Multiple Involvement of Clusterin in Chicken Ovarian Follicle Development

BINDING TO TWO OOCYTE-SPECIFIC MEMBERS OF THE LOW DENSITY LIPOPROTEIN RECEPTOR GENE FAMILY*

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The interaction of the female germ cell with somatic cells during the development of the ovarian follicle in the chicken provides a prime system to study gene expression. Here, we have uncovered the involvement of clusterin, the function(s) of which is still poorly understood, in this complex process. As revealed by molecular cloning, chicken clusterin is a 428-residue protein that migrates at 70 kDa on SDS-polyacrylamide gel electrophoresis and possesses most of the structural features of its mammalian successors. However, in contrast to mammalian clusterin, the chicken protein appears not to be cleaved intracellularly into a disulfide-linked heterodimer; possibly as a consequence thereof, it is not secreted constitutively and is absent from the circulation, where most of clusterin is found in mammals. In the ovary, clusterin is a major product of the somatic granulosa cells, in a pattern correlating with the developmental phases of individual follicles. In that, transcript levels are high not only at onset of vitellogenesis, but also in atretic follicles and in the postovulatory follicle sac, i.e. in situations characterized by apoptotic events. Yolk of growing oocytes contains a 43-kDa truncated form of clusterin that does not appear to be synthesized within the oocyte. Rather, we here show for the first time that 70-kDa clusterin interacts not only with megalin, but also with two chicken oocyte-specific members of the low density lipoprotein receptor (LDLR) gene family. These receptors, termed LDLR-related protein with eight ligand binding repeats (LR8) and LDLR-related protein (380 kDa), likely internalize granulosa cell-derived 70-kDa clusterin, which may subsequently be processed to the 43-kDa product. Thus, chicken clusterin could serve as a marker for follicular atresia and resorption, and, based on its ability to bind several other proteins, it may serve as carrier for the receptor-mediated endocytosis into oocytes of components important for embryonic development, two hitherto unknown functions of this intriguing protein.

Clusterin is a ubiquitous and highly conserved secreted glycoprotein thought to be involved in a variety of biological processes, including lipid transport (1), sperm maturation (2), regulation of the complement cascade (3), apoptosis (4), membrane recycling (5), and possibly others (for review, see Ref. 6). Clusterin has been found in numerous biological fluids, including semen, breast milk, urine, cerebrospinal fluid, and human plasma (3, 7, 8). In addition, the expression of clusterin is induced in several pathological conditions, such as atherosclerosis (9), neurodegenerative processes (10), and testosterone-withdrawn prostate involution (5). Clusterin is obviously identical to the protein(s) previously characterized as apolipoprotein J, sulfated glycoprotein-2, serum protein 40-40, and testosterone repressed prostate message-2. In the present work, we use the term clusterin when referring to this protein. Most recently, in addition to the well characterized, secreted form of the protein, a shorter variant of human clusterin lacking the first 32 amino-terminal residues, including the hydrophobic signal peptide, has been reported (11). This truncated form, produced in response to treatment with transforming growth factor β, is retained intracellularly and targeted to the nucleus via an SV40-like nuclear localization sequence, which is silent in the full-length protein. The biological significance for such a transforming growth factor β-induced nuclear localization is presently unclear.

Despite its purported function(s) in numerous physiological and pathophysiological processes, the sites and mechanisms of action of clusterin remain elusive. To date, the only receptor known to bind and internalize clusterin is megalin/gp330, a member of the low density lipoprotein receptor (LDLR) family (12). Inasmuch as clusterin can also bind to the amyloid β peptide generated from the amyloid precursor protein involved in the development of Alzheimer’s disease (8, 13, 14), to discrete subclasses of high density lipoprotein (1, 15), and to the membrane attack complex C5b-C9 (3, 16), a role for LDLR homologues in mediating the transport of clusterin in complex with these or other yet unidentified molecules appears possible.

Thus, based on our previous work describing LDLR family members in the chicken (17–20), we have become interested in the biology of clusterin in this species. All presently known receptors belonging to the LDLR gene family are represented in this avian model (19, 21, 22), allowing a thorough investiga-

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1 The abbreviations used are: LDLR, low density lipoprotein receptor; LDL, low density lipoprotein; VLDL, very low density lipoprotein; VTG, vitellogenin; PAGE, polyacrylamide gel electrophoresis; LR8, LDLR-related protein with eight ligand binding repeats; LRP, LDLR-related protein, 380 kDa; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; GST, glutathione S-transferase; LRP, LDL receptor-related protein.
tion to their possible roles in clusterin metabolism. As a first step in this direction, we now have molecularly characterized chicken clusterin, and we provide insights into its involvement in the growth and development of chicken oocytes within ovarian follicles.

**EXPERIMENTAL PROCEDURES**

**Isolation of Chicken Clusterin cDNA**—A 180-base pair PCR fragment was produced by reverse transcription-PCR (Life Technologies, Inc.) using chicken testes poly(A)/+ RNA for first-strand cDNA synthesis and two synthetic oligonucleotides corresponding to quail clusterin cDNA (originally reported by Tüür (23)): 5’-GTG GGA GGA GTG CAA GCC CTG CC-3’ and 5’-TGC CGC TGC TCC CGA TCC AGC AG-3’. The fragment was subcloned, and its sequence was found to be 98 and 73% identical to quail and human clusterin, respectively. The fragment was 32P-labeled using the Megaprime DNA labeling kit (Amersham Pharmacia Biotech) and used as probe to screen a random hexamer-primed chicken brain agt11 cDNA library (CLONTECH). Hybridization conditions were as follows: 5 × NET (500 mM NaCl, 75 mM Tris, pH 7.5, 5 mM EDTA), 5 × Denhardt’s solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.2% SDS and 100 μg/ml salmon sperm DNA for 20 h at 65 °C. The membranes were washed twice for 30 min each time in 2 × NET containing 0.2% SDS at 65 °C. Positive clones were identified by PCR using agt11-specific primers, subcloned into the pGEM-T (Promega) vector, and sequenced (Sequenase). The complete open reading frame of chicken clusterin was obtained by ligating two overlapping clones using their common SacI restriction site. Comparison of the 180-base pair PCR product used as probe with the corresponding region in the cDNA clone revealed differences in two positions.

**Chicken Follicles**—Tissue sections from ovarian follicles (small yellow, 5–6 mm in diameter) were prepared for in situ hybridization and immunohistochemistry as described earlier (25, 26). The sections (8 μm) were hybridized overnight at 45 °C with 70% formamide hybridization buffer containing 10 mg/ml bovine serum albumin, 70 mg/ml SDS, 0.5 mM sodium phosphate buffer, pH 6.8, 1 mM EDTA, pH 8.0, and the 32P-labeled DNA probe. Washing was performed at 65 °C in 5 mg/ml bovine serum albumin, 50 mg/ml SDS, 40 mM sodium phosphate buffer, pH 6.8, and 1 mM EDTA, pH 8.0, and then in 10 mg/ml SDS, 40 mM sodium phosphate buffer, pH 6.8, and 1 mM EDTA, pH 8.0. The 10% dextran sulfate was exposed to ReflectionTM film (NEN Life Science Products) with intensifying screens at −80 °C. The relative amounts of RNA loaded were estimated using methylene blue staining of ribosomal RNA.

**Northern Blot Analysis**—For Northern blot analysis, total RNA prepared from various tissues of male and female chickens was denatured using glyoxal and dimethyl sulfoxide, separated by electrophoresis on a 1.2% agarose gel, and blotted onto Hybond N+ nylon membrane (Amersham Pharmacia Biotech) using standard methods (24). The above-described 180-base pair chicken clusterin cDNA fragment was labeled with 32P using the Megaprime DNA labeling kit and used as probe. The probe was hybridized overnight at 65 °C in a solution containing 10 mg/ml bovine serum albumin, 70 mg/ml SDS, 0.5 mM sodium phosphate buffer, pH 6.8, 1 mM EDTA, pH 8.0, and washed three times with 2 × SSPE and twice for 10 min each time in 0.2% Ponceau S in 3% (w/v) trichloroacetic acid and destaining in water. The hybridized membranes were exposed to XAR-5 film (Amersham), and band intensities were measured by phosphorimaging (Fuji). The RNA samples were prepared from various tissues of male and female chickens.

**Antigen Production**—Antiserum against chicken clusterin was prepared against two synthetic peptides corresponding to an amino-terminal (antibody A; residues 106–120 in Fig. 1, HGSSGLVGRQLEELL) or a carboxyl-terminal (antibody B; residues 395–410 in Fig. 1, SLYTVPGDISWDSSPRFM) region of the protein. The peptides were coupled to keyhole limpet hemocyanin and used for immunization of two adult female New Zealand White rabbits. Two injections (day 0 and 21 days later) with the antigens were mixed with Freund’s complete adjuvant and for successive booster injections thereafter (days 21 and 28) with Freund’s incomplete adjuvant (27). Antibodies against LR8 (28) and LRP380 (28) and an antibody recognizing both LR8 and LRP380 (17) are described in the indicated references.

**Preparation of Triton X-100 Extracts**—For preparation of Triton X-100 extracts from chicken ova follicles, the follicles were first punctured, and the majority of the yolk portion was carefully squeezed out, followed by a short wash in phosphate-buffered saline (PBS, pH 7.4). The remaining follicle fragments were then processed as above. Triton X-100 extracts from membrane fractions of chicken tissues were prepared as described previously (28).

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—One-dimensional, 4.5–18% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (29) using a minigel system (Bio-Rad, Mini-PROTEAN® II slab gel system). Samples were prepared either in the absence (nonreducing conditions) or the presence (reducing conditions) of 10 mM dithiothreitol with heating. Electrophoresis was performed at 180 V for 1 h with the inclusion of broad range M, standards (Bio-Rad). Electrophoretic transfer of the proteins to nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech) was performed in transfer buffer (26 mM Tris, 192 mM glycine, 20% methanol) for 1 h at 200 mA, on ice, using the Bio-Rad Mini Transblot system. The transfer was verified by staining the nitrocellulose membrane with 0.2% Ponceau S in 3% (w/v) trichloroacetic acid and destaining in water. Western blotting was performed using specific rabbit antisera at the concentrations indicated in the figure legends, followed by protein A horseradish peroxidase (Sigma, 1:5000) and the chemiluminescence detection method (ECL system, Amersham Pharmacia Biotech).

**Chicken Follicle Expression of Clusterin as a GST Fusion Protein**—A full-length clusterin cDNA was prepared by ligating the two overlapping clones obtained from screening the brain agt11 cDNA library at their common SacI restriction site. The ligated fragment was PCR-amplified using Pfu DNA polymerase (Stratagene) and specific 5’- and 3’-end chicken clusterin oligonucleotides, and subcloned via blunt-end ligation into pGEM-T vector. The purified plasmid was linearized, and the RNA probe prepared and labeled with digoxigenin-UTP by in vitro transcription with SP6 and T7 RNA polymerase (digoxigenin RNA labeling kit (SP6/T7)) according to the manufacturer’s recommendations (Boehringer Mannheim). The slides were then washed three times for 10 min each time with 0.2 × SSC and twice for 10 min each time with 0.1 × SSC at 50 °C. After washing, slides were prepared for immunodetection by incubating them in 150 mM NaCl, 100 mM Tris, pH 7.5 (Buffer A), containing 3% normal goat serum and 1% bovine serum albumin for 30 min at 23 °C. The sections were then exposed to anti-digoxigenin-AP Fab fragments (1:500 dilution, Boehringer Mannheim) in the same buffer for 2 h at room temperature and extensively washed with Buffer A and then with Buffer B (100 mM NaCl, 100 mM Tris, pH 9.5, and 50 mM NaCl). The antigen was detected by incubating the slides overnight with a precipitating BM purple AP substrate (Boehringer Mannheim). The reaction was stopped by incubating the slides in 10 mM Tris, 1 mM EDTA, pH 8.0, and mounting them in Aquamount (BDH, Poole, United Kingdom). Photographs were taken with a Zeiss Axiosvert 10 light microscope.

**Antibody Production**—Antiserum against chicken clusterin was pre-
xylol. The xylol was further substituted for paraffin in two steps at 50 °C, followed by three additional 20-min incubations in paraffin. Tissues were then transferred to plastic moulds and embedded in paraffin. Tissue sections of 6 μm were cut on a Microtome and transferred onto glass slides that had been pretreated with 2% 3-aminopropyltriethoxysilane, followed by heat treatment for 16 h at 60 °C. For immunohistochemistry, the tissue sections were deparaffinized by soaking in xylol two times for 10 min each, followed by treatment with decreasing concentrations of ethanol and a final 5-min incubation in 3% H2O2. The sections were incubated in PBS containing 1% nonfat powdered milk and 3% goat serum (blocking buffer) for 1 h at room temperature. Following five washes with PBS for 1 min each, the sections were incubated in blocking buffer containing either primary antiserum against chicken clusterin, LR8, LRP380, or their corresponding preimmune sera (at the concentrations indicated in the figure legends) and incubated for 16 h at 4 °C in a humid chamber. After being washed five times with PBS, follicle sections were incubated with biotinylated goat anti-rabbit IgG (1:500) in blocking buffer for 1 h at 23 °C. Slides were subsequently washed five times in PBS and incubated with avidin-horseradish peroxidase (1:200) in blocking buffer without goat serum for 1 h at room temperature. Following extensive washing in PBS, the sections were incubated in 0.1 M sodium acetate buffer, pH 5.2, containing 0.03% H2O2 and 0.2 mg/ml 3-amino-9-ethylcarbazole (stock solution: 4 mg/ml in N,N-dimethylformamide). The color reaction was followed under a microscope and terminated by incubating the slides in water. The stained sections were mounted in Aquamount (BDH), and photographs were taken with a Zeiss Axiosvert 10 light microscope.

RESULTS

A full-length chicken clusterin cDNA was isolated from a brain agt11 cDNA library by homology screening. The deduced nucleotide sequence revealed an open reading frame of 1344 nucleotides, encoding a protein of 448 amino acids (Fig. 1). The first translation initiation codon (ATG) is located in a context (ACCATGG) that precisely conforms with the consensus sequence predicted by the compilation of animal initiation start sites (31, 32). At the nucleotide level, chicken clusterin shows 61 and 94% identity to human and quail clusterin, respectively. According to our calculations based on the criteria defined by von Heijne (33), chicken clusterin has a putative signal sequence consisting of 20 amino acids (Fig. 1, boxed), generating an amino-terminal leucine in the mature protein. In reviewing the literature, we noticed that previously, the amino terminus of quail clusterin, identical in that region to chicken clusterin, had been proposed as the glycine 4 residues upstream of this leucine (23) (Fig. 2). Our calculations confirmed, however, the previously reported amino terminus of mature human clusterin (Fig. 2). The predicted mature chicken clusterin polypeptide (428 amino acids) has a calculated Mr of 50,157 and shows 45 and 95% identity to human and quail clusterin, respectively (Fig. 2). All 10 cysteine residues are conserved in the three species (Figs. 1 and 2, black squares), as well as four of the
seven consensus sequences for potential N-linked glycosylation in the human protein (Fig. 1, thin underlines) (7). Chicken clusterin has two additional consensus sequences for N-linked glycosylation that are absent in human clusterin but conserved in quail clusterin (Fig. 1, bold underlines). The four predicted potential heparin binding sites in human clusterin (7) appear not to be strictly conserved in chicken (Fig. 2, light hatched boxes). The dark hatched box indicates the putative nuclear localization sequence (11). Arrows on top mark the reported signal sequence cleavage sites for chicken (C) (this study), quail (Q) (23), and human (H) (6) clusterin, respectively. The upward arrow shows the predicted cleavage site producing the α and β subunits in human clusterin (7).

In order to visualize the chicken protein, we performed immunoblotting of detergent extracts prepared from various tissues, as well as of serum, with rabbit antiserum raised against a synthetic peptide derived from the predicted amino-terminal half of chicken clusterin (antibody A; cf. under “Experimental Procedures”) (Fig. 3). The commonly observed immunoreactive band in all tissues was a ~70-kDa protein. Furthermore, in certain tissues (Fig. 3A), various additional bands were visualized, the identity of which remains unclear. The 70-kDa protein did not change migration when the samples were analyzed under reducing conditions (not shown, but cf. Fig. 8); thus, we conclude that the major 70-kDa protein represents chicken clusterin. Most significantly, chicken serum was devoid of clusterin, in contrast to the situation in mammals, in which clusterin is prominent in the blood compartment (1) (in Fig. 3A, the ~150-kDa protein in serum represents chicken IgG, which cross-reacts, albeit weakly, with the protein A used to detect the bound rabbit antibody). In experiments not shown, we subjected isolated high density lipoprotein fractions (the major clusterin-harboring serum fraction in mammals) from both hens and roosters to the same analysis, but could not detect clusterin there either. Also surprisingly, hepatic levels of the protein were very low compared with those found in mammalian species (34). However, levels of clusterin were significant in brain, heart, kidney, muscle and gonadal tissues. Furthermore, in contrast to reports at the RNA level in mammals (6, 7, 34), there were high levels of clusterin in spleen and lung (Fig. 3). Clusterin was also present in the uropygial and adrenal glands.

Next, we aimed at defining the correlation of the protein data with the expression of chicken clusterin at the transcript level. Northern blotting of total RNA from the same tissues as analyzed for protein content (cf. Fig. 3) confirmed that clusterin is widely expressed (Fig. 4). A single ~1.7-kilobase transcript was present at high levels in brain, lung, testes, follicles (Fig. 4, ovary), ovarian stroma, and oviduct, and at lower levels in heart, muscle, spleen, uropygial gland, and adrenal glands. Thus, there was, in general, good agreement between the tran-
script and protein levels, with the possible exception of kidney and muscle. Importantly, however, only very low, if any, transcript levels were detected in the liver, again in sharp contrast to mammalian species, but in excellent agreement with the protein data of Fig. 3A.

Because of the high levels of clusterin in the ovary and our ongoing research interest in the biology of oocyte growth and follicle development, we studied the pattern of clusterin expression in ovarian follicles in greater detail (Fig. 5). We analyzed follicles in different developmental phases, i.e. with different diameters. Shown in Fig. 5 are phase I small white follicles (1–3 mm diameter); phase I large white follicles (3–5 mm); phase II follicles, including small yellow (5–6 mm) and large yellow (6–9 mm); and phase III follicles, characterized by F7 (10 mm) to F1 (33 mm) follicles, the latter being the next follicle to ovulate. Clusterin transcripts were detectable throughout the entire growth period. The highest levels were observed in phase I follicles (small white and large white), which do not yet contain yellow yolk. There was a gradual decrease in clusterin expression with increasing follicle size (small yellow and large yellow), followed by a moderate to low expression throughout phase III (F7–F1), when committed follicles grow and rapidly acquire yolk.

Interestingly, very high levels of clusterin mRNA were present in atretic follicles (Fig. 5). It has been estimated (35) that only 1 of every 10–20 ovarian follicles that have reached a size of approximately 8 mm (i.e. large yellow) are committed to further growth and entry into the ovulatory pathway; most follicles undergo atresia and become resorbed. Moreover, high levels of clusterin transcripts were also apparent at the opposite end of follicle development, namely in the postovulatory sac, i.e. the follicular tissue that remains following release of the F1 oocyte into the oviduct. Consisting of the somatic cells and the acellular structures that surround and support the oocyte during its growth, the postovulatory sac is destined for apoptosis and resorption (36).

Previous studies in mammals (2, 34) have suggested that clusterin is expressed in somatic cells of gonadal tissue. Thus, in order to identify the cell population(s) within chicken follicles that express clusterin, we performed in situ hybridization analysis on phase II follicles (small yellow, 5–6 mm). These contain high levels of clusterin transcripts (see Fig. 5) and are the easiest to manipulate due to their low yolk content. Within
the cell layers, clearly the highest levels of clusterin transcript were present in the granulosa cells, and much lower levels were present in the vascularized outer thecal layer and peripheral epithelial cells (Fig. 6). The oocyte itself displayed only traces, if any, of clusterin transcripts; the area of the oocyte shown in Fig. 6 is representative of the entire germ cell periphery.

Detailed Western blot analysis for the detection of clusterin in follicles (Fig. 7) confirmed that the 70-kDa protein is present throughout the entire growth phase of chicken follicles, from small white to F1, in agreement with the transcript data (Fig. 5). However, in all follicle extracts, particularly the extracts of larger follicles that harbor yolk-rich oocytes (F4–F1), a smaller band of ~43 kDa was observed in addition to full-size clusterin, suggesting that the 43-kDa protein might be yolk-derived. Analysis of yolk from vitellogenic follicles confirmed that the 70-kDa protein is absent from and the 43-kDa protein is present in yolk (data not shown). These results are compatible with the 43-kDa protein being a truncated form of clusterin, possibly derived by proteolytic processing following uptake of 70-kDa clusterin into the yolk compartment of the oocyte. In this context, many yolk precursors, e.g. very low density lipoprotein (VLDL), vitellogenin (VTG), and α2-macroglobulin, bind to oocyte-specific LDL receptor family members, are internalized, and postendocytotically cleaved into defined fragments (19, 37–39).

These findings prompted us to attempt the identification of candidate receptors for clusterin on the oocyte surface. Thus far, two oocyte-specific LDLR family members have been described. One is the chicken homologue of the mammalian so-called VLDL receptor, termed LR8 for its harboring 8 LDL receptor ligand binding repeats, that serves as a receptor for a broad spectrum of ligands (19, 25). The other receptor is an LDL receptor-related protein (LRP) with an apparent diameter of ~380,000 (28), here termed LRP380. To date, only mammalian megalin, another large member of the LDLR gene family, (12), is known to be a receptor capable of binding clusterin.

In order to identify putative chicken clusterin receptors, we performed ligand blotting analysis on oocyte membrane-enriched material (the membrane fraction had been prepared from pooled, yolk-rich F4–F1 follicles) with recombinant chicken clusterin-GST fusion protein, followed by detection of bound ligand with anti-chicken clusterin antiserum (1:250) (antibody A) as described under “Experimental Procedures,” and anti-chicken clusterin antiserum (1:250) (antibody B) as described under “Experimental Procedures,” and lane 6 was incubated with anti-chicken LR8 antiserum (1:500) that also cross-reacts with LRP380 (17). Numbers on the left correspond to the positions of migration of marker proteins (in kDa).

family members may indeed be involved in the metabolism of clusterin, at least within the oocyte.

Finally, in order to obtain further evidence for the possibility that LR8 and LRP380 mediate clusterin uptake from the extracellular space, we localized clusterin and the receptors in the follicle by immunohistochemistry (Fig. 9). Whereas the Northern blot and in situ hybridization results of Figs. 5 and 6 strongly suggest, but do not show, that clusterin protein is produced in granulosa cells, Fig. 9, A and B, clearly reveals strong immunoreactivity in these cells and lower levels in the intraovocytic (yolk) region. The immunoreactive material in the yolk possibly represents, at least in part, the 43-kDa protein (cf. Fig. 7). Importantly, both LR8 (Fig. 9, C and D) and LRP380 (E and F) are conspicuously absent from the granulosa cells but present in the plasma membrane of the oocyte and in the region of endocytic activity underlying the oolemma (40).

DISCUSSION

Despite extensive studies about clusterin, its function(s) has not been identified unambiguously. To our knowledge, published reports on genetic manipulation at the clusterin locus are not available; however, transgenic mice overexpressing human clusterin under the control of the phosphoglycerate kinase promoter become obese.2 This finding, if confirmed, is in

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2 L. E. French, personal communication.
Clusterin is a normal component of human blood, and it has been detected in breast milk, urine, cerebrospinal fluid, and seminal plasma (3, 7, 8); accordingly, clusterin expression is almost ubiquitous, with the possible exception of adult lung and intestine. Although particularly high levels of clusterin are found in epithelial cells, it is not confined to these cells (34). An interesting case of cell type specificity is the distribution of clusterin in mammalian gonads. In the male genital tract, the Sertoli cells lining the seminiferous tubules and the epithelial cells lining all tubules in the head and tail of the epididymis contain clusterin transcripts (41). In the mammalian female, much of the epithelium of the genital tract expresses clusterin, including the granulosa cells that line the follicles in the ovary (34).

The chicken ovary harbors a complete series of growing oocytes, i.e. an identifiable hierarchy of follicles containing oocytes with diameters ranging from 1 to 35 mm. The development of chicken oocytes can be divided into three phases; in the first phase, numerous oocytes, characterized by the absence of typical yellow yolk, increase in diameter from 60 μm to 1–3 mm (here referred to as small white follicles) over several months. The second phase begins with the entry of certain of these oocytes into a slow growth phase, and at reaching a size of 6–7 mm, an estimated 90% of them undergo atresia, whereas the remaining follicles enter the final, third phase. The oocytes selected into the third phase grow rapidly due to the accumulation of yolk precursors and, at reaching a diameter of 30–35 mm, are ovulated. This final phase, a dramatic, 7-day growth spurt, results in the deposition of up to 250 mg of cholesterol, 4 g of triglycerides, and ~5 g of protein into the yolk of the oocyte. Ovulation occurs every 25 h and is thought to mark the beginning of the rapid growth phase of an oocyte (in the F7 follicle) that will be laid as component of the egg 7 days later. Therefore, at any one time during the reproductive period, the chicken ovary contains numerous, macroscopically distinct follicles either positioned in the developmental cascade or undergoing atresia. Thus, follicular growth in the chicken is a complex process, in which the oocyte, the granulosa cells juxtaposed to the oocyte, and the thecal cells must act together to ensure growth of the female germ cell and ovulation. In this powerful model system, gene expression can be studied at the single-cell germ line level (the oocytes) and in a pure somatic cell population (the granulosa cells).

In the present investigation, we have indeed obtained evidence for an important involvement of clusterin in the interactions between these cell types. First, clusterin is synthesized in the granulosa cells, which surround the oocytes. Second, levels of transcripts, but not necessarily of translation product, appear to correlate with the developmental status of the follicle. Third, receptors for clusterin, here identified as such for the first time, are expressed on the surface of the oocyte, i.e. immediately adjacent to the cells that produce clusterin. Fourth, possibly related to this finding, a truncated form of clusterin appears to accumulate in yolk.

The restriction of clusterin expression in the follicles to granulosa cells is of interest. These cells are thought to support the oocyte during its entire growth and development (42, 43). In the rapid oocyte growth phase, granulosa cells presumably undergo a crucial step in their differentiation program in preparation for ovulation. Ovulation occurs when the granulosa, thecal, and epithelial cell layers rupture along an avascular region termed stigma and release the germ cell with its surrounding perivitelline (sperm binding) layer (44). Following ovulation, the granulosa cells remain in the cavity of the so-called postovulatory sac, which subsequently becomes resorbed from the peritoneum. We have found clusterin transcripts and protein in granulosa cells throughout the life span of the follicle (Figs. 5–7). However, at the transcript level, there are peaks of expression in early previtellogenic follicles, as well as in atretic follicles and in the postovulatory sac. As mentioned above, only about 10% of all follicles proceed from the previtellogenic to the vitellogenic stage; the remaining follicles undergo atresia. Thus, the high levels of clusterin expression in small follicles may mirror their predominantly atretic fate. The process of follicle atresia presumably resembles that of resorption of the postovulatory sac, and includes apoptotic mechanisms (45–47). Our finding of elevated transcript levels in these two situations indeed points to similarities between atresia and resorption at the molecular level and is compatible with the frequently proposed involvement of clusterin in apoptosis (4, 5, 48, 49). Moreover, the anti-apoptotic gene bcl-xl is expressed in granulosa cells in a pattern opposed to that observed here for clusterin (50).

The apparent lack of differences at the protein level in situations where there are obvious differences in clusterin tran-
script levels in follicles (compare Figs. 5 and 7) will need further attention. Discordance of transcriptional and translational activities is a trivial possibility; it is also conceivable that at elevated synthetic levels, clusterin protein is processed to a form that becomes unrecognizable by our antibody or that it is produced in a secretable form. The former possibility is supported by findings of a truncated form of human clusterin that can be transported to the nucleus (11), the latter by the presence of an immunoreactive 43-kDa protein in the yolk of growing oocytes. This form of clusterin might be the end product of receptor-mediated endocytosis of granulosa cell-derived clusterin by the adjacent oocyte, as discussed below. Alternatively, the 43-kDa form could be the product of oocyte-specific expression of a differentially spliced transcript; however, we have not obtained any evidence for the presence of alternative transcripts in oocytes in Northern blot or PCR-based analyses.

An important difference between avian and mammalian clusterins is the lacking posttranslational proteolytic cleavage into two sulfhydryl-linked subunits of the chicken protein, a conclusion based on the following observations. First, a major 70-kDa protein is detected in immunoblots under both reducing and nonreducing conditions; the larger than predicted size (70 compared to 50 kDa) suggests extensive glycosylation of chicken clusterin, analogous to that of mammalian homologues (51–53). Second, the protein sequence around the known cleavage site is different from that in human clusterin (RIVRcleavage- SL M) but identical in quail and chicken clusterins. Third, the cleavage site is different from that in human clusterin (RIVR-70-kDa protein is detected in immunoblots under both reducing conditions). Fourth, a major clusterin-carrying lipoprotein fraction in mammals) (55) has shown that granulosa cells produce and secrete apolipoprotein B moiety of VLDL (38), VTG (38), and possibly riboflavin-binding protein (57), as well as α2-macroglobulin (39), during their delivery into yolk. It will be interesting to determine whether the same or other enzyme(s) produces the 43-kDa fragment of clusterin. Fourth, we and others will need to address the question of whether clusterin and/or the 43-kDa form is necessary for normal embryo development in a variety of species. Fifth, in the context of embryo development, another possibility is that clusterin functions as the mediating receptor-ligand in a “piggy-back” transport mechanism that leads to the uptake of essential embryonic factors. Not only has such a piggy-back mechanism been described previously in the chicken system, i.e. VTG as carrier for riboflavin-binding protein (57), but also several proteins are known to be bound by and complexed with clusterin (1, 8, 16). The identification of such partners of clusterin, in particular those produced by granulosa cells in the chicken, is a prime goal in our current research efforts.

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