Title: Zona Pellucida Protein ZP2 is Expressed in the Oocyte of Japanese Quail (*Coturnix japonica*).

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Abstract

The avian perivitelline layer, a vestment homologous to the zona pellucida of mammalian oocytes, is composed of at least three glycoproteins. Our previous studies have demonstrated that the matrix's components, ZP3 and ZPD, are synthesized in ovarian granulosa cells. Another component, ZP1, is synthesized in the liver and is transported to the ovary by blood circulation. In this study, we report the isolation of cDNA encoding quail *ZP2* and its expression in the female bird. By RNase protection assay and *in situ* hybridization, we demonstrate that ZP2 transcripts are restricted to the oocytes of small white follicles. The expression level of *ZP2* decreased dramatically during follicular development, and the highest expression was observed in the small white follicles. Western blot and immunohistochemical analyses using the specific antibody against ZP2 indicate that the 80-kDa protein is the authentic ZP2, and the immunoreactive ZP2 protein also present in the oocytes. Moreover, ultrastructural analysis demonstrated that the immunoreactive ZP2 localizes in the zona radiata, the perivitelline space and the oocyte cytoplasm in the small white follicles. By means of Western blot analysis and immunofluorescence microscopy, we detected a possible interaction of the recombinant ZP2 with ZP3 and that this interaction might lead to the formation of amorphous structure on the cell surface. These results demonstrate for the first time that the avian zona pellucida gene is expressed in the oocyte, and that the ZP2 protein in the oocyte might play a role for the PL formation in the immature follicles of the ovary.

Introduction

The egg envelope is an extracellular matrix that surrounds the oocyte of vertebrate animals and plays essential roles in gamete recognition during the initial process of fertilization, including species-specific sperm-egg binding, induction of the acrosome reaction and prevention of polyspermy (McLeskey *et al.*, 1998). 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 or four glycoproteins (*i.e.*, ZP1, ZP2, ZP3 and ZP4) in mammalian species (Litscher and Wassarman, 2007; Lefievre *et al.*, 2004; Ganguly et al., 2007; Hoodbhoy *et al.*, 2005; Izquierdo-Rico *et al.*, 2009). In the cases of non-mammalian vertebrate 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). The ZP gene family proteins share a highly conserved amino acid sequence near the C-terminus called the ZP domain, consisting of about 260 amino acid residues with 8 or 10 conserved cys residues (Bork & Sander, 1992).

The avian ovum is enveloped by multiple layers of different extracellular matrices, including an inner layer of vitelline membrane, a continuous membrane, and an outer layer of vitelline membrane (Bellairs *et al.*, 1963). At the time of ovulation, the avian oocyte is 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. 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; Sasanami *et al.*, 2007). During follicular development, because avian oocytes dramatically increase their size by the accumulation of egg-yolk delivered from blood circulation (Wyburn *et al.*, 1965), their egg envelope must extend quickly throughout the rapid growing period of the oocyte.

In avian species, 3 glycoproteins have been identified as the component of PL, ZP1, ZP3 and ZPD in both quail (Pan *et al.*, 2001; Sasanami *et al.*, 2003b; Sato *et al.*, 2009) and chickens (Waclawek *et al.*, 1998; Takeuchi *et al.*, 1999; Bausek *et al.*, 2000; Okumura *et al.*, 2004). These glycoproteins coordinately assemble into the fibrous matrix on the surface of the oocyte (Sasanami *et al.*, 2004), and one of its major components, ZP1, has the ability to induce the sperm acrosome reaction in Japanese quail (Sasanami *et al.*, 2007).

It has been suggested that in mouse zona pellucida, the three glycoproteins, ZP1, ZP2 and ZP3, are organized into long filaments and that these filaments are comprised of the long chains of the ZP2/ZP3 hetero-dimmer that are interconnected by ZP1 with disulfide bonds giving rise to a three-dimensional matrix (Greve & Wassarman, 1985). Among these glycoproteins, it has been suggested that ZP3 behaves as a primary sperm-binding ligand and that this relatively species-specific binding leads to the successful induction and completion of the sperm acrosome reaction (Wassarman & Litscher, 2008). In addition, the ZP2 protein has been proposed to play a role in secondary binding of the sperm that assists

the weak association of the acrosome-reacted sperm to the egg during fertilization (Wassarman & Litscher, 2008). It has also suggested that proteolytic cleavage of ZP2 after cortical reaction contributes to the blockade of polyspermy (Dean, 2003).

It has been reported that the avian PL is comprised of two major glycoproteins, ZP1 and ZP3, because after the separation of the PL constituents by SDS-PAGE, only two major bands (175 kDa and 35 kDa in the quail and 183 kDa and 32 kDa in the chicken) identified as ZP1 and ZP3, respectively, were detected with Coomassie brilliant blue (CBB) staining (Mori & Masuda, 1993; Kido & Doi, 1988). Thus, if other constituents are present in the PL, including ZP2, they might be present in lower amounts than in mammalian ZP. A recent analysis of chicken genome and EST sequence data has revealed that the ZP2 gene is expressed in the ovary and that the cDNA encoding chicken ZP2 has been cloned (Hughes, 2007; GenBank Accession number: AB197938). However, whether or not the ZP2 proteins actually present in the ovary is not known, and the question of which cells express this gene is still unanswered. In the present study, we cloned quail ZP2 cDNA, and show that the *ZP2* gene is expressed in the oocytes of the small white follicles (SWF). Moreover, we also provide details about the localization of the ZP2 protein in the follicles 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*. The animals were decapitated, and the preovulatory follicles were dissected and placed in physiological saline. The follicular walls were peeled off the large-yolk filled follicles by fine forceps, and the adhering yolk materials were washed away by a gentle stream of water from a Pasteur pipette. 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, 20-11).

cDNA cloning of quail ZP2

Total RNA was extracted from the small white follicles (SWF, apporoximately 1 mm in diameter) 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'-GGMATVYDYSYKYRGTGGAT-3', antisense primer; 5'-GTCCWRDTYRTAYKCACASCCATC-3') designed from the alignment of available sequences of chicken (GenBank accession number: AB197938), mouse (GenBank accession number: M34148), rat (GenBank accession number: AB000929), human (GenBank accession number: BC096305), dog (GenBank accession number: U05779), cat (GenBank accession number: U05776) and cow *ZP2* (GenBank accession number: AB042653). PCR was performed in a 50 μl volume with PCR buffer, 5 m mol ml^{-1} $MgCl_2$, 0.4 m mol ml^{-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 65.3 ℃, and 1.5 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 *ZP2* 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'-GCTTTCATGTCTGGTGACAAGG-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'-AACAAAGACAGGAGCGATGATTT-3'), and a 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'-CCGTGCTCCAGGCTGGTGCAGTT-3'. This amplicon was re-amplified with the nested gene-specific primer (5'-AGAGATCCCATTGCCCTGAGGCAG-3') in combination with adaptor primers. 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

Total RNA was extracted from brain, heart, lung, liver, spleen, kidney, oviduct, adrenal gland and SWF of laying female birds as described above.

PCR-amplified fragments derived from quail *ZP2* cDNA (350 nt; 148-494 of GenBank accession number AB295393) was subcloned into a pGEM-3Z vector (Promega Corporation, Madison, WI, USA). After linearization with the 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

The birds were decapitated, the follicles 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 1078-1122 of GenBank accession number AB295393) 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. After the exposure, the slides were coated with Kodak NTB-2 emulsion (Kodak), and exposed for 7 days at 4C in a dark box. After exposure, the slides were developed in Kodak Dektol and mounted in glycerol. The specimens were examined under a dark-field microscopy with an X 40 objective (Nikon, Tokyo, Japan).

Production of antiserum against quail ZP2

Rabbit polyclonal anti-quail ZP2 antibody was raised against bacterially expressed His-tag fused with partial ZP2 protein (from Thr^{134} to Leu^{236}). Quail *ZP2* cDNA was amplified by PCR (cycling conditions: 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min for 30 cycles) in order to introduce *XhoI* and *EcoRI* sites upstream of the Thr^{134} and downstream of the Leu^{236}, respectively. The sense and antisense primers used were 5′-AAAACTCGAGACAAAGGATTCCATGGCAGTT-3′ and 5′-CGGAATTCAAGCATTCTTGACTCCATGGT-3′, respectively. The PCR product containing the partial quail *ZP2* cDNA was digested with *XhoI* and *EcoRI*, 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 an ampicillin-resitant clone was selected after the nucleotide sequence

analysis was performed. Recombinant quail ZP2 was expressed as the 63 kDa fusion protein (11 kDa of the partial ZP2 protein with 52 kDa of the trigger factor plus the His tags) in the presence of 1 m mol ml^{-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. The purity of the recombinant ZP2 was verified by separating the protein by SDS-PAGE followed by CBB staining.

A single female New Zealand White rabbit (SLC, Hamamatsu, Japan) was immunized with the recombinant quail ZP2 (300 μg of protein) as described previously (Kuroki & Mori, 1995).

Gel electrophoresis and Western blot analysis

The SWF was homogenized in SDS-Tris (1% (w/v) SDS buffered at pH 6.8 with 70 m mol ml^{-1} Tris-HCl) and the proteins were extracted with vigorous shaking overnight at 4C. Insoluble materials were removed by centrifugation at 14 500 x *g* for 15 min, and clear supernatants served as SWF extracts. The protein concentration in each sample was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).

SDS-PAGE under non-reducing conditions was carried out as described previously (Laemmli 1970), using 10 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 ZP2 antiserum (1:1 000) 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.

Culture of granulosa cells

The granulosa layers isolated from the largest follicles were placed into a 6-well culture plate (Falcon Plastics, Los Angels, CA, USA) filled with 3 ml of Ham's F-12 (Dainippon Seiyaku, Osaka, Japan) supplemented with 10% (v/v) fetal calf serum, penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) at 39 °C, and were incubated for 8 h in a humidified atmosphere with 5% CO_2. After the incubation, the conditioned medium was collected and centrifuged at 10 000 x *g* for 10 min to remove cellular debris. The conditioned medium was analyzed with Western blot analysis using anti-quail ZP2 antiserum (1:500), anti-quail ZP3 antiserum (Kuroki & Mori, 1995) (1: 10 000) or normal rabbit serum (1:500) antiserum (Ohtsuki *et al.*, 2004) as described above.

Expression of ZP2 in a mammalian cell line

Quail ZP2 cDNA was amplified by PCR (the cycling conditions: 94 °C for 30 sec, 51.3 °C for 30 sec and 72 °C for 2 min, for 35 cycles) to introduce *HindIII* and *XbaI* sites upstream of the initiator methionine and downstream of the chain termination codons, respectively. The sense and antisense primers used were 5'- AAAAAAGCTTGCAGCTCTGGCACTTTGAGGA -3' and 5'- GCTCTAGATTAATGTACCACATTTACC -3', respectively. The PCR product containing the full-length quail ZP2 cDNA was digested with *HindIII* and *XbaI*, and ligated into the mammalian expression plasmid vector pcDNA3.1(+) (Invitrogen, San Diego, CA, USA) treated with the same restriction enzymes. The resulting quail ZP2 expression construct was transformed into competent *Escherichia coli*, strain *DH5α* (Takara, Osaka,

Japan). The sequence of the constructs was verified with DNA sequence analysis.

Chinese hamster ovary cells (CHO-K1 cells; generously provided by Dr. Kazuhiko Imakawa, Department of Animal Breeding, University of Tokyo, Tokyo, Japan) were cultured in Ham's F-12 (Dainippon Seiyaku) supplemented with 10% fetal calf serum, penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C in a humidified atmosphere with 5% CO_2. Before transfection, cells were plated into a 6-well culture plate (Falcon Plastics) and cultured for 48 h until they had grown to approximately 75% confluency. DNA (2.5 μg) was introduced into the CHO-K1 cells using Lipofectamine Plus Reagent (Gibco BRL) as suggested by the manufacturer, and the cells were cultured in fresh medium (3 ml/well) for an additional 48 h to express recombinant ZP2. After the culture, the conditioned medium was collected and centrifuged at 10 000 x *g* for 10 min to remove cellular debris. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), scraped from the plates into SDS-Tris (500 μl/well) and dissolved by vigorous shaking. After centrifugation at 10 000 x *g* for 10 min, clear supernatants were stored as total cell lysates. The protein concentration was determined as described above.

A quail ZP1 expression construct was generated and expressed in CHO-K1 cells as described previously (Sasanami *et al.*, 2006).

The cell lysates from ZP2-transfected cells and the conditioned medium derived from ZP1-expressing cells were concentrated to approximately 1/10 volume by using Amicon Ultra-4 centrifugal filter device (molecular weight cut off: 10 kDa, Millipore), and then analyzed with Western blot analysis using anti-quail ZP2 antiserum (1:500) or anti-quail ZP1

antiserum (1:500) as described above.

Analysis of the interaction of ZP2 and ZP3

CHO-K1 cells transfected with vector alone or ZP2 expression construct were cultured for 48 h on 0.1% gelatin-coated coverslips in the conditioned medium of the granulosa cells (3ml/well) at 37 °C in a humidified atmosphere with 5% CO_2. After the culture, the cells were fixed in 3.7% (v/v) formaldehyde in PBS at room temperature for 30 min, and were then permeabilized in cold acetone (-20 °C) for 5 min. After being washed with PBS, the cells were incubated with PBS containing 3% (w/v) gelatin for 1 h and were then incubated with anti-quail ZP2 antiserum (1:50), or anti-quail ZP3 antiserum (1:100) for 2 h at 4 °C. After several washings with PBS, the specimens were reacted with FITC-conjugated goat anti-rabbit IgG (1:50, Cappel) for 1 h at 4 °C. After several washings with PBS, the samples were embedded in glycerol, and were examined under a fluorescent microscopy (BX 50, Olympus Optics, Tokyo, Japan).

For the Western blot analysis, the cells after the culture were washed twice with PBS, scraped from the plates into SDS-Tris (500 μl/well) and dissolved by vigorous shaking. After centrifugation at 10 000 x *g* for 10 min, clear supernatants were detected using anti-quail ZP2 antiserum or anti-quail ZP3 antiserum as described above. The protein concentration was determined as described above.

Immunohistochemical observation

For localization of ZP2 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 ZP2 antiserum (1:300) or pre-immunoserum (1:300) were described previously (Sasanami *et al.*, 2002). The immunolabeled sections were examined under an interference-contrast photomicroscope (BX 50, Olympus Optics).

Immunoelectron microscopy

For immunoelectron microscopy, relatively small and large SWF (approximately 0.7 mm and 1.3 mm in diameter, respectively) were fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol ml^{-1} cacodylate buffer (pH 7.4) overnight at 4C. The specimens were embedded in Lowicryl K_4M resin (Polysciences, Warrington, PA, USA). Thin sections were first treated with anti-ZP2 antibody (1:100) and then with a gold-conjugated goat antiserum against rabbit IgG (1:30) (E-Y Laboratories, San Mateo, CA, USA). The samples were next stained with uranyl acetate and observed with a model H-8000 electron microscope (Hitachi, Tokyo, Japan)

The quantitative evaluation of the labeling density was performed as described previously (Kohsaka et al., 1993). Randomly selected electron micrographs taken at a magnification of X6,000-X10,000 were enlarged to X19,200-X32,000. Each photographic paper (25 cm x 15.5 cm) was weighed and the surface area of the photographic paper was calculated as $\mu m^2/g$. The number of the immuno-gold particles was counted manually, and the area of the printing paper contains oocytes or granulosa cells was scissored off and weighed. The labeling density was expressed as the number of particles per unit of surface area (μm^2). Data was expressed as means ± SD and was subjected to students' t-test using Excel (Microsoft, Redmond WA). Differences were considered statistically significant when $P < 0.05$.

Results

Cloning of quail ZP2

The degenerate PCR and RACE procedures led to the isolation of the cDNA covering the open reading frame and untranslated regions of quail ZP2 (Fig. 1A). The sequences have been deposited in the GenBank database (accession number: AB295393). The amplified sequence of ZP2 is 2196 nucleotides long and contains a single open reading frame. This sequence contains an ATG initiation codon and a stop codon (TAA) in positions at 1-3 and 2083-2085, respectively and a polyadenylation signal (AATTAAA) is found in positions at 2107-2113.

The isolated cDNA encoded 694 amino acids with a theoretical molecular weight of 75.585 kDa, including a predicted 21-amino acid signal peptide (Fig. 1A). As with other ZP proteins, ZP2 contains a ZP domain (Val^{347}-Leu^{613}) and a consensus furin-cleavage site (Arg^{615}-Ser^{616}-Lys^{617}-Arg^{618}) near the C terminus, indicating the possibility that the C-terminal region of the newly synthesized precursor protein could be proteolyticaly processed by furin-like protease. The molecular mass of the processed peptide (with no signal peptide and cleavage at Arg^{618} in the C-terminal region of ZP2 protein) was calculated to be 65.891 kDa. The sequences showed a high degree of hydrophobicity at the C-terminal region corresponding to the transmembrane domain between Val^{664} and Leu^{680}, which is followed by a short cytoplasmic tail. Quail ZP2 was compared with ZP2 protein from domestic fowl (Fig. 1B). The quail ZP2 is closely related to its chicken counterpart and the amino acid sequence show a high degree of homology (84.6 %). There are 6 putative *N*-glycosylation sites in the translated

protein that are completely conserved with chicken homologue. 10 Cys residues were seen in the ZP domain of the quail ZP2 and all of the Cys are completely conserved in the ZP domains of chicken and quail ZP2, suggesting that their 3-dimensional structures are similar to each other. These common features suggest that our clone is an authentic member of ZP2.

mRNA expression of ZP2 in quail

To investigate the tissue distribution of *ZP2* transcripts, we analyzed the total RNA isolated from various tissues of laying quail using a gene-specific RNase protection assay. As shown in Figure 2A, a single protected probe fragment was detected only in the samples prepared from SWF (lane 10, Fig. 2B). No detectable signals were seen in the RNA samples isolated from the other tissues tested. No such signal above the background was observed when the same sample was hybridized with sense probe, confirming the specificity of the hybridization (data not shown). It should be noticed that the size of the protected fragment was smaller than that of the probe itself (lane 1) because the RNase treatment can remove the unhybridized regions, which are derived from the pGEM-3-z vector during the synthesis of RNA probes. These results demonstrated that the expression of the ZP2 gene is restricted to the ovary of the quail.

The temporal accumulation of the *ZP2* transcripts during follicular development was observed by an RNase protection assay. As shown in Fig. 2B, the band of the protected fragment in the SWF as well as LWF was seen (lanes 1 and 2), whereas the intensity of the band dramatically decreased when the RNA derived from the small yellow follicles (SYF) was detected

(lane 3). Because mature follicles like F1 and F2 are large, we could not isolate the RNA from the whole follicles; however, the protected fragment was not detected when the sample was prepared from the follicular wall of F2 (lane 4) as well as F1 (lane 5). These results are in accord with the *in situ* hybridization data (Fig. 3A), where the intense signal was detected in the SWF (less than 1 mm in diameter). No detectable signals were seen in the case of the mature follicles including F1, F2 or F3. Again, no such intense signal was detected in any other specimens hybridized with the sense probe (Fig. 3B). These results clearly suggest that the expression of the *ZP2* gene in the follicles is progressively decreased during follicular development and that the high expression is seen in the immature follicles, especially in SWF.

To further localize the expression of ZP2 transcripts in the follicles, we observed the hybridized-specimens under dark-field microscopy (Fig. 3C). As shown in the figure, the intense signals were observed in most of the oocyte of the SWF (asterisks in Fig. 3C), suggesting that the ZP2 mRNA appears to be transcribed in the oocyte itself. The specimens that had been hybridized with the sense probe did not contain these radio-labeled signals (insert in Fig. 3C). These results suggest that the expression of the ZP2 gene is restricted in the oocyte of the SWF and that the expression is decreased during follicular development.

Presence of ZP2 protein in the oocytes

To confirm the presence of the ZP2 protein in the oocytes, we produced an antiserum against bacterially expressed quail ZP2. As shown in the figure 4, the purified recombinant ZP2 showed a single band migrating

approximately 63 kDa in molecular weight after the gels were stained with CBB (Fig. 4A, lane 2). We immunized this protein to a rabbit, and the antiserum against quail ZP2 was obtained. To visualize the ZP2 protein in the SWF extract, we performed Western blot analysis. As shown in Figure 4B, our anti-ZP2 antibody reacted well with the band with 80 kDa in the SWF extract under non-reducing conditions (Fig. 4B, lane 1). No band was detected when the sample was reacted with preimmunoserum (Fig. 4B, lane 2), which suggests that our anti-ZP2 antibody is specific to 80-kDa ZP2 proteins. These results suggest that the immunoreactive materials that reacted with anti-ZP2 antibody exist in the SWF extract.

To investigate the localization of ZP2 protein in the follicles, we prepared paraffin sections of LWF and analyzed them by immunohistochemistry. As shown in Figure 5A, the immunoreactive material recognized by anti-quail ZP2 antibody accumulated in the region beneath the apical surface of the granulosa cells, and was observed as a narrow line (arrow). Positive immunoreactions were also seen in the cytoplasm of the oocyte (arrowheads). The immunoreactive materials are not yolk-derived materials, because the yolk granules seen inside the oocyte were not stained with anti-quail ZP2 antibody. No positive immunostaining was seen when the sections were incubated with preimmunoserum (insert, Figs. 5). These results indicate that ZP2 protein is localized in the oocyte and that it could be selectively transported toward the surface of the cells.

In order to further investigate the localization of ZP2 protein in the oocyte of the SWF, we performed ultrastructural observations on the SWF of quail ovary (Fig.6). In accordance with the results of the

immunohistochemistry, immunoelectron microscopy revealed the presence of immunoreactive ZP2 in the oocyte. In SWF of 1.3 mm in size, the gold particles are mainly localized in the region called "zona radiata", which located just below the follicular granulosa cells (panel A, arrows). Although limited in number, the gold particles were found in the perivitelline space between the granulosa cells and oocyte (panel A, PS). In addition, the particles were also observed in the cortical region of the cytoplasm in the oocyte (arrowheads). However, no such accumulations of the gold particles are seen in the granulosa cells. In the case of the SWF that were 0.7 mm in diameter, the border between the follicle cells and oocytes was obscure, but the immuno-gold particles appeared to be present dominantly in the oocyte (Fig. 6B). This statement is also supported by the quantitative analysis in that the number of the immuno-gold particles in the oocyte ($7.26 \pm 3.23/\mu m^2$, n=10) is significantly greater than that in the granulosa cells ($4.37 \pm 1.13/\mu m^2$, n=10) ($p<0.05$). These results are consistent with the notion that the ZP2 proteins are mainly present in the oocyte but not in the granulosa cells of the quail ovary, and that the results obtained here are in accord with that of the light microscopy.

Expression of recombinant ZP2 in the cultured CHO-K1 cells

Consistent with our previous report (Sasanami *et al.*, 2006), quail ZP1 expressed in the cultured CHO-K1 cells was secreted from the cells during the culture, and the immunoreactive 97-kDa band was seen in the conditioned medium of the transfected cells (panel A, lane 3). Although the nature is unknown, we also detected the immunoreactive 45-kDa band in addition to the 97-kDa ZP1 in the cell lysate (panel A, lane 4). In

contrast, in the ZP2-expression experiments, we found that the immunoreactive materials that migrated approximately 80 kDa and over 200 kDa in molecular weight were present in the cell lysate (panel B, lane 4), and no detectable band reacting with the anti-quail ZP2 antiserum was seen in the conditioned medium (panel B, lane 3). These results indicate that the ZP2 that expressed in the cultured CHO-K1 cells is not secreted from the cells during the culture.

We next investigated whether the recombinant ZP2 expressed in the cultured CHO-K1 cells possessed the ability to interact with ZP3 protein. To achieve this goal, we prepared the conditioned medium of the granulosa layers which containing ZP3 protein. As shown in Fig. 8A, when the conditioned medium of the granulosa cells was detected with anti-ZP3 antiserum, the 35-kDa ZP3 band was detected (lane 1). On the other hand, our anti-ZP2 antiserum (lane 2) as well as normal rabbit serum (lane 3) did not cross-react with ZP3 protein. To test the possible interaction of the cellular ZP2 and the ZP3, we cultured the ZP2-expressed cells or the mock-transfected cells in the presence or absence of the conditioned medium of the granulosa cells. The cell lysates after the culture were detected with Western blot analysis using anti-ZP3 antiserum (Fig. 8B). As shown in the figure, when the CHO-K1 cells that had been transfected with the ZP2 gene were cultured in the presence of the conditioned medium, the immunoreactive ZP3 band was detected (lane 4). However, we did not detect the 35-kDa band when the same cells were cultured in the absence of the conditioned medium (lane 3). In addition to the ZP3 band, the sample prepared from ZP2-transfected cells contained additional band migrating around 31 kDa (lanes 3 and 4), however, it appeared to be a

non-specific protein because it was also detected in the mock-transfected cells cultured in the presence or absence of the conditioned medium (lanes 1 and 2). To further localize the ZP3 protein in the cells, we observed these cells by means of immunofluorescence microscopy. As shown in the Figure 9, when the ZP2 transfected cells cultured with the conditioned medium were detected, the immunoreactive materials with anti-ZP3 antiserum were seen as agglomerate on the surface of the cells (panel B). In the case of the cells that were cultured without the conditioned medium of the granulosa cells, we failed to detect such structure that reacted with anti-ZP3 antiserum (insert). Taken collectively with the data in Figure 8, these results are consistent with the notion that the ZP3 protein in the conditioned medium of the granulosa cells could interact with cellular ZP2 that expressed in the CHO-K1 cells, and that these complex might lead to the formation of an amorphous structure on the cells.

Discussion

In a previous study, we found that quail granulosa cells produce ZP3, which is one of the major components of the PL (Pan *et al.*, 2001), whereas another constituent, ZP1, is synthesized in the liver (Sasanami *et al.*, 2003b). A recent study revealed that another minor constituent, ZPD, was produced and secreted from the granulosa cells in Japanese quail (Sato *et al.*, 2009). In the present study, we clearly demonstrated that both the mRNA for ZP2 and the ZP2 protein are present in the oocytes of immature follicles in the quail ovary. To our knowledge, this is the first demonstration that avian ZP glycoprotein is expressed in the oocyte

itself. The sources of ZP glycoproteins are different in different species (Menkhorst & Selwood, 2008). In most mammalian species including mice, there is clear evidence showing that ZP glycoproteins are synthesized by the growing oocytes (Bleil & Wassarman, 1980). In *Xenopus* laevis, they are also expressed in the oocytes (Yamaguchi *et al.*, 1989). In addition to the oocytes-restricted expression, the follicular granulosa cells also contribute to the synthesis of ZP3 in rabbits (Lee & Dunbar, 1993) and in cynomolgus monkeys (Martinez *et al.*, 1996). On the other hand, glycoproteins homologous to ZP glycoproteins in chorion, the fish egg envelope, are produced in the liver and transported to the ovary by the blood circulation, like vitellogenin (Hamazaki *et al.*, 1985; Hamazaki *et al*., 1989), whereas the egg envelope glycoproteins of carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) appear to be derived from an ovarian source (Conner & Hughes, 2003). Our experimental evidence strongly suggests that female germ cells (*i.e.*, the oocyte) also participate in producing the ZP glycoprotein in addition to the ovarian granulosa cells (ZP3 and ZPD) and the liver (ZP1) in avian species. This is a unique case in which three types of cells (hepatocytes, ovarian granulosa cells and oocytes) participate in the production of ZP glycoproteins in vertebrate animals (Menkhorst & Selwood, 2008). It might be due to the size of the avian oocytes that are far larger than those of fish, amphibian and mammalian species, and that the glycoproteins synthesized and secreted from oocyte and granulosa cells are insufficient to cover the surface area of the big oocytes in birds. We suppose that an active organ in protein synthesis, such as liver, should participate in the synthesis of the ZP

glycoproteins in avian species.

The immunological studies with anti-quail ZP2 antiserum showed that immunoreactive material accumulates in the zona radiata located between the granulosa cells and oocyte (Fig. 6), and that the apparent molecular mass is approximately 80 kDa (Fig. 4B). The immunoreactive materials are also present in the cortical region of the cytoplasm of the oocyte (Figs. 5 and 6), supporting our statement that the ZP2 proteins are produced by the oocyte. It is interesting to note that the ZP2 expressed in the cultured CHO-K1 cells is not secreted, but instead remains in the cell lysate (Fig. 7), and that this phenomenon could be related to the localization of the ZP2 protein in zona radiata, which is constructed from the microvilli of oocyte and the surrounding follicle cells (Ito *at al.*, 2003). We think, therefore, that after translation, the ZP2 proteins are selectively transported toward the apical surface of the oocyte, which are apposed to the perivitelline space where the fibers of the PL start to accumulate when the follicles begin to grow (Ito *at al.*, 2003).

In the present study, we found that the transcripts of ZP2 protein in the follicles decreased during follicular development, and the mRNA for ZP2 was not detected at the latter stages of folliculogenesis (Fig. 2C). This pattern is very different from that for the other ZP glycoproteins in quail, in that immunoreactive ZP3 appears as early as in the PL obtained from the SYF, and the ZP1 and ZPD accumulate thereafter (Pan *et al.*, 2001; Sato *et al.*, 2009; Sasanami *et al.*, 2004). In mice, Epifano *et al.* (1995) investigated the accumulation of ZP1, ZP2 and ZP3 transcripts in oocyte lysates during oogenesis using an RNase protection

assay. They showed that the transcript of ZP2 was detected as early as in the lysates of the resting oocyte at the prophase stage, whereas the accumulation of the ZP1 and ZP3 transcripts became detectable after the oocytes began to grow. Although the origins of the ZP glycoproteins are different from each other, the expression of quail ZP glycoproteins are also likely regulated in a coordinate manner, as in the case of the mouse ZP glycoproteins.

In the mouse ZP, ZP2 and ZP3 are present in roughly equimolar amounts and polymerise to form a long chain of heterodimer (Wassarman & Litscher, 2008), whereas ZP2 protein in the PL of the largest follicles in quail ovary was at an undetectable level (data not shown). As was mentioned earlier, the PL in quail as well as in chicken is constructed mainly by ZP1 and ZP3, and these components specifically bind to form the PL fiber (Ohtsuki *et al.*, 2004; Sasanami *et al.*, 2006; Kinoshita *et al.*, 2008; Okumura *et al.*, 2007a; Okumura *et al.*, 2007b). In fact, in vitro incubation of ZP1 with ZP3 spontaneously produced fibrous aggregates, which were visible under optical microscopy (Okumura *et al.*, 2007b). In addition, Okumura *et al.* (2007a) reported that the ZP1 and ZP3 were observed to colocalize on the surface of ZP3-expressing transfectants cultured in the presence of the hen serum and that they suggested that ZP1 transported via blood circulation into the ovary encounters and associate with ZP3 secreted from granulosa cells, resulting in the formation of heterocomplexes around the oocyte. In our previous study, however, when the ZP3 expression construct had been introduced into the CHO-K1 cells, we observed that the most of the ZP3 were secreted away into the medium, and the immunoreactive substances were not localized on

the cell surface (Sasanami *et al.*, 2003a; Sasanami *et al.*, 2003c). In the present study, we observed a detectable interaction of ZP2 and ZP3 by means of Western blot analysis (Fig. 8) and immunofluorescence microscopy (Fig. 9). Although the direct evidence is not available, based on the fact that we could observe the interaction of ZP2 and ZP3, we propose that the ZP2 protein localized on the surface of the oocyte might be one of a target molecule for ZP3 binding, and that this interaction might in fact trigger the ZP glycoprotein accumulation (*e.g.*, ZP3 and ZP1 heterocomplex formation) for the construction of the PL in the follicles. This hypothesis is also supported by the observation of the coordinate expression patterns of ZP glycoproteins described in the present study (ZP2) and our previous studies (ZP1, ZP3 and ZPD). From this view, we believe that the interaction of ZP1 and ZP3 might be important for the formation of the PL in the latter stage of the follicular development, especially in the yolk-filled yellow follicles and that our present finding that consistent with the interaction of ZP2 and ZP3 might shed new light on the mechanisms of the initiation step for the PL formation in the immature follicles of the quail ovary. Further studies are needed to uncover the mechanism of the formation of PL fibers during follicular maturation.

Acknowledgments

The authors are grateful to Miss Tsukasa Sato and Miss Ayako Katayama for technical assistance. This work was supported in part by financial assistance from Ito Scientific Foundation (Tokyo, Japan) and Hamamatsu Scientific Foundation (Hamamatsu, Japan) to T. Sasanami.

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Figure captions

Fig. 1 (A) Nucleotide and deduced amino-acid sequences of quail ZP2 cDNA. The deduced amino acid is represented as a single-letter abbreviation shown below the nucleotide sequence (GenBank Accession Number: AB295393). 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. Primer positions used for amplifying the probe for RNase protection assay (hatched underlines) and the antigen production for anti-ZP2 antiserum (double underlines) and probe for in situ hybridization (bold underline) are shown. (B) Alignment of amino acid sequence of quail and chicken ZP2. Conserved residues are shown in asterisk and the consensus site for *N*-glycosylation in bold letters; the consensus furin cleavage site is underlined. Conserved Cys residues are indicated with a shaded box.

Fig. 2 Tissue-specific expression of ZP2 mRNA. (A) RNase protection assays were performed to detect the mRNA expression of the *ZP2* gene. We hybridized 15 μg of total RNA isolated from brain (lane 2), heart (lane 3), lung (lane 4), liver (lane 5), spleen (lane 6), kidney (lane 7), oviduct (lane 8), adrenal gland (lane 9), and SWF (lane 10) with riboprobes specific to *ZP2*. As a reference, the antisense probe used in this experiment was run on the left lane (lane 1). Protected fragments were detected by autoradiography. The results shown are representative of repeated experiments. (B) Expression pattern of ZP2 mRNA during

follicular development. The 15 μg of total RNA prepared from SWF (lane 1), large white follicles (lane 2), small yellow follicles (lane 3), the follicular wall of the second (lane 4), or the largest follicles (lane 5) were hybridized with riboprobes specific to *ZP2*. Representative autoradiograms of triplicate experiments are shown.

Fig. 3 *In situ* hybridization of quail ovary. Autoradiograms of ovarian sections after hybridization with ^{33}P-labeled antisense probe specific to ZP2 are shown (A). Arrowheads, *, ** or *** indicate SWF, the third, the second and the largest follicles, respectively. Control hybridization with ^{33}P-labeled sense probe is depicted in (B). Scale bar, 5 mm. The results are representative of repeated experiments. The slides hybridized with antisense (C) or sense probe (insert in C) were coated with Kodak NTB-2 emulsion, and the specimens were examined under a dark-field microscopy. Asterisks in (C) indicate the follicles having the silver grains visualized in the oocytes of SWF. Scale bar, 100 μm. The results are representative of repeated experiments.

Fig. 4 SDS-PAGE analysis of the purified recombinant ZP2 protein and Western blot analysis of ZP2 protein in the extract of the SWF. (A) The purified recombinant ZP2 protein for immunization (3 μg) was separated on SDS-PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue R 250 (lane 2). A molecular weight marker was run in the next lane (lane 1). (B) The extracts prepared from the SWF (20 μg /lane) were separated on SDS-PAGE under non-reducing conditions, transblotted onto PVDF membrane, and detected with anti-quail ZP2 antiserum (lane 1, 1:1 000) or preimmunoserum (lane 2, 1:1 000). The results representative of repeated experiments are shown.

Fig. 5 Immunohistochemical analysis of ZP2 in follicular wall. Sections of follicular wall obtained from LWF were processed for immunohistochemical observation using anti-quail ZP2 antiserum (1:250) or preimmunoserum (insert, 1:250). Arrow and arrowheads indicates the positive staining in the region beneath the apical surface of the granulosa cells and in the cytoplasm of the oocyte, respectively. The results representative of repeated experiments are shown. Bar = 50 μm.

Fig.6 Immunoelectron micrographs of SWF. Ultra thin sections of follicular wall of 1.3 mm (A) or 0.7 mm (B) follicles were stained with anti-quail ZP2 antiserum. (A) Accumulations of the gold particles are frequently found in zona radiata (arrows), perivitelline space as well as cortical region of the oocyte below the zona radiata (arrowheads). In contrast, no such accumulation are not seen in the granulosa cells (B) In 0.7 mm follicles, immuno-gold particles are evenly localized in the cytoplasm of the oocyte (arrows). Shown are the results representative of repeated experiments. Bar = 1 μm. PS: perivitelline space, ZR: zona radiata, N: nucleus.

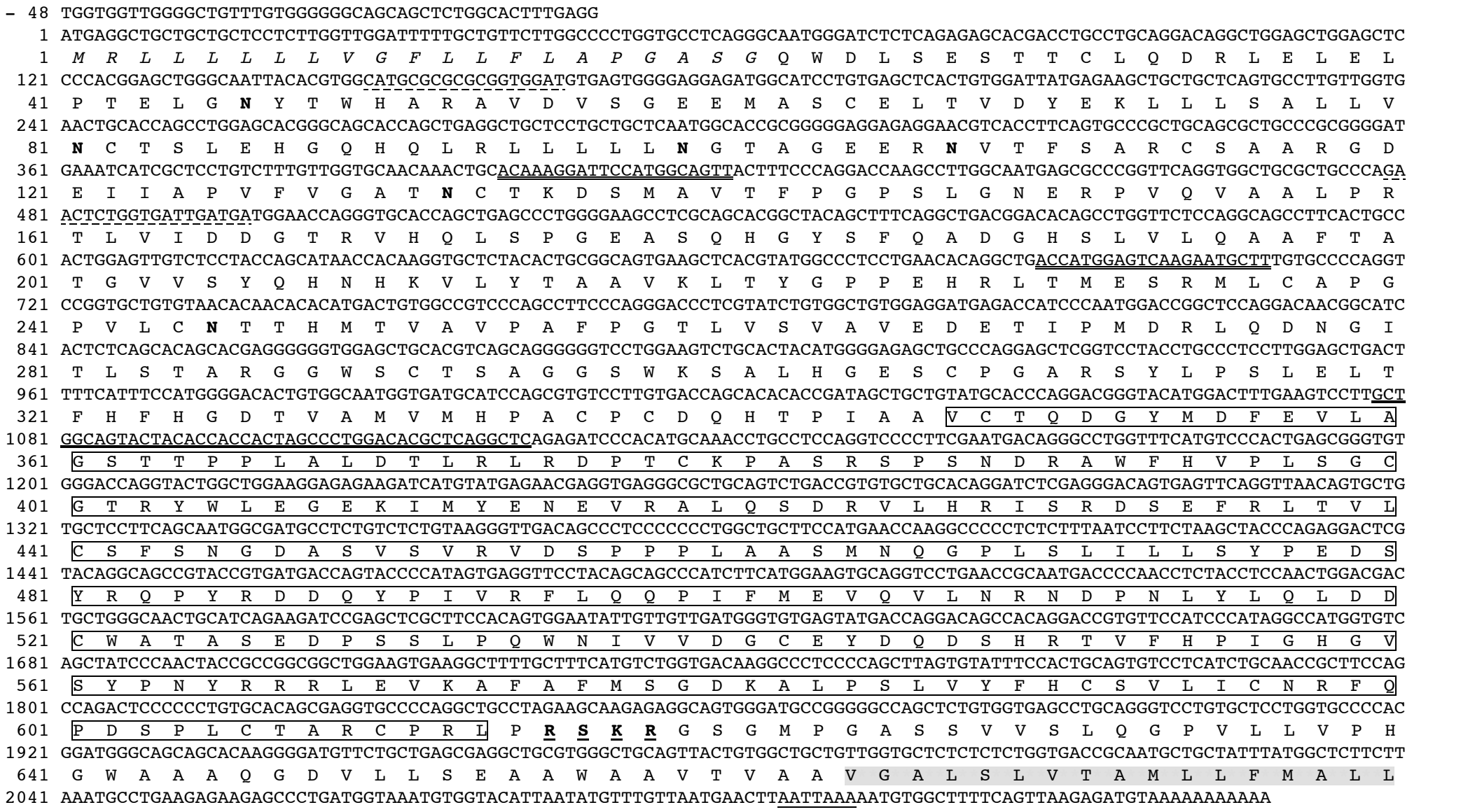
Fig. 7 Western blot analysis of recombinant ZP1 and ZP2 expressed in the cultured CHO-K1 cells. (A) CHO-K1 cells transfected with vector alone (lanes 1 and 2) or quail ZP1 expression construct (lanes 3 and 4) were cultured for 48 h and the medium and cell lysates were recovered. They were then concentrated and the medium (lanes 1 and 3; 16 μl per lane) and cell lysates (lanes 2 and 4; 10 μg protein per lane) were probed with anti-quail ZP1 antiserum (1:1 000 dilution). (B) Quail ZP2 expression construct (lanes 3 and 4) or vector alone (lanes 1 and 2) were introduced into CHO-K1 cells and the medium (lanes 1 and 3; 16 μl per lane) and the

cell lysates (lanes 2 and 4; 10 μg protein per lane) after 48 h of culture were detected with anti-quail ZP2 antiserum as described above. The immunoblots shown are representative of 3 experiments.

Fig. 8 Western blot analysis of ZP3 protein interact with recombinant ZP2 (A) Granulosa layers were cultured for 8 h, and the ZP3 protein in the medium (14.4 μl of the culture medium per lane) were detected by using anti-quail ZP3 antiserum (lane 1, 1:10 000), anti-quail ZP2 antiserum (lane 2, 1:500) or normal rabbit serum (lane 3, 1:500). Immunoblots shown are representative of at least three experiments. (B) CHO-K1 cells transfected with vector alone (lanes 1 and 2) or quail ZP2 expression construct (lanes 3 and 4) were cultured for 48 h in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of the conditioned medium of the granulosa cells, and the cell lysates were recovered. They were then detected with Western blotting as described in Materials and methods (10 μg protein per lane). The representative results of 3 experiments are shown.

Fig. 9 Immunocytochemical localization of ZP3 protein incorporated into the CHO-K1 cells. The CHO-K1 cells transfected with ZP2 expression construct were cultured for 48 h in the presence or absence (inserts) of the conditioned medium of the granulosa cells, and the cell were stained with anti-quail ZP3 antiserum by means of immunofluorescence microscopy as described in Materials and methods. The representative results of 3 experiments are shown. Bar = 50 μm.

Kinoshita *et al.*, Fig. 1A



Kinoshita *et al.*, Fig. 1B

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Quail           MR--LLLLLLVGFLLFLAPGASGQWDLSESTTCLQDRLELELPTELGNYTWHARAVDVSG
Chicken         **GR******F***********E******M**********R*****V*******

Quail           EEMASCELTVDYEKLLLSALLVNCTSLEHGQHQLRLLLLLNGTAGEERNVTFSARCSAAR
Chicken         ***M***HA******************Y********************Y**H****H

Quail           GDEIIAPVFVGATNCTKDSMAVTFPGPSLGNERPVQVAALPRTLVIDDGTRVHQLSPGEA
Chicken         *******L***E*****************SD*HL****V*TG**T****IK*****L***

Quail           SQHGYSFQADGHSLVLQAAFTATGVVSYQHNHKVLYTAAVKLTYGPPEHRLTMESRMLCA
Chicken         M******L****H**F***********K****A*****L**M*********V******V

Quail           PGPVLCNTTHMTVAVPAFPGTLVSVAVEDETIPMDRLQDNGITLSTARGGWSCTSAGGSW
Chicken         ****F*********I*******MA**********Q***K****KTTV*VELH-VSRRVL

Quail           KSALHGESCPGARSYLPSLELTFHFHGDTVAMVMHPACPCDQHTPIAAVCTQDGYMDFEV
Chicken         **T*******RVQ***S**K******EE********Q*****L*****A**R********

Quail           LAGSTTPPLALDTLRLRDPTCKPASRSPSNDRAWFHVPLSGCGTRYWLEGEKIMYENEVR
Chicken         *********V******************L*********************************

Quail           ALQSDRVLHRISRDSEFRLTVLCSFSNGDASVSVRVDSPPPLAASMNQGPLSLILLSYPE
Chicken         **R**S*************A*****************N*******T**************

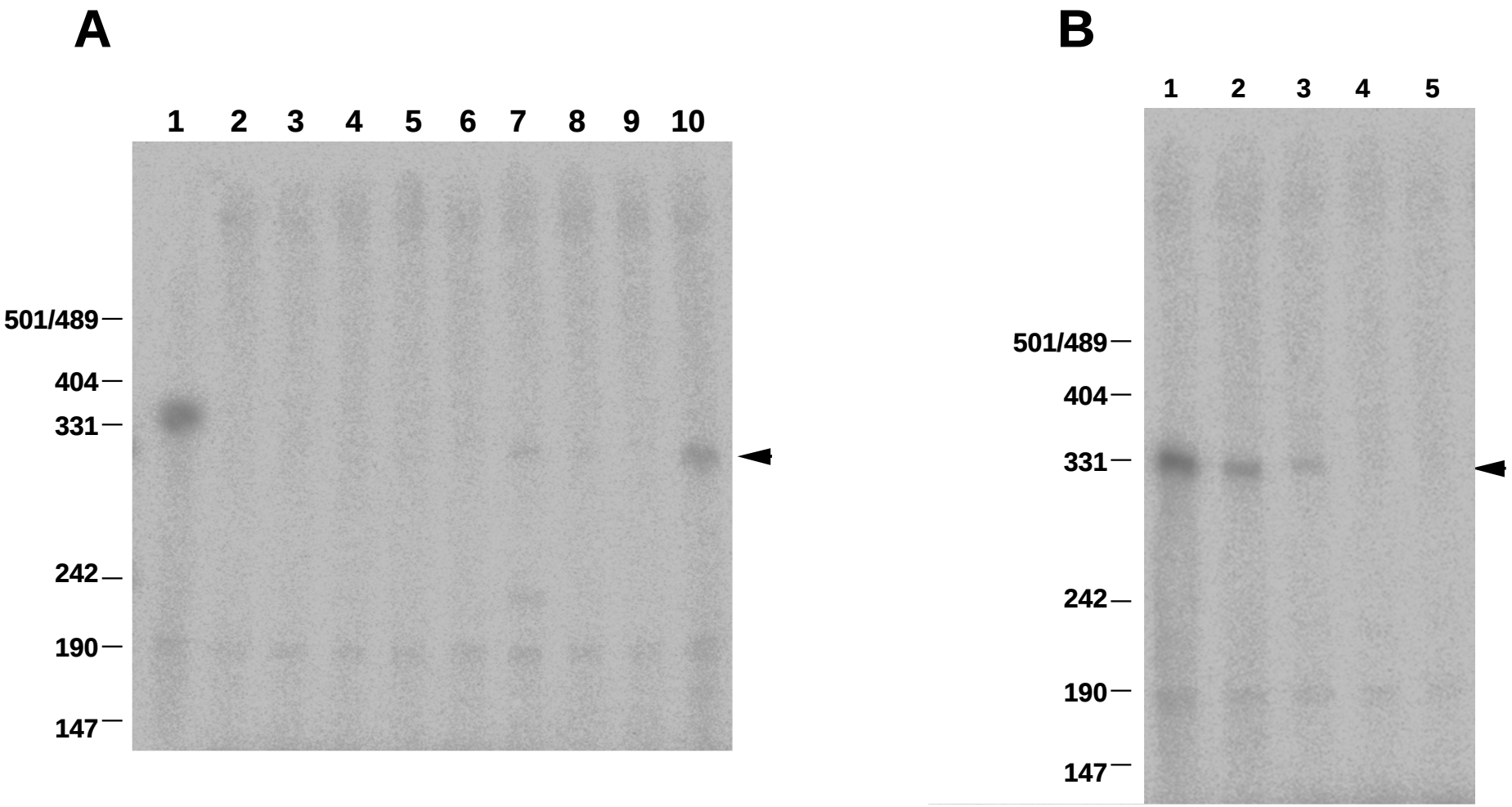
Quail           DSYRQPYRDDQYPIVRFLQQPIFMEVQVLNRNDPNLYLQLDDCWATASEDPSSLPQWNIV
Chicken         *******H********Y*****************************L***T********

Quail           VDGCEYDQDSHRTVFHPIGHGVSYPNYRRRLEVKAFAFMSGDKALPSLVYFHCSVLICNR
Chicken         *******E***Y******V**********Q*********V*******G***********S*

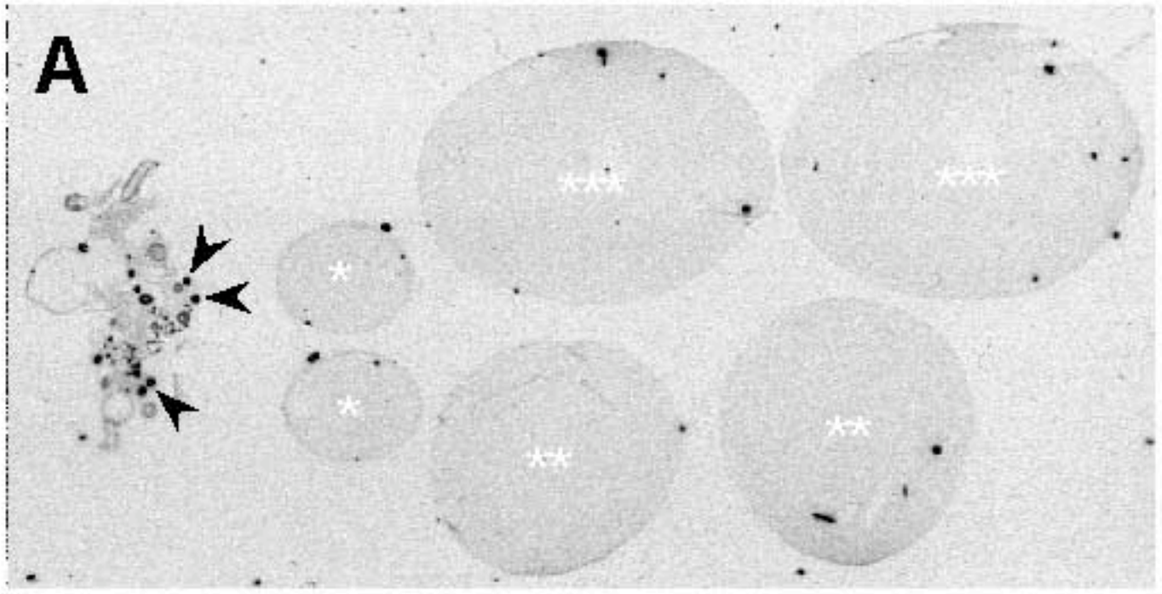
Quail           FQPDSPLCTARCPRLPRSKRGSGMPGASSVVSLQGPVLLVPHGWAAAQGDVLLSEAAWAA
Chicken         **L*************R**********L**********************R*GT***KVV***

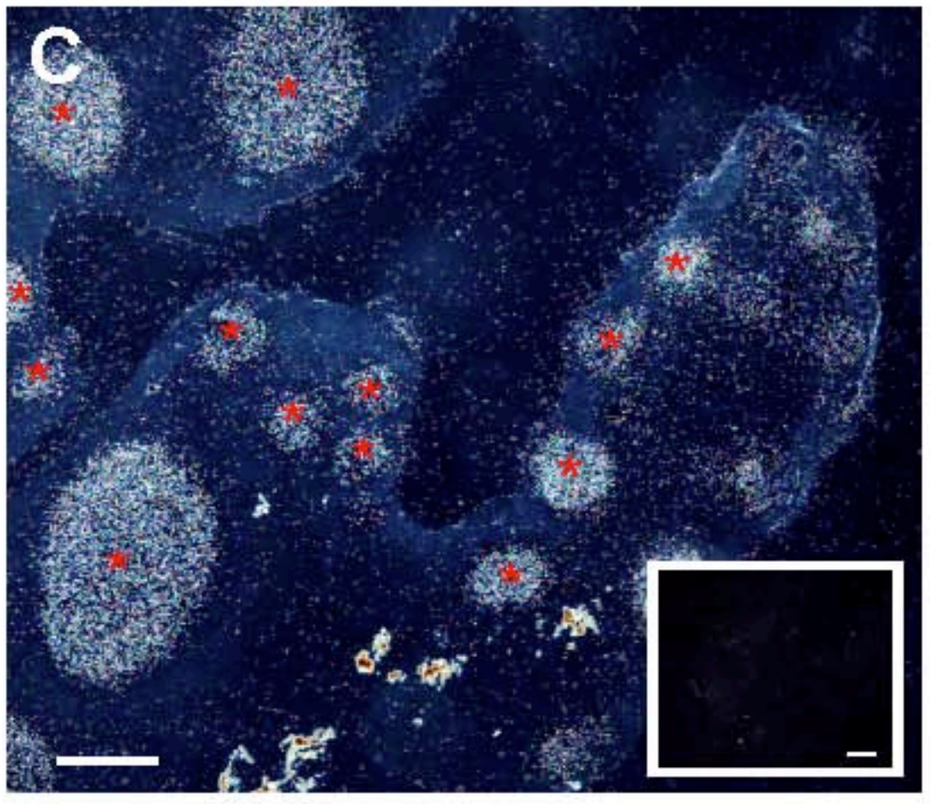
Quail           VTVAAVGALSLVTAMLLFMALLKCLKRRALMVNVVH
Chicken         **AT***VF**TAI*****D***********A***Y
```

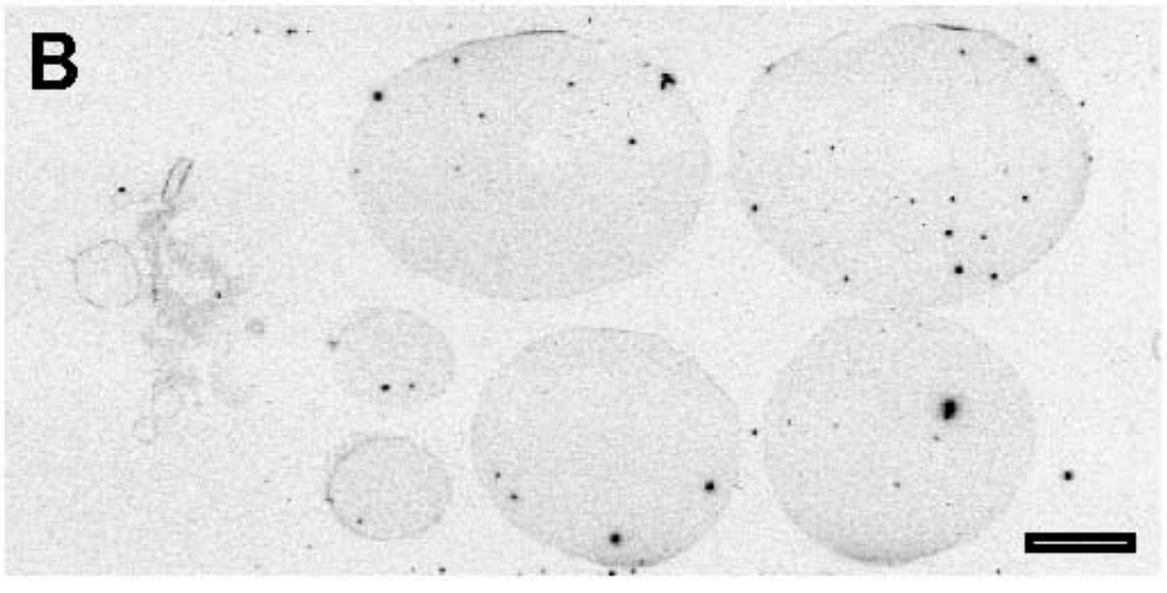
Kinoshita ***et al.,*** Fig. 2



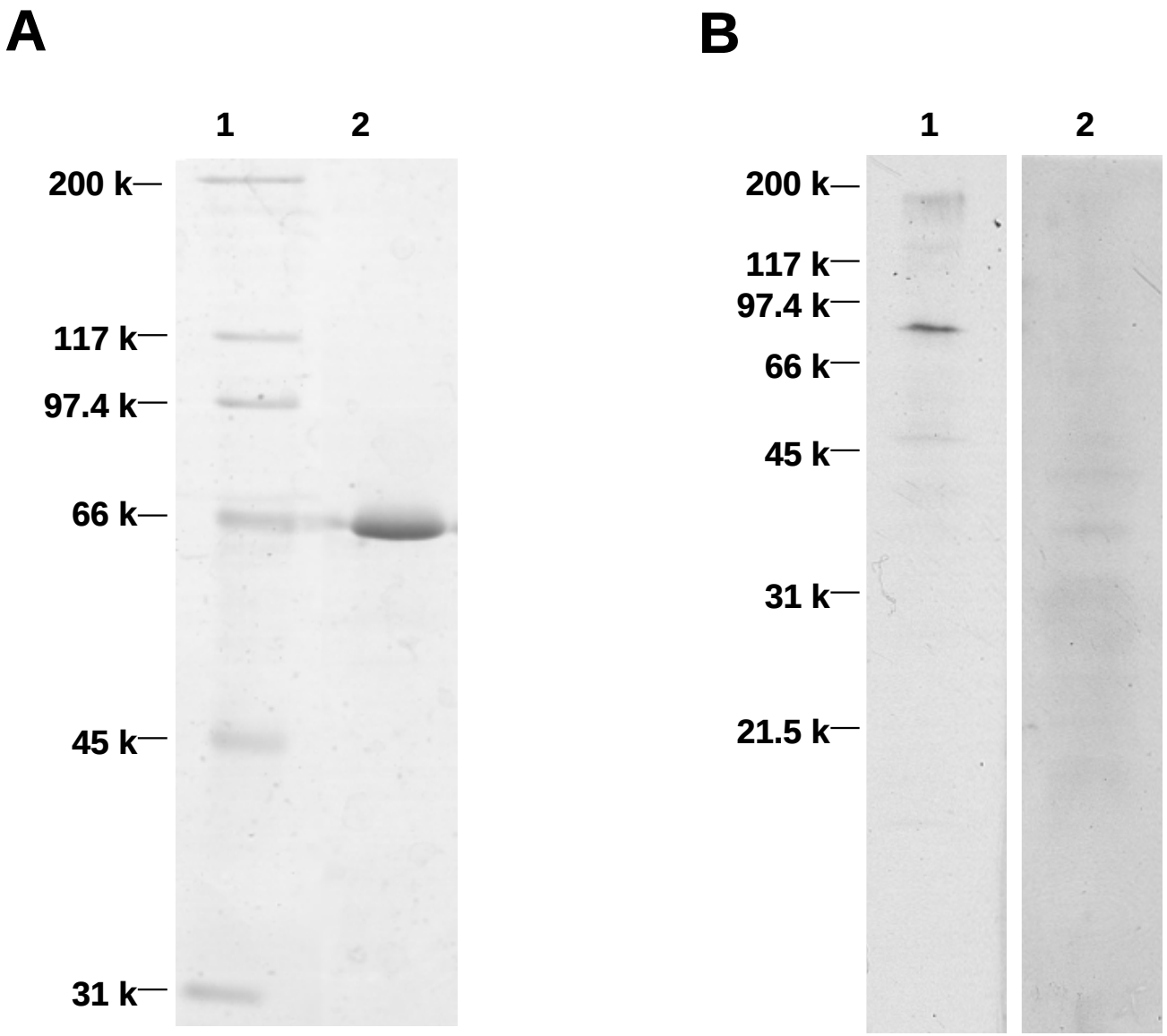
Kinoshita *et al.*, Fig. 3



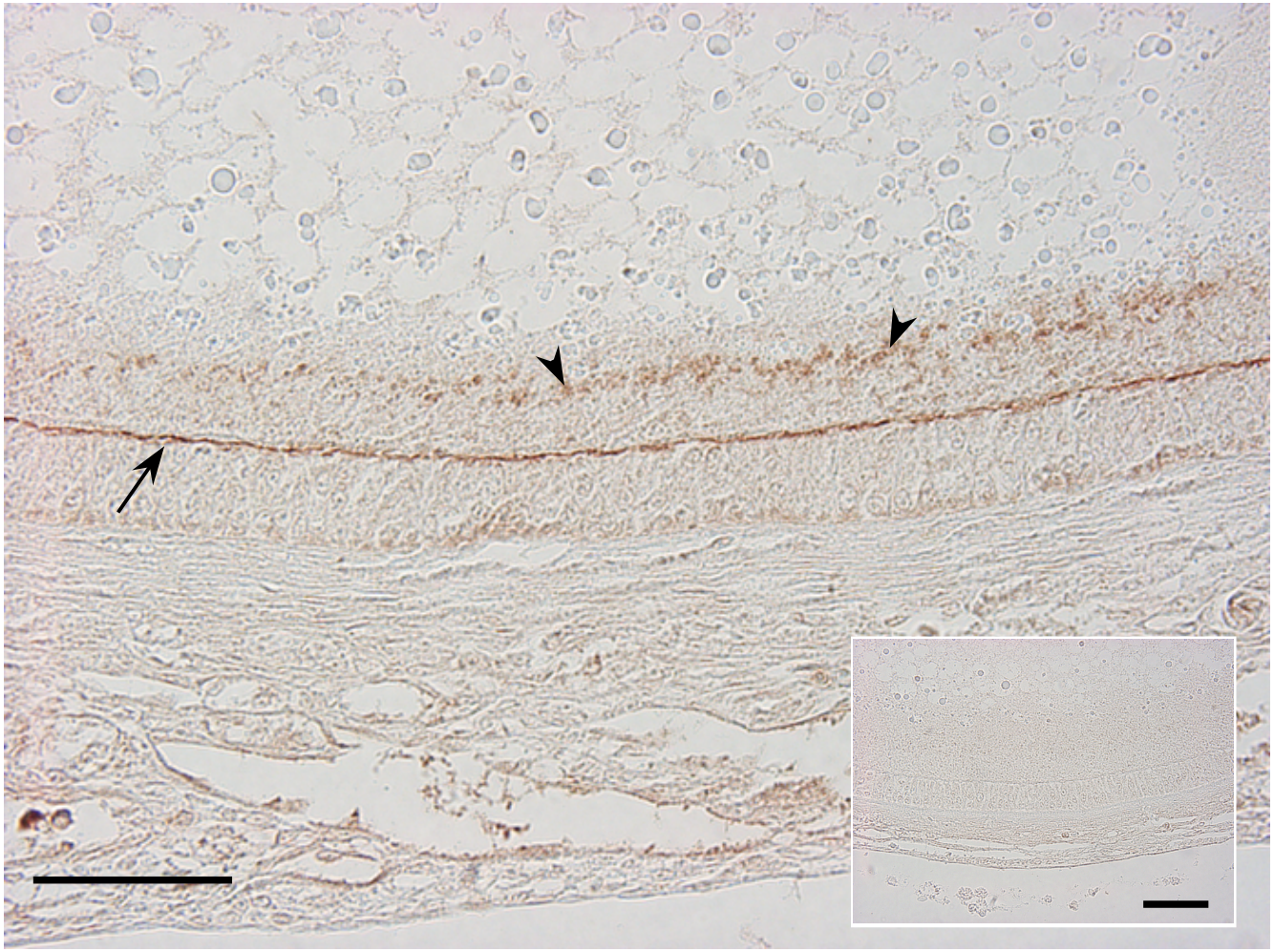




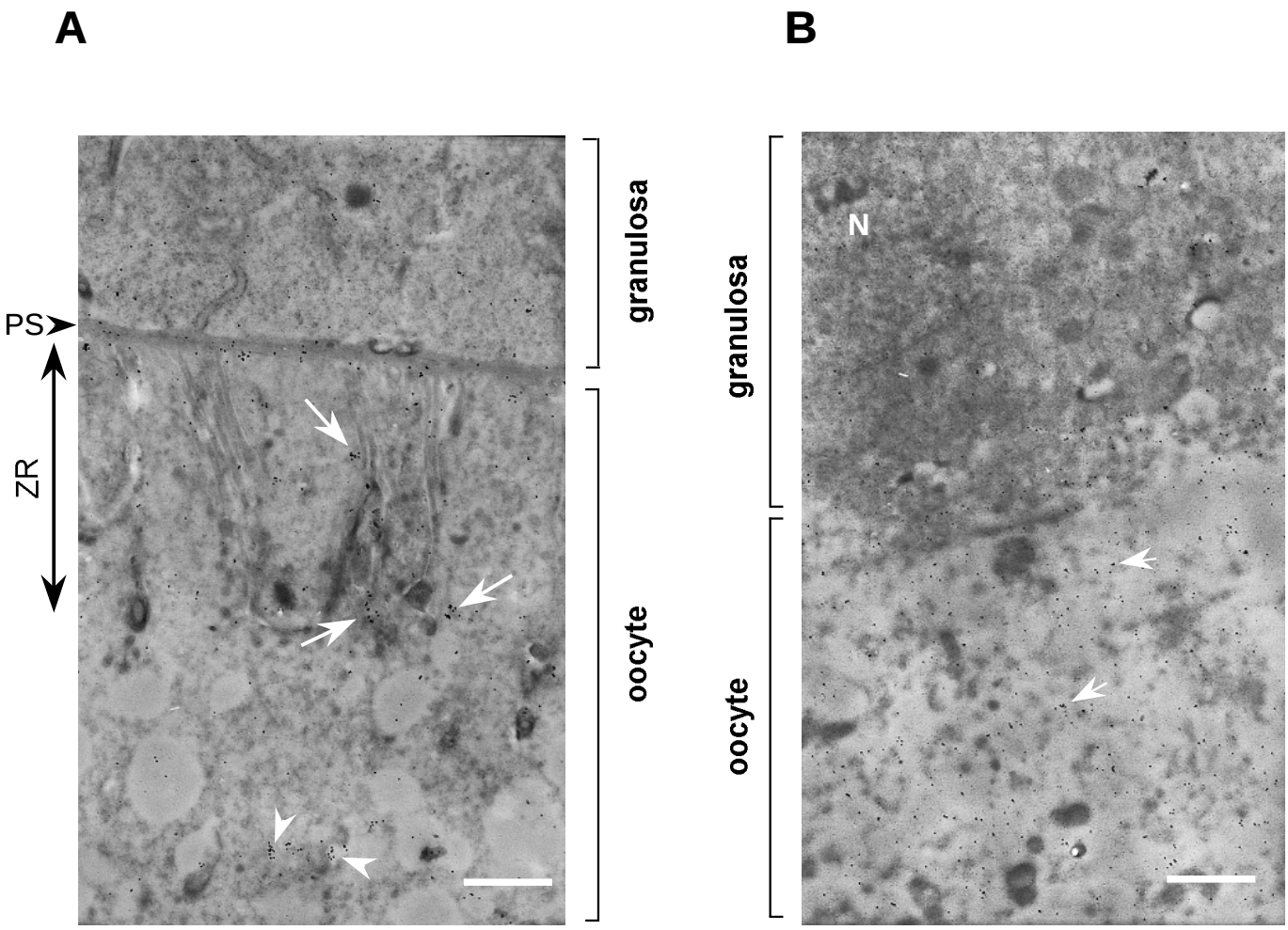
Kinoshita ***et al.,*** Fig. 4

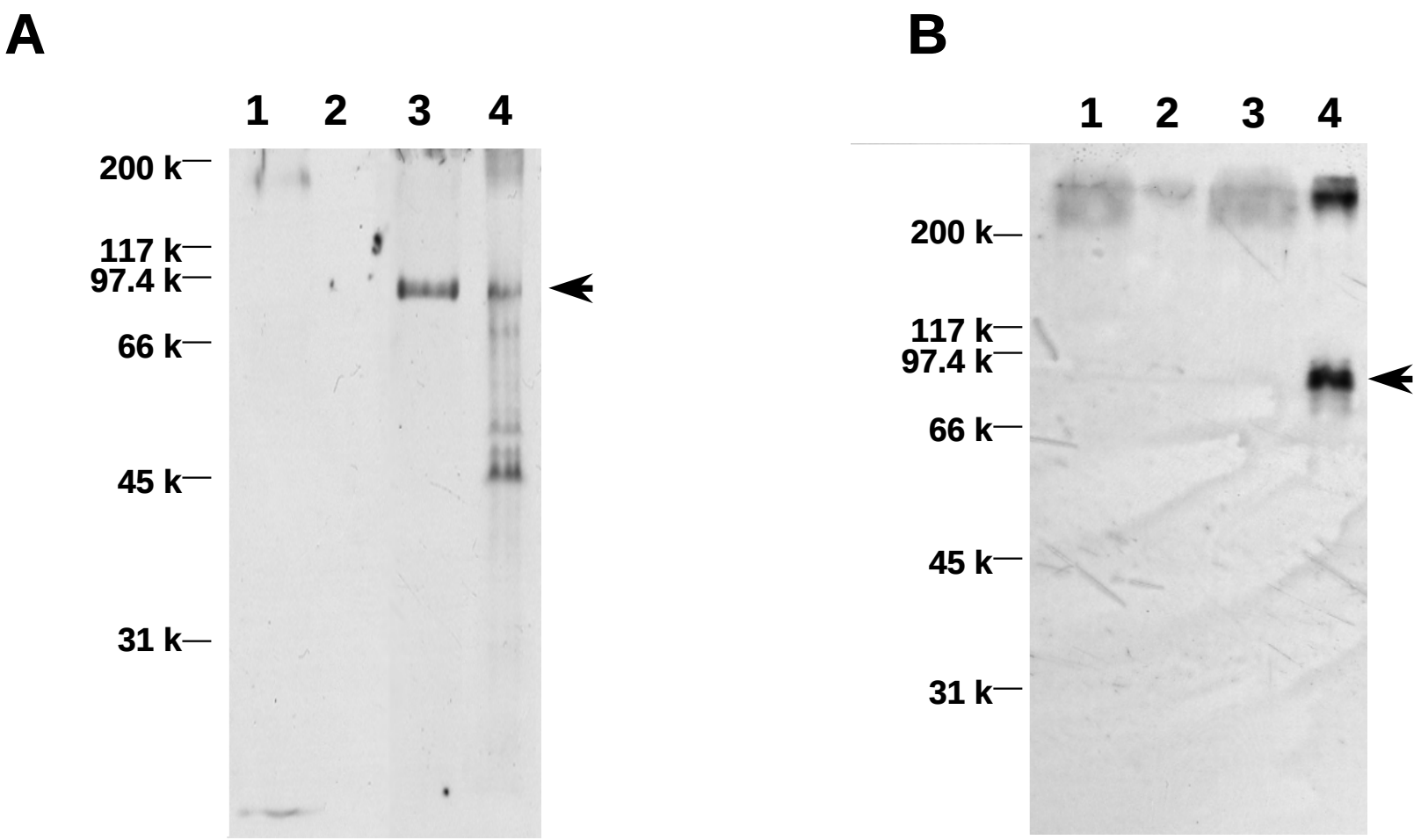


Kinoshita ***et al.,*** Fig. 5



Kinoshita ***et al.,*** Fig. 6

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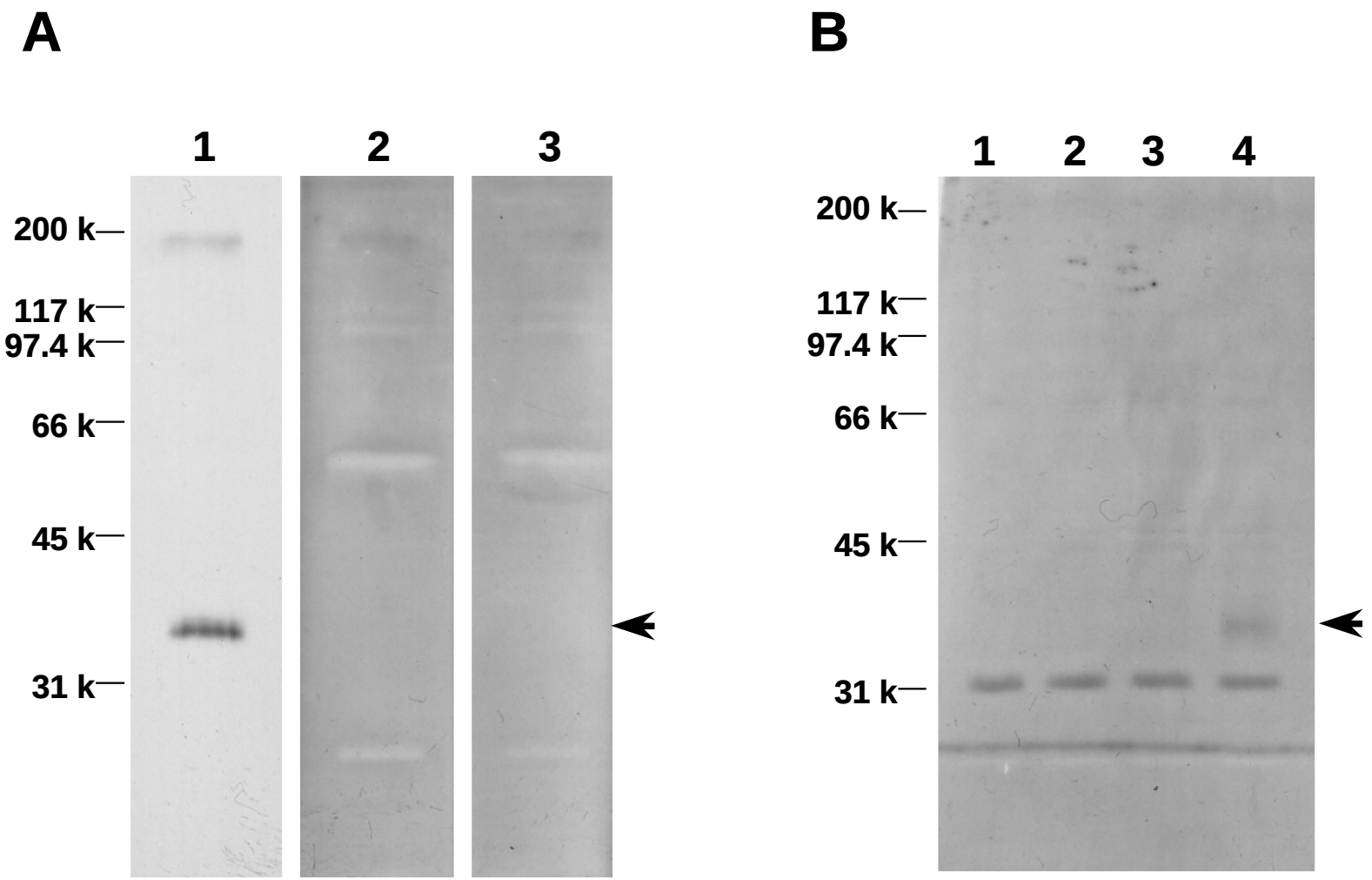
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Kinoshita ***et al.,*** Fig. 9