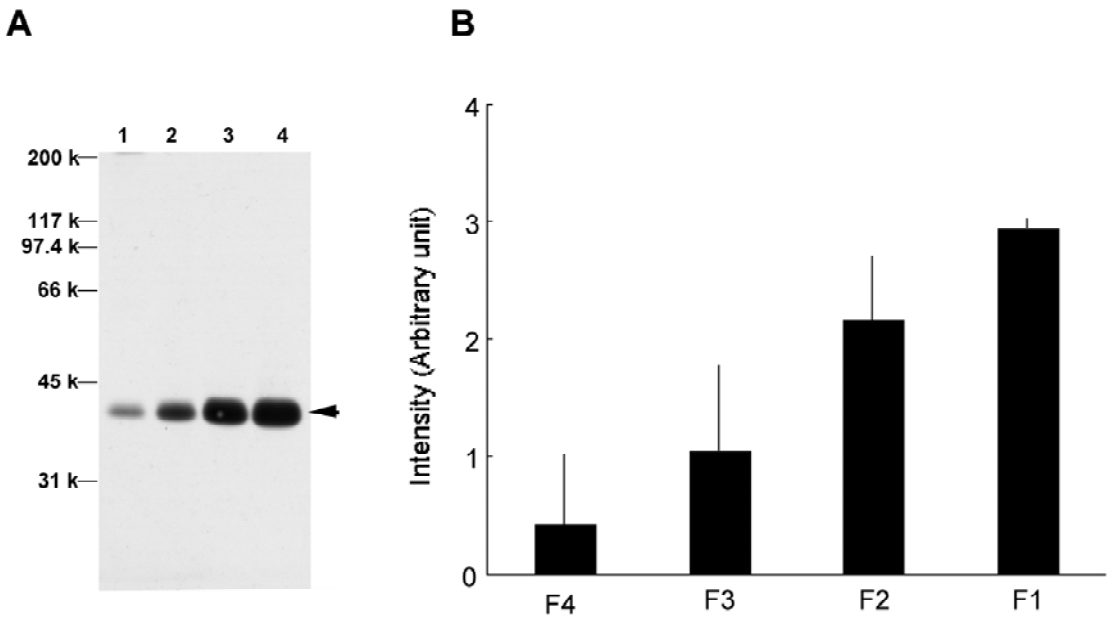
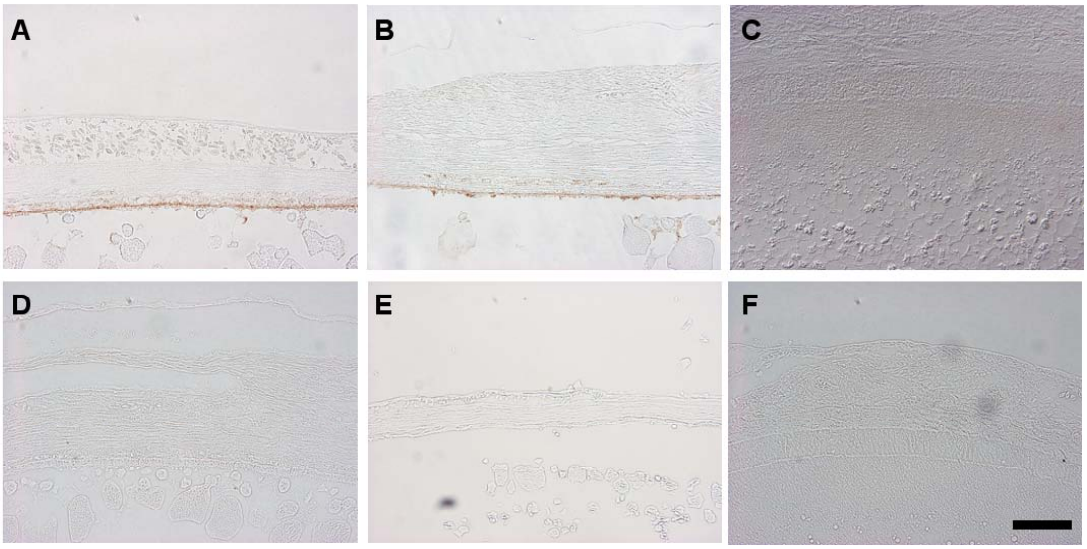


Sato et al., Fig 5



Sato et al., Fig. 6



Sato et al., Fig 7