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The Chicken Homologue of Zona Pellucida Protein-3 Is Synthesized by Granulosa Cells[1]

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ABSTRACT

Oocyte development within avian ovarian follicles is an intricate process involving yolk deposition and the formation of extraoocytic matrices. Of these, the perivitelline membrane (pvm) not only plays a role in sperm binding but also provides mechanical support for the large oocyte's journey through the oviduct after ovulation. To date we have focused on the mechanisms for uptake of yolk precursors into oocytes of the chicken; now we extend our studies to a detailed analysis of the pvm. In the course of characterization of its major components, we obtained partial protein sequences; comparison with the GenBank database revealed that one of the pvm proteins is the homologue of mammalian zona pellucida glycoprotein 3 (ZP3), a key component in sperm binding. Following a nomenclature based on gene structure, the protein is referred to as chicken ZPC (chZPC). The chicken protein (444 residues) and murine ZP3 (424 residues) are highly conserved, with 41% of the amino acids identical. As shown by Northern blot analysis, the avian ZPC gene is expressed exclusively in the granulosa cells surrounding the oocyte, in contrast to murine ZP3, which is synthesized by the oocyte. Upon reaching a size larger than 1.5 mm in diameter, follicles accumulate chZPC in highly polarized fashion, i.e., in the space intercalated between the oocyte and the granulosa cells, as revealed by immunohistochemistry of follicle sections. ChZPC synthesis and secretion by granulosa cells was demonstrated directly by metabolic labeling and immunoprecipitation from the culture medium of granulosa cell sheets isolated ex vivo from follicles. Immunoblot analysis and glycosidase treatment of chZPC from preovulatory and freshly ovulated oocytes, as well as laid eggs, revealed that the primary product undergoes a two-step decrease in size from follicle to laid egg that is unlikely to be due to modification of the carbohydrate moiety.

INTRODUCTION

The perivitelline membrane (pvm) of the chicken egg is a highly organized meshwork of fibrils surrounding the oocyte. It is composed of two layers, an inner layer and an outer layer, separated by a thin so-called continuous membrane [1]. The inner layer serves as an adhesion site for the addition in the oviduct of the continuous membrane and of the outer layer following ovulation [2, 3]. During migration of the oocyte through the oviduct, the assembly of the pvm is completed in the infundibulum by addition of the major components ovomucin, lysozyme, and the ''vitelline membrane outer proteins'' I and II (VMOI and VMOII; [2, 3]). For successful fertilization to occur, sperm must penetrate the pvm [4]; highly species-specific sperm-binding determinants of this specialized extracellular matrix ensure exclusion of foreign sperm [5]. Furthermore, the outer layer appears to contain components that are important for the prevention of polyspermy via processes mediated by acrosin, a trypsin-like sperm enzyme [6].

Although there is considerable ultrastructural and chemical knowledge about the pvm of chickens, delineation of the molecular structure of the components of the pvm and details of their site(s) of synthesis have not been reported. However, there is significantly more information available with regard to analogous proteins and genes in mammals [7–9], frogs [10], and fish [11]. The extracellular matrix of mammalian oocytes, which are small in comparison to those of oviparous species, is termed the zona pellucida (ZP) [12]. The elucidation of molecular details of ZP components and our understanding of the fertilization process in mammals are most advanced in the mouse [13, 14]. (Glyco)proteins involved in the process of sperm binding to the ZP have been identified and characterized [15–17]. Upon making contact with the ZP, the sperm releases acrosomal enzymes into the extraoocytic environment [18]. Acrosome-intact but not acrosome-reacted sperm recognize and interact with the species-specific *O*-linked oligosaccharides present on zona pellucida glycoprotein 3 (ZP3) [19]. Furthermore, small murine ZP3 glycopeptides as well as the isolated *O*-linked oligosaccharides of ZP3 do not induce the acrosome reaction but can serve as inhibitors of sperm binding [20]. These data suggest that the ZP3 molecule plays a trifunctional role in that it 1) serves as a structural component of the ZP, 2) functions as a sperm receptor via binding of the male gamete to its *O*-linked oligosaccharides, and 3) triggers the acrosome reaction of the sperm.

In the course of our work on the receptor-mediated growth of chicken oocytes [21, 22], we have become interested in the contribution of the acellular follicular structures, basement membrane and pvm, to the overall development of the giant oocyte. As the surface area of a fully grown chicken oocyte is at least 35 cm^2, the chicken system appears to provide an ideal setting for biochemical and molecular genetic studies on the formation of oocyte-supporting structures. We have initiated such studies by generating protein sequence information from one of the major proteins in the chicken pvm. We herein report on the structural characterization, sites of expression, biosynthesis, postovulatory modification, and localization of this protein, which was identified by comparison with a cDNA sequence submitted to the database (accession no. D89097, Takeuchi Y. et al.) as chicken ZPC (chZPC).

MATERIALS AND METHODS

Animals

Derco-brown laying hens (Heindl Co., Vienna, Austria), 30–40 wk old and maintained as described previously [23],

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were used as source for eggs as well as for the isolation of follicles and liver. For tissues from males, Derco-brown roosters were used. Antibodies were produced in adult female New Zealand White rabbits.

Preparation and Solubilization of Chicken pvm

Perivitelline membranes were obtained either from laid eggs or from ovarian follicles. The egg white of freshly laid eggs was separated from the oocyte, which was punctured to drain the yolk. The membranes were washed in TBS (137 mM NaCl, 2.5 mM KCl, 2.5 mM Tris-HCl, pH 7.6) to remove adhering yolk material and incubated for 1 h at 4°C in 200 mM Tris maleate (pH 6.5), 2 mM $CaCl_2$, 0.5 mM PMSF, 2.5 mM leupeptin, and 1.4% Triton X-100, followed by ultracentrifugation at 300 000 × *g* for 40 min at 4°C. Then the detergent-pretreated membranes were solubilized in TBS, 2% SDS, and 50 mM dithiothreitol (DTT; equivalent of 5 eggs/ml) for 16 h at 45°C with constant shaking. Insoluble constituents were sedimented by centrifugation at 12 000 rpm for 20 min in an Eppendorf (Hamburg, Germany) centrifuge and discarded.

For pvm isolation from follicles, ovaries were dissected from mature chickens immediately after decapitation. Granulosa cell sheets (inner pvm with adhering granulosa cells and basement membrane) were prepared as described previously [24]. Then they were either processed for biosynthetic studies (see below) or subjected to the same solubilization procedure as described above for egg pvm.

Protein Sequencing and Immunological Procedures

Chicken pvm proteins were separated by preparative SDS-PAGE (4.5–18%) under reducing conditions. The most prominent bands were cut out and electrophoretically eluted at 180 V for 8 h in electrophoresis buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). Microsequencing of the proteins was carried out essentially as described previously [25]. The amino acid sequence of chZPC was analyzed with the Gene Works for Macintosh (2.5.1.) program (Apple Computers, Cupertino, CA).

Rabbit anti-chZPC antibodies were raised by three series of intradermal injections each of 500 μg chZPC in 400 μl elution buffer, mixed with an equal volume of Freund's complete (Day 0) or incomplete (Days 14 and 35) adjuvant. Preimmune serum and immune sera were stored at −80°C.

Electrophoretic and Western Blot Analysis of the Chicken pvm

Chicken pvm proteins were separated by SDS-PAGE (4.5–18%); molecular weight standards were from Bio-Rad (Hercules, CA; Kaleidoscope Prestained Standard). After electrophoresis, proteins were either visualized with Coomassie brilliant blue or transferred to nitrocellulose (Hybond-C extra; Amersham, Arlington Heights, IL) for immunoblotting. Transfer was performed in 26 mM Tris, 192 mM glycine, 20% methanol overnight at 40 mA at 4°C. For immunoblotting, the membrane was blocked in a solution of 0.1% Tween 20, PBS, and 10% nonfat dry milk for 1 h, followed by incubation with anti-chZPC antiserum (1:2000) in blocking solution for 1 h. After three washes for 10 min each with washing buffer (0.1% Tween 20, PBS), the blot was incubated with protein A-horseradish peroxidase (1:5000) as second antibody for 1 h and washed three times with washing buffer. Bands were visualized by the chemiluminescence procedure as suggested by the manufacturer (Pierce, Rockford, IL).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis and Northern Blotting

Total RNA was prepared from granulosa cell-enriched material obtained by mechanical removal from the cavity of postovulatory sacs, granulosa cell sheets of different follicular stages (F1-F5; numbered in sequence to size, starting with F1 as the largest preovulatory follicle), small follicles (2–8 mm in diameter), embryonic ovary (18-day and 15-day incubation), whole embryo (10-day incubation), and livers of hens or estrogen-treated roosters as well as testes from estrogen-treated and control roosters. Tissues were extracted with Tri Reagent, including phenol and guanidine thiocyanate (Molecular Research Center, Inc., Cincinnati, OH). Total RNA (10 μg; 30 μg for embryonic RNAs) was subjected to electrophoresis on a 1.2% agarose gel (glyoxal-method; [26]), followed by blotting onto a nylon filter (Hybond-N; Amersham). RNA was covalently bound to the dried membrane by UV cross-linking. The hybridization probe was obtained by reverse transcription (Moloney murine leukemia virus-reverse transcriptase; Promega, Madison, WI) of 500 ng total RNA obtained from granulosa cell sheets for 45 min at 37°C, followed by PCR with an oligonucleotide pair according to the cDNA sequence of chZPC (GenBank accession no. D89097). The PCR conditions were 30 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 40 sec, and primer extension at 72°C for one min; the sense primer was 5′-GACATCCTCAACATCCAGGC-3′, and the antisense primer was 5′-GACAGCAGTTGCAGACATCC-3′, corresponding to the peptide sequences in Figure 2, marked with arrows. The PCR product was sequenced and confirmed to represent the chZPC cDNA. The resulting 390-base pair (bp) probe was labeled with [α-^{32}P]dCTP by random priming, and hybridization was performed in buffer containing 1% BSA, 7% SDS, 0.5 M sodium phosphate (pH 6.8), and 1 mM EDTA at 65°C for 20 h (10^6 cpm/ml). Washing was performed at 65°C, first in 40 mM sodium phosphate buffer (pH 6.8), 0.5% BSA, 5% SDS, and 1 mM EDTA and then in 1% SDS, 40 mM sodium phosphate (pH 6.8), and 1 mM EDTA. The blot was exposed to Reflection (Eastman Kodak, Rochester, NY) film with intensifying screen at −80°C for the times indicated in the figure legends.

Biosynthetic Labeling of ZP Glycoproteins and Immunoprecipitation of chZP3

Granulosa cell sheets were isolated from F2 follicles [24] and incubated in methionine-free medium (RPMI 1640, supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1 mg/ml L-glutamine, 5% fetal calf serum) for 2 h at 37°C. After addition of 250 μCi/ml [^{35}S]methionine and 2% (v:v) medium containing 30 μg/ml methionine, the sheets were incubated for another 16 h for metabolic labeling. Then the granulosa cell sheets were sedimented by centrifugation at 100 × *g* for 4 min, and the medium was collected. The pelleted sheets were washed two times with methionine-free medium (RPMI 1640) and extracted with a solution of 200 mM Tris maleate (pH 6.5), 0.5 mM PMSF, 2.5 mM leupeptin, 2 mM $CaCl_2$, and 1.4% Triton X-100. Insoluble material was sedimented by ultracentrifugation at 300 000 × *g* for 20 min and discarded. The detergent extract of the cells and the medium were subjected to immunoprecipitation with anti-chZPC antise-

rum as well as with preimmune serum at 4°C for 2 h, followed by incubation with protein A immobilized on Sepharose CL-4B (Pharmacia, Piscataway, NJ) for 4 h at 4°C. The Sepharose was sedimented and washed two times with washing buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 0.5% Triton X-100, and 100 μg/ml methionine), resuspended in 20 μl SDS-PAGE sample buffer containing 50 mM dithiothreitol, heated to 95°C for 3 min, and centrifuged for 2 min. SDS-PAGE was performed as described above by using a 12% separating gel under reducing conditions. The gel was treated for 20 min with EN^{3}HANCE (DuPont, Brussels, Belgium) and then soaked in water, dried on 3 MM Whatman (Clifton, NY) paper, and exposed to Reflection film for 20 h.

Confocal Microscopy

Isolated granulosa cell sheets were attached to adhesion slides (Bio-Rad) and fixed in methanol:acetone (1:1) for 5 min at −20°C. The samples were incubated in PBS containing 0.2% gelatine for 30 min and then in antisera (1:200 dilution) in PBS/0.2% gelatine for 2 h at 30°C. After three washes in PBS, samples were incubated with goat anti-rabbit IgG conjugated to a 1:50 dilution of BODIPY FL (Molecular Probes, Leiden, Netherlands) for 1 h at 23°C. After several washes in PBS, samples were incubated in 1 mg/ml RNase (Boehringer, Mannheim, Germany) in PBS + 5 mM $MgCl_2$ for 30 min, stained with 0.1 μg/ml propidium iodide for 10 min, washed in distilled water, and embedded in Moviol (Hoechst, Frankfurt, Germany). Samples were viewed in a Zeiss Axiophot (Thornwood, NY) microscope and the MRC 600 laser scanning microscope (Bio-Rad) equipped with a krypton laser and a double-channel filter set.

Immunohistochemistry

Follicles were fixed for 20 h in fixation buffer containing 75 mM L-lysine, 2% paraformaldehyde, 11.2 mM sodium *meta*-periodate, 75 mM sodium phosphate (pH 7.3); this was followed by soaking in increasing concentrations of ethanol (50–96%) and XEM 200 (Vogel GmbH, Giessen, Germany). Follicles were then embedded in paraffin, and sections were prepared (3 μm). Sections were baked at 50°C for 16 h onto silanized slides. Paraffin was removed by soaking in XEM 200, two times for 15 min each, followed by immersion in decreasing concentrations of alcohol. Sections were blocked in a humid chamber with PBS containing 3% goat serum and 1% nonfat dry milk for 1 h. Incubation with anti-chZPC antiserum or preimmune serum (dilution 1:1000) in blocking solution was followed by five washes in PBS and incubation with the secondary antibody (biotinylated goat anti-rabbit IgG, 1:500; Sigma Chemical Co., St. Louis, MO) in the same buffer. Slides were washed again in PBS and incubated in avidin-horseradish peroxidase (1:200). All incubations were performed for 1 h except the staining with 9-ethylcarbazol-3-ylamin (0.2 mg/ml in acetate buffer, pH 5.2, plus 0.03% hydrogen peroxide). Staining was controlled under the microscope and stopped by soaking in water.

Glycosidase Digestion of chZPC

Extracts of pvm of follicles and laid eggs were subjected to SDS-PAGE under nonreducing conditions. Bands corresponding to ZPC from follicles and laid eggs, respectively, were cut out and electroeluted (elution buffer, 15 mM ammonium bicarbonate) at 180 V for 6 h. All digestions of chZPC with glycosidases were carried out at 37°C. Incubation of 10 μg protein was performed with 1) sialidase (from *Vibrio cholerae*; Boehringer Mannheim) in 50 mM sodium acetate, 4 mM $CaCl_2$ (pH 5.2) for 1 h; 2) sialidase, followed by ultracentrifugation at 125 000 × *g* for 1 h at 4°C, and subsequently with *O*-glycosidase (from *Diplococcus pneumoniae*; Boehringer Mannheim) in 20 mM Tris phosphate (pH 7.4) for 16 h; or 3) *N*-glycosidase F (from *Flavobacterium meningosepticum*; Boehringer Mannheim) in 20 mM sodium phosphate, 10 mM EDTA, 2% octylglucoside, and 0.2% SDS (pH 7.2) for 16 h. Digestions were terminated by boiling samples for 5 min. SDS-PAGE was performed on 15% gels; this was followed by Western blotting as described above.

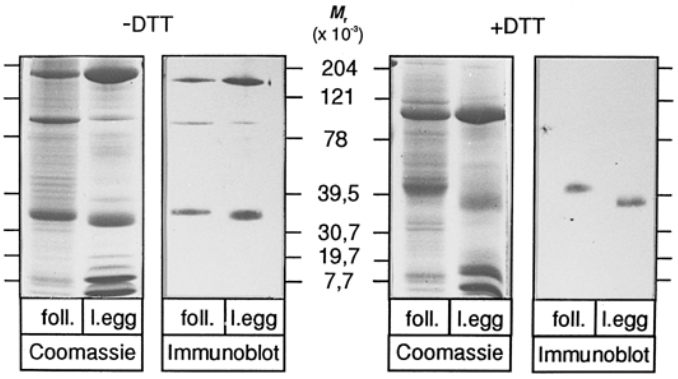

FIG. 1. Electrophoretic analysis of chicken pvm and immunoblotting of chZPC. Extracts of chicken pvm prepared from laid eggs (l.egg) and follicles (foll.) were subjected to SDS-PAGE (4.5–18%) under nonreducing conditions (left) and reducing conditions (right). The proteins were either stained with Coomassie blue (30 μg protein each) or transferred to nitrocellulose (0.2 μg protein each) for immunoblotting with anti-chZPC antiserum as described in *Materials and Methods*. The positions of migration of molecular weight standards (Kaleidoscope Prestained Standard; Bio-Rad) are indicated.

RESULTS

Isolation of Chicken pvm and Characterization of chZPC

In accordance with its function as a protective, stabilizing, and fertilization-competent envelope of the oocyte, chicken pvm shows a high degree of chemical stability. Thus, complete solubilization of the pvm required the application of an SDS-containing buffer; prior washing of the pvm with Triton X-100 appeared to remove certain minor components, which were not characterized further. Under reducing conditions, the electrophoretic pattern of the Triton X-100-washed, SDS-dissolved pvm from laid eggs showed four major protein bands with apparent molecular weights of ~5000, ~13 000, ~34 000, and ~95 000, respectively (Fig. 1, l.egg, +DTT).

We obtained amino acid sequences of the N-termini and of tryptic peptides of these four proteins and compared them to the EMBL and GenBank databases. This analysis confirmed previous reports that the 5-kDa and the 13-kDa proteins represent chicken VMOI [2] and lysozyme [27], respectively. Sequencing of fragments of the 95-kDa protein revealed that it represents a novel protein, which will be subjected to future analysis. Interestingly, the 34-kDa protein contained sequences that showed a high degree of homology to mammalian ZP3s. We indeed identified these peptide sequences as identical to those deduced from a recently reported chicken cDNA (GenBank accession no.

A

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MLGELEAGGM QGGRVVLGLL CCLVAGVGSY TPWDISWAAR GDPSAWSWGA  50
EAHSRAMAGS HPVAVQCQEA QLVVTVHRDL FGTGRLINAA DLTLGPAACK 100
HSSLNAAHNT VTFAAGLHEC GSVVQVTPDT LIYRTLINYD PSPASNPVII 150
RTNPAVIPIE CHYPRRENVS SNAIRPTWSP FKLRTVSRGE AVFSLRLMSD 200
DWSTERPFTG FQLGDILNIQ AEVSTENHVP LRLFVDSCVA ALSPDGDSSP 250
HYAIIDFNGC LVDGRVDDTS SAFITPRPRE DVLRFRIDVF RFAGDNRNLI 300
YITCHLKVTP ADQGPDPQNK ACSFNKARNT WVPVEGRRDV CNCCETGNCE 350
AACALRRLNP WRDGEAAFRR DPGKEVRADV VMPVLLSADP GAVGQQEEGG 400
DGPAVMVPSV GTGLVCVTVP VALAAVGVAV GIARKGCTRT STAV        444
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B

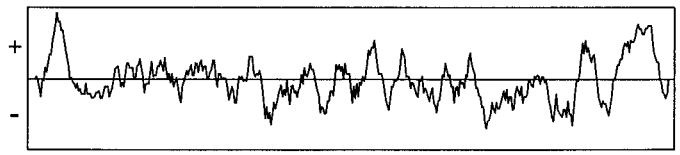

FIG. 2. Amino acid sequence of the chZP3 homologue. **A**) The nucleotide sequence from which the protein sequence was deduced is deposited in GenBank, accession no. D89097; the numbering starts with the initiator methionine. Peptide sequences obtained by microsequencing are printed in bold letters, the amino terminus of the mature protein is indicated by an asterisk, and the hydrophobic domain at the C-terminus is underlined. The sequences corresponding to the primers used for RT-PCR (see *Materials and Methods*) are indicated by arrows; a putative *N*-glycosylation site is doubly underlined, di-arginines are boxed, and 12 conserved cysteines are circled. **B**) Computer analysis of the amino acid sequence of chZPC was performed as described in *Materials and Methods*. Hydrophobic domains are indicated by (+), hydrophilic domains by (−). The hydropathy plot (Kyte/Doolittle) of chZPC illustrates two prominent hydrophobic domains of the protein, representing the signal sequence at the N-terminus and a putative membrane-anchoring domain at the C-terminus.

D89097, Takeuchi Y. et al.), which we tentatively designated as chicken ZPC. The cDNA sequence predicts a 444-residue precursor protein with a mass of 47 502 daltons, whose features are highlighted in Figure 2A. Our protein sequencing data (bold letters) establish the amino terminus of the protein in the laid egg as alanine, 56 residues downstream of the initiator methionine. Other features are a single consensus site for *N*-linked glycosylation (doubly underlined) and 17 cysteines, 2 of which are present in the signal sequence. Of the remaining 15 cysteines, 12 are conserved between chZPC and its homologues in other species (circled in Fig. 2A, and cf. Fig. 3). In fact, pairwise comparison of the primary sequences of chZPC with homologues from a variety of species (Fig. 3) revealed that identities over the entire length of the proteins range from 32% (vs. carp) to 44% (vs. human) and that the central regions are from 49% (vs. *Xenopus laevis*) to 57% (vs. human) identical. These core regions include so-called ZP domains [28] of approximately 260 residues, which contain 8 absolutely conserved cysteines (residues 67, 99, 120, 161, 238, 260, 304, 322 in chicken). Significantly, there is a hydrophobic stretch of 41 residues at the carboxy-terminus that contains a potential membrane-spanning domain, typical of most zona proteins [14] (underlined in Fig. 2B).

In the context of these structural features, and since at least some of the pvm proteins are thought to be synthesized in the ovarian follicles, i.e., before ovulation and not in the oviduct, we next tested for the presence of chZPC in follicles. The results in Figure 1 show that by electrophoretic and Western blotting analysis, chZPC is indeed present in follicles; these results revealed that lysozyme and VMOI were absent from the pvm of follicles. Interestingly, however, chZPC appeared larger in the follicle than in the laid egg; the difference in electrophoretic mobility was particularly obvious under reducing conditions. This difference was further analyzed as described below. Under nonreducing conditions, the antibody raised against chZPC showed weak cross-reaction with the 95-kDa component and a 190-kDa band. The latter may represent a disulfide-linked dimer of the 95-kDa protein (cf. reduced vs. nonreduced gels); alternatively, the cross-reactivity may result from disulfide linkage of ZPC to other pvm proteins; e.g., in mice, ZP3 has been reported to be linked to ZP2, another major protein of the ZP [29].

Analysis of chZPC Transcripts

In previous studies it was found that synthesis of the murine sperm receptor mZP3 occurs exclusively in the oocyte [14, 30, 31]; in rabbits a participation of follicle cells was suggested [32], whereas evidence was provided that in certain spawning female fishes the genes specifying zona proteins are expressed in the liver [33–36].

In order to examine the site of ZPC production in the chicken, we performed Northern blot analysis of various tissues with a 390-bp ^{32}P-labeled chZPC cDNA fragment as probe (the positions of primers used to amplify the fragment are indicated in Fig. 2A). Figure 4 shows that this probe hybridized to a single transcript of ~1.9 kilobases (kb). In granulosa sheets from follicles of different growth stages, the strongest signal was detected for the two largest follicles. In pooled small whole follicles (SF; diameter, 2–8 mm), transcripts were clearly detectable; but in the ovaries of 15- and 18-day-old embryos, chZPC transcript was barely visible. Under the same conditions, chZPC transcripts were undetectable in 10-day-old whole embryos (Fig. 4, left). We also tested the livers of laying hens and estrogen-treated roosters (LH and ER, Fig. 4, left) as well as the testes of untreated (CR) and estrogen-treated roosters; in none of these tissues could chZPC transcripts be observed. Upon closer inspection of the hybridization data in follicles, it is obvious that with decreasing size of the follicles (F3 to F5), transcript levels in the granulosa cells decrease. Furthermore, in contrast to the high levels of transcripts in granulosa cells of F1 and F2 follicles, granulosa cells isolated from postovulatory follicles showed only very low levels of ZPC transcript (Fig. 4, right). The result of the experiment shown in Figure 4 (right) is an example for our frequent finding that the granulosa cells of the largest follicle (F1) appear to contain less ZPC message than the follicle to ovulate thereafter (F2), possibly reflecting a regulatory event in the preovulatory phase. Next, we performed metabolic labeling experiments in order to test directly for ZPC synthesis in granulosa cells.

Biosynthesis of chZPC

Preliminary studies showed that the antibody we had raised was able to precipitate chZPC from pvm extracts. Thus, granulosa cell sheets of F2 follicles (see *Materials and Methods*) were metabolically labeled by incubation in the presence of [^{35}S]methionine for 16 h, and cell extracts and medium were subjected to immunoprecipitation. Figure 5 shows that the antiserum specifically precipitated a single

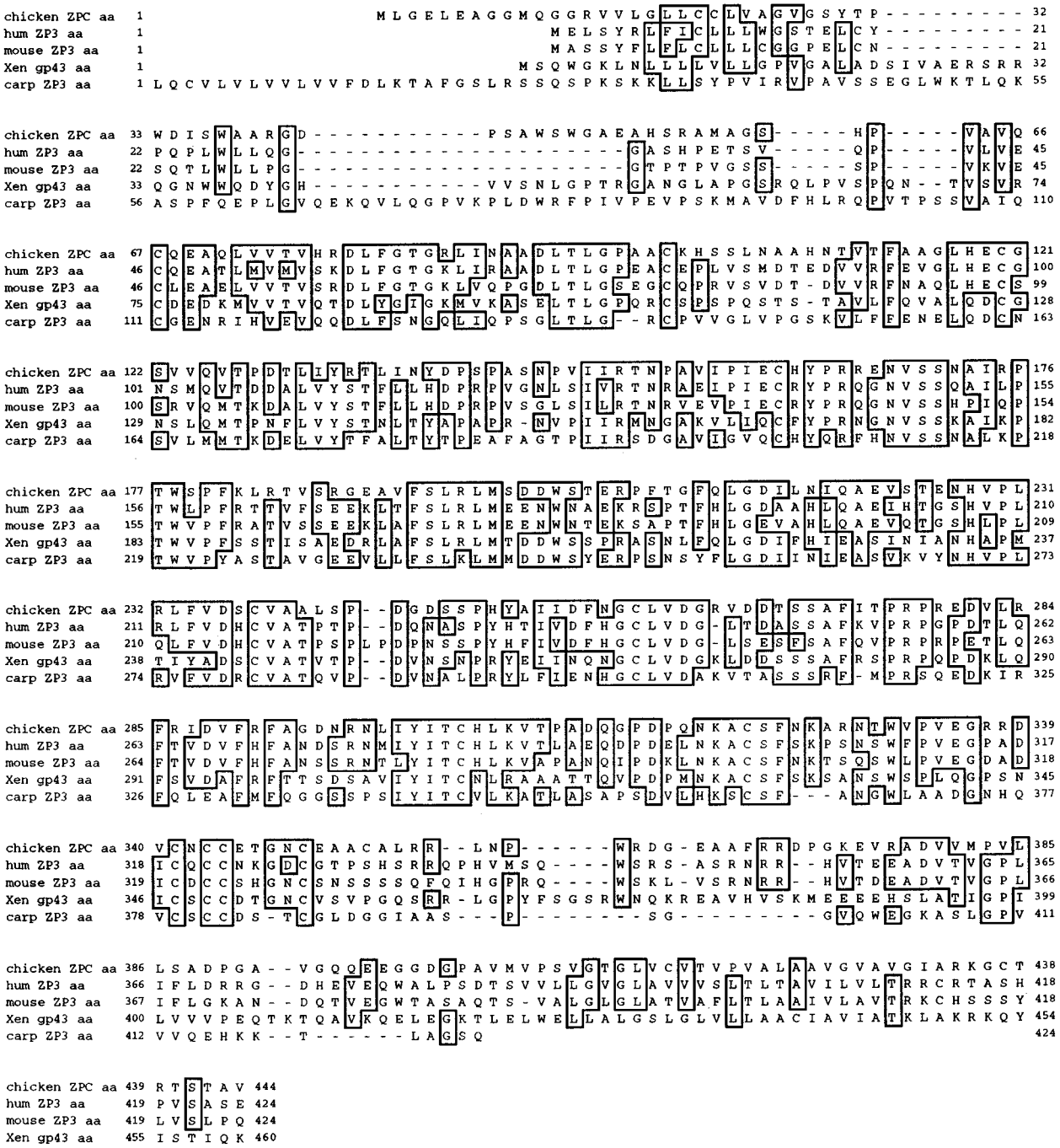

FIG. 3. Multiple amino acid sequence comparison of ZP3 homologues from the chicken (this paper), the human [52], the mouse [53], *Xenopus* [10], and carp [54]. Alignment was performed with the Mac Vector 6.0.1.§ for Macintosh program. Identical residues are boxed (at a minimum of three residues identical).

protein secreted into the culture medium. Preimmune serum (C.) did not react with any protein present in the incubation medium. Under our conditions, only a very small amount of biosynthetically labeled chZPC was observed in Triton X-100 extract of the granulosa cells.

Taken together with the transcript analysis, these data demonstrate that ZPC, a major component of the avian pvm, is produced exclusively by the surrounding granulosa cells during the rapid growth phase of the oocyte. Furthermore, these data show the exquisite monospecificity of our anti-chZPC antibody, allowing its use in the immunolocalization studies described below.

Confocal Microscopy and Immunohistochemistry

Analysis by confocal microscopy with anti-chZPC antibody of the pvm associated with F2 granulosa cell sheets revealed that the protein is present in and/or forms a fibrillar meshwork (Fig. 6A). Given the limitations of resolution of the technique, we can say that the fibrils have an average thickness of ~300 nm. Identical results were obtained with pvm preparations of F1-F4 follicles (not shown). The granulosa cells in follicles at these stages display a high degree of polarization; the apical side with the pvm corresponds to the side apposed to the oocyte, and the basal or baso-

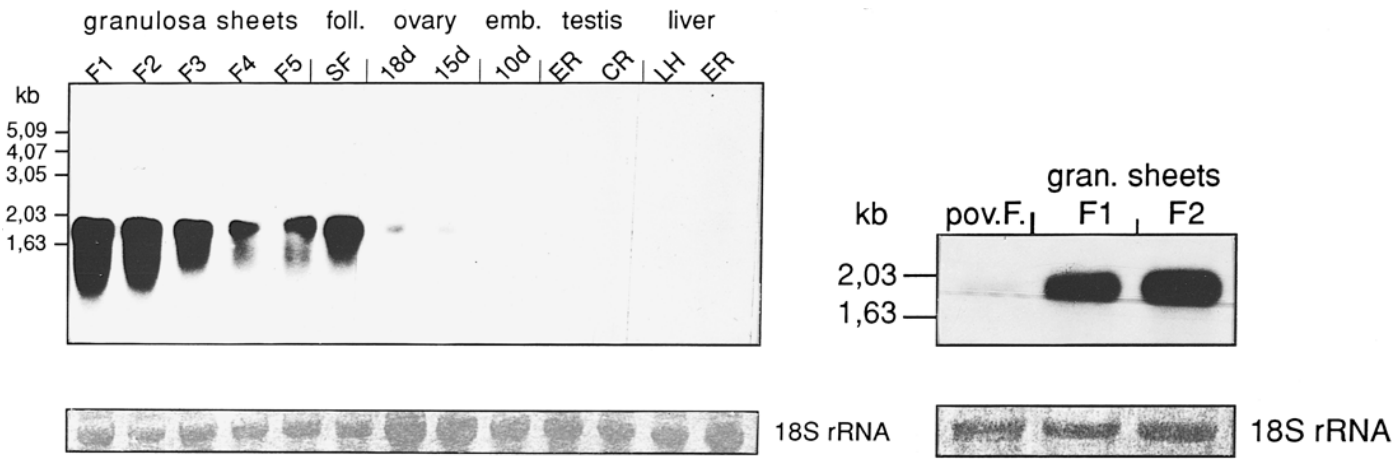

FIG. 4. Expression pattern of the chZPC mRNA. Left: Total RNA (10 μg) isolated from granulosa sheets of follicles of different stages (F1-F5), from pooled small follicles (SF; diameter 2–8 mm), from testes of estrogen-treated (ER) and control (CR) roosters, and from livers of laying hen (LH) and estrogen-treated rooster (ER), as well as 30 μg total RNA isolated from ovaries of 15-day and 18-day-old embryos (ovary) and from 10-day-old whole embryos (emb.), were denatured and separated by electrophoresis on a 1.2% agarose gel. A ^{32}P-labeled 390-bp chZPC cDNA fragment was used as probe (see *Materials and Methods*). Right: Total RNA (10 μg) from a granulosa cell-enriched fraction of a postovulatory follicle (pov.F.) and from granulosa cell sheets of F1 and F2 follicles was analyzed under the same conditions as described above. Autoradiography at −70°C was for 5 h (F1-F5), 7 days (remaining lanes of left panel), and 12 h (right panel), respectively, with an intensifying screen. One-kilobase ladder was used as size marker for both blots. Methylene blue staining of the avian homologue of 18S rRNA was used as loading control (bottom).

lateral aspect is bordered by the basement membrane. A confocal microscopy image of a cross section through the granulosa cell sheet (Fig. 6B) demonstrates the strictly apical localization of ZPC, i.e., within the pvm.

The chicken pvm in follicles was also visualized by immunohistochemistry using the anti-chZPC antibody (Fig. 7). The localization of the immunoreactive material, forming a sharp band between the oocyte plasma membrane and the granulosa cells, illustrates that the result of secretion of chZPC by granulosa cells is the presence of the protein in exquisitely polarized fashion. Immunohistochemistry also revealed that chZPC protein is undetectable in follicles smaller than 1.5 mm in diameter (Fig. 7D), although chZPC mRNA appears already at late embryonic stages (Fig. 4).

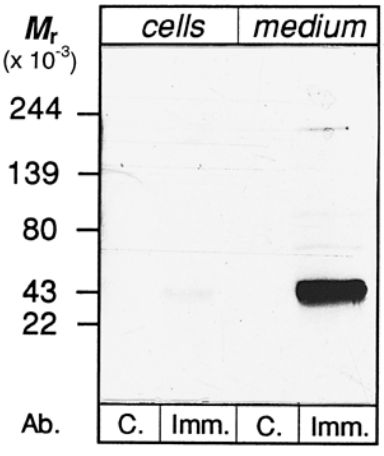

FIG. 5. Immunoprecipitation of metabolically labeled chZPC. Granulosa sheets isolated from F2 follicles were incubated for 16 h in [^{35}S]methionine-containing medium (250 μCi/ml), followed by immunoprecipitation of the medium and of detergent extracts of the cells with anti-chZPC antiserum (Imm.) or preimmune serum (C.) as described in *Materials and Methods*. Immunoprecipitates were analyzed by SDS-PAGE (12%) and autoradiography (see *Materials and Methods*) for 16 h. The positions of migration of molecular weight standards are indicated.

ChZPC Isoforms in Follicles and Laid Eggs

With detailed information on its expression available, we next addressed the apparent size difference between chZPC in follicles and laid eggs (cf. Fig. 1). We performed two sets of experiments. First, in order to determine the site of generation of this size difference, we subjected extracts of follicles, of the pvm of a freshly ovulated oocyte (isolated from the infundibulum), and of a laid egg to immunoblotting with anti-chZPC antibody (Fig. 8). This analysis revealed two apparently stepwise increases in electrophoretic mobility of chZPC from the follicular stage to the egg, likely occurring at ovulation and during deposition of the outer pvm layer, respectively.

One reason for the size difference(s) could be modifications of the carbohydrate moiety of ZPC after ovulation. In order to test for this possibility, deglycosylation analyses were performed on SDS extracts of pvm derived from both follicles and laid eggs. The results shown in Figure 9 suggest that the size difference is not due to partial or total deglycosylation of ZPC after ovulation, as the two forms decrease equally after *N*-glycosidase F treatment and indicate that the single consensus Asn (see Fig. 2A) is indeed modified by *N*-glycosylation. Treatment with sialidase and *O*-glycosidase resulted in an almost undetectable decrease in size (data not shown).

DISCUSSION

The most significant finding in the current studies is the realization that chZPC, despite its extensive homology to related proteins from mammals, frogs, and fish, is synthesized in granulosa cells but not in liver or oocytes, as is the case for fish or for mice and frogs, respectively. Our studies indicate that the glycoprotein is synthesized as a larger precursor directed toward the apical aspect of the granulosa cells within ovarian follicles. We have identified the amino terminus of the form of ZPC present in the laid egg by amino-terminal sequencing. However, structural features of the precursor, as well as our finding of a two-step modification of the apparent size of chZPC from the follicular

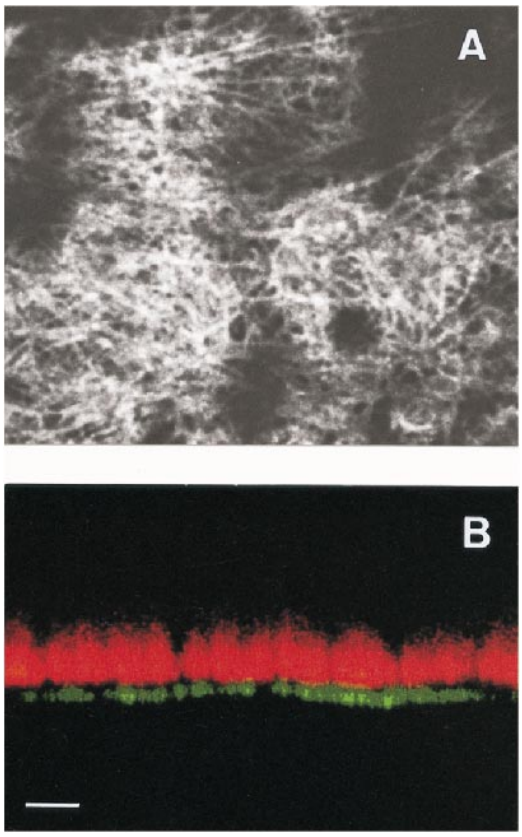

FIG. 6. Confocal microscopy of immunostained chZPC. Isolated granulosa sheets (from F2 follicles) were processed for immunofluorescence microscopy using anti-chZPC antiserum. **A**) Confocal image of an optical section through the plane of the granulosa sheet's pvm. Area shown is 41 × 49 mm. **B**) Confocal image of an optical section perpendicular to the granulosa cell layer stained with anti-chZPC antiserum (green) and propidium iodide (red) to stain nuclei. Bar = 5 μm.

stage to the laid egg, suggest that several posttranslational events lead to the final product. The precursor form of chZPC is presumably modified at or immediately following release of the oocyte from the follicle, and subsequently when the oocyte has passed the site where fertilization normally takes place. In any case, all experimental material in this study was derived from virgin hens, and thus the modifications of chZPC take place independently of contact with sperm. We propose that in the chicken, the first modification might prepare the pvm, and/or ZPC itself, for sperm binding, and that ZPC is then further processed to become part of the durable pvm that surrounds the yolk in the laid egg. Such a scenario is compatible with the general view that envelopes of oocytes in egg-laying species develop in stages: for instance, in *Xenopus*, there are four forms. The so-called oocyte envelope surrounds the oocyte in the ovary; the coelomic egg envelope surrounds ovulated eggs that have not yet entered the oviduct; the vitelline envelope surrounds oviposited eggs; and the final envelope encloses extracorporeally fertilized eggs [37].

In the mouse, a function of *O*-linked carbohydrates of ZP3 in fertilization has been proposed [13], and modification of ZP3 during sperm binding seems to take place [38]. However, in the chicken, equal size reductions of the precursor and the mature form of chZPC upon enzymatic deglycosylation (Fig. 9) suggest that the processing step is unlikely to involve sugar modification(s). Rather, similar to the situation in *Xenopus* [37, 39], the size reduction might be due to a proteolytic clip. Analysis of the hydrophobic regions (Fig. 2B) of the protein shows a potential transmembrane region at the C-terminus, in addition to the signal peptide at the N-terminal end of the precursor protein. Murine ZP proteins ZP1, ZP2, and ZP3 [14, 40], porcine ZP1 [41] and ZP3 alpha [42], human ZP2 [43] and ZP3 [44], and gp43 (a ZP3 homologue from *Xenopus* [10]) contain hydrophobic regions close to the carboxy-terminus as well. Such regions are characteristic of ''zona pellucida domains'' [28] of approximately 260 residues with 8 absolutely conserved cysteines (Fig. 3), common to a number of matrix-forming proteins that may be involved in the formation of filaments [45], such as those shown here for chZPC.

Recently, ZP domains have been identified in chicken and murine β-tectorin [10, 46], extracellular fibrillar matrix molecules of the inner ear. The ZP domains almost invariably share, in addition to their carboxy-terminal hydrophobic region, a furin cleavage consensus sequence upstream of the potential membrane domain and eight strictly conserved cysteine residues. All of these features appear to be conserved in the chZPC protein, with the exception of the potential furin site [47]; this appears to be replaced by three di-arginines, one of which is separated by 33 residues from the putative membrane-spanning domain. Since, to our knowledge, cleavage at the furin site has not been demonstrated for any ZP domain, the significance of the potential furin site in terms of ZP processing remains to be demonstrated. However, a serine protease with specificity for lysyl-X and arginyl-X bonds has been isolated from *Xenopus* oviduct and has been reported to cleave a 43-kDa glycoprotein of the egg envelope [37]. Similar protease(s) may be responsible for the modification of ZP proteins in other species, including the chicken, where cleavage could occur at di-arginine(s). Alternatively or in addition, proteolytic processing of chZPC may involve truncation at the amino terminus. The first residue determined by microsequencing of chZPC isolated from the laid egg is at position 56, while analysis of the sequence according to von Heijne [48] reveals the most likely cleavage site for the signal peptide between positions 29 and 30, which would result in a size difference of about 3 kDa between the mature form and that in the laid egg.

Of particular interest is the presence of the above-mentioned hydrophobic domain close to or at the carboxy-terminus of ZP proteins. In chicken β-tectorin, the hydrophobic domain has been suggested to constitute a glycosyl phosphatidylinositol (GPI) anchor; such lipid moieties direct newly synthesized proteins in epithelioid cells to the apical cell surface, where they become transiently anchored and then further processed [49, 50]. It appears entirely possible that such signals in ZP proteins generally effect their highly polarized expression. In the chicken follicle, our immunohistochemical data demonstrate the exclusive presence of chZPC on the apical aspect of the granulosa cells (Fig. 7). In addition, further modification of GPI-anchored proteins frequently includes their rapid co-translational proteolytic processing, including cleavage of the hydrophobic terminal domain. Indeed, the results of Figure 5 show that newly synthesized chZPC is secreted from the granulosa cells. Finally, it is interesting to note that the sizes of chZPC in the pvm of follicles, ovulated oocytes, and laid eggs are smaller than expected on the basis of the calculated size of the precursor molecule. These forms could conceivably indeed arise by proteolysis at the carboxy-terminus, since our

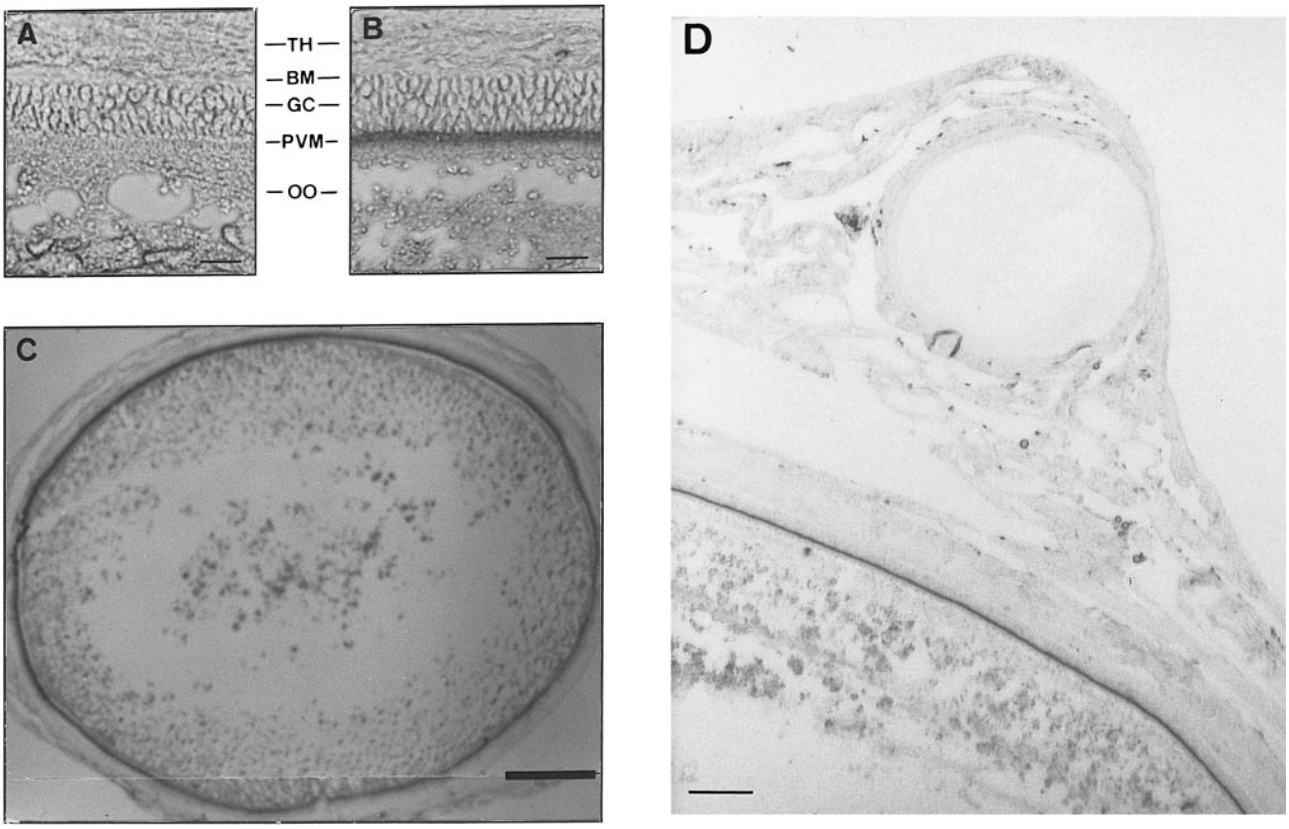

FIG. 7. Immunohistochemical localization of chZPC. Sections of small follicles (diameter, 1–6 mm) were prepared and processed for immunohistochemistry as described in *Materials and Methods*. Higher magnification shows treatment with preimmune serum (**A**) or anti-chZPC antiserum (**B**), clearly staining the chicken pvm (PVM) between the oocyte (OO) surface and the granulosa cell layer (GC), which in turn is followed by the basement membrane (BM) and the thecal cell layer (TH). Lower magnification (**C**) shows staining of a ~4-mm follicle with anti-chZPC antiserum; (**D**) shows absence of chZPC in a 1.5-mm follicle next to a clearly stained larger follicle. Bars = lengths of 20 μm (**A, B**), 675 μm (**C**), and 400 μm (**D**), respectively.

results show that extensive modification of the carbohydrate moiety is unlikely to occur (Fig. 9).

From an evolutionary point of view it appears intriguing that the known homology in primary sequence between zona proteins in mammals, amphibia, and fish extends to an avian species. This is compatible with the possibility that the carbohydrate moieties, rather than the protein backbone of ZP3, may contribute to its interaction with sperm components [19]. However, the recent result showing that the ZP3 polypeptide itself induces the acrosome reaction in human sperm was interpreted as a challenge to this notion [51]. The difference in site(s) of synthesis of ZP proteins may be related to the differences in the biology of the reproductive efforts of these species. In mammals, which de-

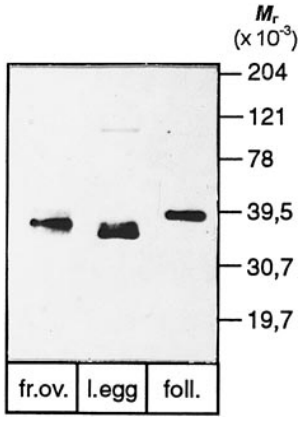

FIG. 8. Chicken ZPC undergoing postovulatory modifications. Perivitelline membranes were prepared from freshly ovulated eggs (fr.ov.), laid eggs (l.egg), and follicles (foll.); and 0.2 μg each were subjected to SDS-PAGE (4.5–18%) and Western blotting under reducing conditions using anti-chZPC antiserum as described in *Materials and Methods*. The Bio-Rad Kaleidoscope Prestained Standard set was used as size marker.

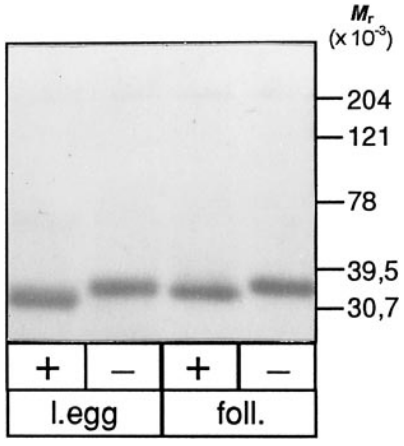

FIG. 9. Glycosidase treatment of chZPC. Chicken ZPC derived from laid egg (l.egg) or follicle (foll.) pvm was eluted from the gel after SDS-PAGE (4.5–18%) as described in *Materials and Methods*. The eluted proteins (2 μg each) were subsequently treated with (+) or without (−) *N*-glycosidase F (0.4 U/10 μg) for 16 h at 37°C and analyzed by SDS-PAGE (15%) under nonreducing conditions, followed by staining with Coomassie blue. The Bio-Rad Kaleidoscope Prestained Standard set served as size marker.

velop relatively small oocytes, ZP3 appears to be synthesized by the germ cells themselves; in fish, where rather large oocytes become fertilized extracorporeally, at least certain ZP proteins are synthesized in the liver; and in the chicken, where oocytes become extremely large and are fertilized in the oviduct, ZPC is produced by the adjacent granulosa cells, as shown here. The apposition of the granulosa cell layer to the extremely large surface of the oocyte in its rapid growth phase makes the granulosa cells the ideal source of ZPC, which must become deposited evenly around the oocyte. In this context, it will be interesting to determine the site(s) of synthesis of other components of the pvm of the chicken follicle, in studies that are now under way in our laboratory.

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