

# 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 prostatic 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  $\beta$ , 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  $\beta$ -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)<sup>1</sup> family (12). Inasmuch as clusterin can also bind to the amyloid  $\beta$  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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<sup>1</sup> 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; LRP380, LDLR-related protein, 380 kDa; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; LRP, LDL receptor-related protein.

tion into 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)<sup>+</sup> RNA for first-strand cDNA synthesis and two synthetic oligonucleotides corresponding to quail clusterin cDNA (originally reported as T64 (23)): 5'-GTG GGA GGA 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 <sup>32</sup>P-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 amplified by PCR using Agt11-specific primers, subcloned into the pGEM-T (Promega) vector, and sequenced on both strands (Sequenase). The complete open reading frame of chicken clusterin was obtained by ligating two overlapping clones using their common *Sac*I 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.

**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<sup>+</sup> nylon membrane (Amersham Pharmacia Biotech) using standard methods (24). The above-described 180-base pair chicken clusterin cDNA fragment was labeled with <sup>32</sup>P using the Megaprime DNA labeling kit and used as probe. The membrane was hybridized overnight at 65 °C in a solution containing 10 mg/ml bovine serum albumin, 70 mg/ml SDS, 0.5 M sodium phosphate buffer, pH 6.8, 1 mM EDTA, pH 8.0, and the <sup>32</sup>P-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 Hybond filter was exposed to Reflection<sup>TM</sup> 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.

**In Situ Hybridization**—Tissue sections from ovarian follicles (small yellow, 5–6 mm in diameter) were prepared for *in situ* hybridization as described earlier (25, 26). The sections (8 µm) were hybridized overnight at 45 °C with prehybridization solution containing 10% dextran sulfate and ~300 pg/µl of the digoxigenin-labeled antisense or sense RNA probes. The RNA probes were prepared as follows: a 180-base pair PCR fragment (Fig. 1, nucleotides 333–512), prepared from the chicken clusterin cDNA by PCR amplification using the primers 5'-GTG GGA GGA GTG CAA GCC CTG CC-3' (sense) and 5'-TGC CGC TGC TCC CGA TCC AGC AG-3' (antisense), was subcloned into the pGEM-T vector (Promega). 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 MgCl<sub>2</sub>). The bound antibody 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 Axiovert 10 light microscope.

**Antibody Production**—Antiserum against chicken clusterin was pre-

pared against two synthetic peptides corresponding to an amino-terminal (antibody A; residues 106–120 in Fig. 1, HSGSGLVGRQLEELL) or a carboxyl-terminal (antibody B; residues 395–410 in Fig. 1, SLTVPGLDISWDDPRFM) region of the protein. The peptides were coupled to keyhole limpet hemocyanin and used for immunization of two adult female New Zealand White rabbits. For the first injection (day 0), 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 tissues, freshly obtained samples were placed in ice-cold solubilization buffer (4 ml/g of wet weight) containing 200 mM Tris-maleate, pH 6.5, 2 mM CaCl<sub>2</sub>, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 µM leupeptin, and 1% Triton X-100 and homogenized with an Ultra Turrax T25 homogenizer at medium setting for 30 s, and then at high setting for a further 30 s. The extraction mixture was kept on ice for 15 min and then centrifuged at 300,000 × *g* for 40 min at 4 °C. The resulting supernatant was stored at -20 °C in aliquots until used. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad). For preparation of Triton X-100 extracts of chicken ovarian 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 tissue was 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 according to Laemmli (29) using a minigel system (Bio-Rad, Mini-Protein<sup>TM</sup> II slab cell). 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<sub>r</sub>* 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).

**Prokaryotic 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 *Sac*I 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 pCR-Script<sup>TM</sup> SK(+) (Stratagene). The identity of the PCR-generated fragment was verified by sequencing. The obtained full-length clusterin cDNA was then directionally cloned in-frame into a pGEX-5X-1 expression vector (Amersham Pharmacia Biotech) followed by transfection of *Escherichia coli* XL1 Blue subcloning-grade competent cells (Stratagene). The GST fusion protein was induced by incubating the cells in the presence of 0.1 mM isopropylthio-β-D-galactoside and purified according to the method described by Frangioni and Neel (30).

**Combined Ligand and Western Blotting with GST-Clusterin Fusion Protein**—Nitrocellulose strips containing SDS-PAGE-separated oocyte membrane proteins were blocked for 1 h in TBS (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 2 mM CaCl<sub>2</sub>) plus 5% skim milk and 0.1% Tween (blocking buffer). Strips were then incubated for 1 h in the same buffer with or without purified GST-clusterin fusion protein at the concentrations indicated in the figure legends. The strips were washed three times for 20 min each time with TBS containing 0.1% Tween and 2 mM CaCl<sub>2</sub> (washing buffer) and then incubated for 1 h in blocking buffer containing either anti-chicken clusterin antiserum, anti-chicken LR8 antiserum, or anti-chicken LRP380 antiserum at the concentrations indicated in the figure legends. After three 20-min washes, the bound antibodies were detected with protein A-horseradish peroxidase (1:5000) using the chemiluminescence detection method.

**Immunohistochemistry**—Chicken follicles (small yellow, 5–6 mm in diameter) were fixed overnight with 4% paraformaldehyde in PBS, pH 7.4, at 4 °C. Following three successive washes with PBS, the follicles were treated with increasing concentrations of ethanol (50–100%), and then, within a series of five steps, the ethanol was replaced by 100%

|                                                                                                    |      |
|----------------------------------------------------------------------------------------------------|------|
| GGGCTCTGGG ACGGGACCAT GGCCTGCT CTGCTCGCCC TGCTCAGCCT GGGGCTCGTC TGCCAGGGGG GGCAAGGCCT CGTGCCCCC    | 90   |
| <u>M A L P L L A L L S L G L V C Q G G Q G</u> L V P P                                             | 4    |
| AGCGAGCTGA AACAGCTCTC AGCAGCCGGC AGCAAGTACA TTGACACTGA AGTGGAAGAC GCCATCAATG GGTGAAGCA GATGAAGACA  | 180  |
| S E L K Q L S A A G S K Y I D T E V E N A I N G V K Q M K T                                        | 34   |
| CTGATGGACA AGACACGCAA GGAGCACCAG GCCATGTGCT ACACGCTGGA GGAGACCAAG AGGAGGAAGG AGGAGGCAGT GAAGCTGGCC | 270  |
| L M D K T S K E H Q A M L H T L E E T K R R K E E A V K L A                                        | 64   |
| TTGGAGAAGG AGAAGCAGCT GGCAGAGAAG CAGGAGGTGT GCAACGAGAC GATGCTGTCA CTGTGGGAGG AGTGCAAGCC CTGCCTCAAA | 360  |
| L E K E K Q L A E K Q E V C <u>N E T</u> M L S L W E E C K P C L K                                 | 94   |
| CACACCTGCA TGCCTGCTA CTCGAAGATA TGCCACACGG GCTCGGGGCT GGTGGGCCGC CAGCTGGAGG AGCTCTCTAA CCGTCTCTCG  | 450  |
| H T C M R V Y S K I C H S G S G L V G R Q L E E L L N R S S                                        | 124  |
| CCCTTCTCCA TCTGGGTGAA CGGCGAGCGC ATCGATGCGC TGCTGGATCG GGAGCAGCGG CAGGAGCGGC GCTTCGAGGA CCTGGAGGAG | 540  |
| P F S I W V N G E R I D A L L D R E Q R Q E R R F E D L E E                                        | 154  |
| CGCTTTGGAC TGATGGAGGA CGGAGTGAGG GACATCTTCC AGGACAGCAC GCAGCTCTAC GGGCCTGCTT TCCCTTCTT CCGAACGCCT  | 630  |
| R F G L M E D G V E D I F Q D S T Q L Y G P A F P F F R T P                                        | 184  |
| CCCTTGGGTG GTTTCCTGTA AGCTTTCGTC CCACCCGTGC AACGTGTCCG CCTGGTGCCA CCGCGCAGGA GGCTGTCCCG AGAGCTGCAC | 720  |
| P F G G F R E A F V P P V Q R V R L V P P R R R L S R E L H                                        | 214  |
| CCCTTCTTGC AGCACCCGGT GCATGGCTTC CACCGCTCTC TCGAGATGAC GCAGCGCATG CTGGATGGGG GGCACGGCGC CTGGGACCAC | 810  |
| P F L Q H P V H G F H R L F E M T Q R M L D G G H G A W D H                                        | 244  |
| CTGCTGGGGG GCTTTGAGTC AGAATCCCTG AACTTCAGCA CCGATCGCAT GGTGTGCGCT GAGATCCGGC GCAACTCGGC CGGCTGCCTG | 900  |
| L L G G F E S E S R <u>N F S T</u> D R M V C R E I R R N S A G C L                                 | 274  |
| CGGATCGGGG ATGAGTGCGA GAAGTGCCTG GAGATCTTGG CTGTGGACTG CTGCAGACA GACCCAGTGC AGAGCCAGCT GAGGGAGCAG  | 990  |
| R M R D E C E K C R E I L A V D C S Q T D P V Q S Q L R E Q                                        | 304  |
| TTTGAGGATG CCCTGCGCCT GGCCGAGCGC TTCACCCGCC GCTACGATGA CCTCCTCAGC GCCTTCCAGG CCGAGATGCT CAACACCAGC | 1080 |
| F E D A L R L A E R F T R R Y D D L L S A F Q A E M L <u>N T S</u>                                 | 334  |
| AGCCTCCTGG ATCAGCTCAA CCGTCAGTTC GGTGTGGTCT TACGCCTGGG CAACCTCAGC CAGGGCACCG ATGGGTTCCT GCAGGTCAAC | 1170 |
| S L L D Q L N R Q F G W V L R L G <u>N L T</u> Q G T D G F L Q V T                                 | 364  |
| ACGGTGTCT CCAAAATCC CAACCTCAG GACCCGTGAG CCCCAGCGCA CACGCAAGTG ACGGTGCAGC TCTTTGACTG GGAGCCCTCG    | 1260 |
| T V F S K T P N L E D P S A P A D T Q V T V Q L F D S E P L                                        | 394  |
| TCGCTCACC TGCTGGGGA CATCTCTGTG GACGATCCAC GCTTCATGGA GATCGTGGCC GAGCAGGCGC TGCAGCACTA CAAGCAGAAC   | 1350 |
| S L T V P G D I S W D D P R F M E I V A E Q A L Q H Y K Q <u>N</u>                                 | 424  |
| AACACCATAG AGTAGCAGTC CCTCTCTGT CCCCACGTCC CACATCAGTG GGTCAAACGC CGTACCACG TCCCGTGCT GGGCCCCCCC    | 1440 |
| <u>N T I E</u> ***                                                                                 | 428  |
| TTCCCCAGGG CCCGAAGCAG <u>GCAATAAGG</u> TAGAGTGAAC CCCC                                             | 1484 |

FIG. 1. Nucleotide and deduced amino acid sequence of the cDNA for chicken clusterin. The amino acid sequence of chicken clusterin is presented in single-letter code beneath the cDNA sequence. Numbering of the amino acids refers to the mature protein, generated by removal of the putative 20-residue signal peptide (boxed). Ten conserved cysteines are indicated by black squares. Six potential N-linked glycosylation sites are underlined, where a thin underline indicates conservation in human clusterin and a thick underline indicates absence in human clusterin. Three asterisks represent the termination codon, and a polyadenylation signal is indicated by a dashed underline.

xylo. The xylo 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 xylo two times for 10 min each, followed by treatment with decreasing concentrations of ethanol and a final 5-min incubation in 3% H<sub>2</sub>O<sub>2</sub>. 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 23 °C. Following extensive washing in PBS, the sections were incubated in 0.1 M sodium acetate buffer, pH 5.2, containing 0.03% H<sub>2</sub>O<sub>2</sub> 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 Axiovert 10 light microscope.

## RESULTS

A full-length chicken clusterin cDNA was isolated from a brain λgt11 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  $M_r$  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

FIG. 2. **Comparison of the clusterin protein sequences of chicken, quail, and human.** Identical residues in all three species are boxed. Numbering starts with the respective initiator methionines, and gaps introduced to optimize the alignment are indicated by dashes. Black squares mark the 10 conserved cysteines. Four potential heparin binding sites in the human sequence (7) are underscored by 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  $\alpha$  and  $\beta$  subunits in human clusterin (7).

|         |             |            |             |             |             |     |
|---------|-------------|------------|-------------|-------------|-------------|-----|
| Chicken | M-ALEHLLALL | SLGLVC-QG- | QGLVPPSEL   | KQLSAAGSKY  | IDTEVENATIN | 47  |
| Quail   | M-ELLELLALL | SLGLVC-QG- | QGLVPPNEL   | KQLSAAGSKY  | IDAEVENATIN | 47  |
| Human   | MKTLLLVFVGL | LLTWESGVVL | GDQTVSDNEL  | QEMSNQGSKY  | VNKEICQNAVN | 50  |
| Chicken | GVKQMKTLMD  | KTSKEHQAML | HTLEETKRRK  | EEAVKLALAK  | EKCLAEKQEV  | 97  |
| Quail   | GVKQMKTLMD  | KTSKEHQAML | HTLEETKRRK  | EEAVKLALAK  | EKCLAEKQEV  | 97  |
| Human   | GVKQMKTLTE  | KTNEERKTLT | SNLEBAKKKK  | EEALNETRES  | ETKLEKELPGV | 100 |
| Chicken | CNETMLSLWE  | ECKPCLKHTC | MRVYSKICH   | SGSLVGRQLE  | EELNRSPPFS  | 147 |
| Quail   | CNETMLSLWE  | ECKPCLKHTC | MRVYSKICH   | SGSLVGRQLE  | EELNRSPPFS  | 147 |
| Human   | CNETMMLLWE  | ECKPCLKHTC | MKFYARVCH   | SGSLVGRQLE  | EELNRSPPFY  | 150 |
| Chicken | IMWNGERIDA  | LLDREQQRER | RFEDLEERFG  | LMEDGVEDIF  | QDSTQLYGPA  | 197 |
| Quail   | IMWNGERIDA  | LLDREQQRER | RFEDLEERFG  | LMEDGVEDIF  | QDSTQLYGPA  | 197 |
| Human   | FMWNGRIDDS  | LLDENDRQTH | MLDVMQDFH   | RASSIIDELF  | QDR-----    | 193 |
| Chicken | FFFRIRIPFPG | GFREAFVPPV | QRVRLMPHRR  | -----RL     | SRFL-----   | 233 |
| Quail   | FFFRIRIPFPG | GFREAFVPPV | QRVRLMPHRR  | -----L      | SRFL-----   | 232 |
| Human   | F-FTIRFPQ-D | TYH--YIP-- | --FSL--PHRR | PHFFFPKSRI  | VRSLMPFSPY  | 234 |
| Chicken | -----H      | PFLQHPVHG  | HR-----LFEM | TQRLMDGGHG  | AWHLLGGFE   | 270 |
| Quail   | -----H      | PFFQHPMHG  | HRLFQPLFEM  | TQHMLDGGHG  | AWHPLGGFA   | 273 |
| Human   | EPLNFHAMFQ  | PFLE-MIHEA | QQ-----AM   | DIH-FHS--P  | AFCHPPTFTI  | 274 |
| Chicken | SESRNFSIDR  | MVCREIRFNS | AGCLRMDHC   | EKCREILAVD  | CSQTLPMQSQ  | 320 |
| Quail   | TESRNFSIDR  | MVCREIRFNS | AGCLRMDHC   | EKCREILAVD  | CSQTLPMQSQ  | 323 |
| Human   | REGDD---DR  | TVCREIRFNS | TGCLRMDHC   | EKCREILAVD  | CSTNNPSQAK  | 321 |
| Chicken | LREQFEDALR  | LAERFTRRYD | LLLSAFQAEH  | LNTSSLLQL   | NFOFGWILRI  | 370 |
| Quail   | LREQFEDALR  | LAERFTRRYD | LLLSAFQAEH  | LNTSSLLQL   | NFOFGWISRI  | 373 |
| Human   | LRRELDSELO  | VAERLTRRYN | ELLSKYQWEM  | LNTSSLLQL   | NFOFGWISRI  | 371 |
| Chicken | GNLTQGDGFG  | L-QVTTVFSK | TPNLEIPSPAP | ACTQVITVCLF | DSEPLSITVP  | 419 |
| Quail   | GNLTQGDGFG  | L-QVTTVFSK | TPNLEIPSPAP | ACTQVITVCLF | DSEPLSITVP  | 422 |
| Human   | ANLTQGEDQY  | YLRVTTVASH | TSDSDVPSGV  | --TEVWVCLF  | DSEPLSITVP  | 419 |
| Chicken | GDLSWDLPRF  | MEIVAHQALQ | HYKQNTTIE   |             |             | 448 |
| Quail   | GDLSWDLPRF  | MEIVAHQALQ | HYKQNTTIE   |             |             | 451 |
| Human   | VEVSRKNPKF  | MEIVAHKALQ | EYRKKHREE   |             |             | 448 |

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*). However, a potential nuclear localization signal (Fig. 2, *dark hatched box*), present in human clusterin (LEEAKKKK) and proposed to target a truncated form of the protein to the nucleus (11), is also present in the chicken protein (LEET-KRRK), as well as several potential in-frame translation initiation sites (corresponding to codons 52, 56, and 66 in the avian precursor protein; Fig. 2).

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-

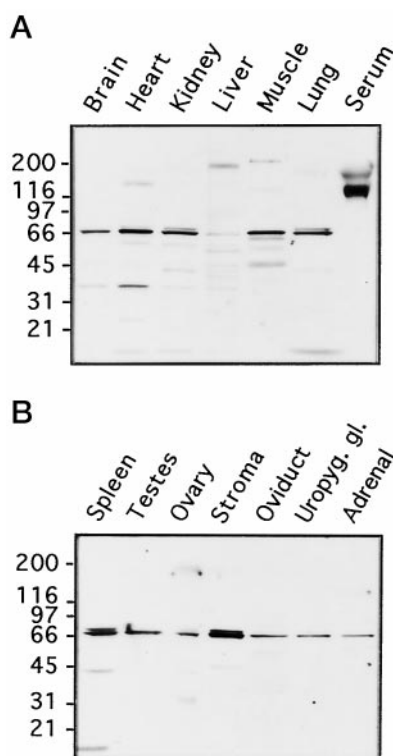


FIG. 3. Western blot analysis of clusterin in different chicken tissues. Crude Triton X-100 extracts (30  $\mu$ g protein/lane) prepared from the indicated chicken tissues (uropyg. gl., uropygial gland), as well as 1.5  $\mu$ l of serum, were subjected to 4–18% SDS-PAGE under non-reducing conditions and processed for immunoblotting with rabbit serum (1:250) raised against a synthetic chicken clusterin peptide (antibody A) (see under “Experimental Procedures”). Numbers on the left correspond to the positions of migration of marker proteins (in kDa).

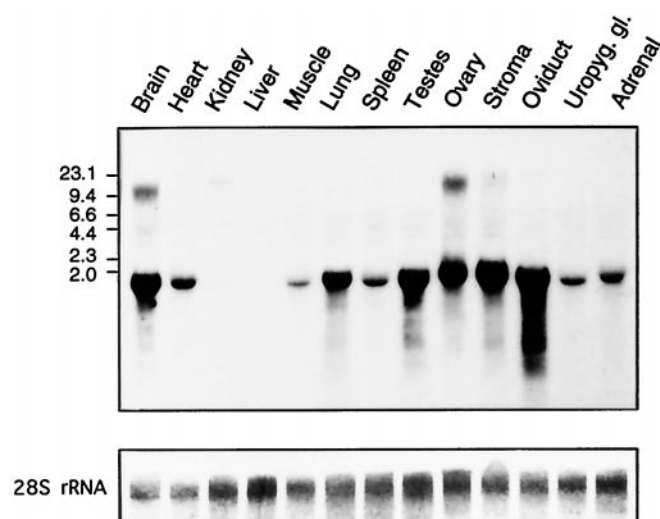


FIG. 4. Northern blot analysis of clusterin transcripts in chicken tissues. Total RNA (25  $\mu$ g/lane) was isolated from the indicated chicken tissues (uropyg. gl., uropygial gland), denatured, and separated by electrophoresis on a 1.2% agarose gel. Hybridization was performed with a 180-nt chicken clusterin fragment (nucleotides 333–512 in Fig. 1).  $\lambda$  DNA digested with *Hind*III was used as a size marker (in kilobase pairs). The blot was standardized by methylene blue staining of 28S rRNA (bottom panel).

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.

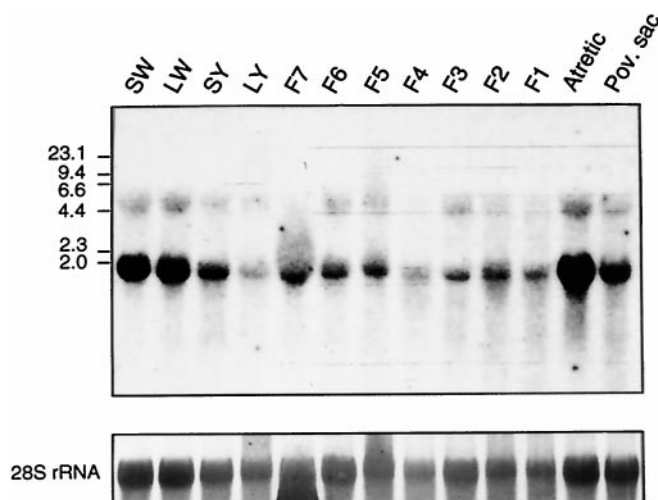


FIG. 5. Northern blot analysis of clusterin expression during chicken follicle development. Total RNA (25  $\mu$ g/lane) isolated from follicles of different sizes was denatured and separated by electrophoresis on a 1.2% agarose gel. SW, small white; LW, large white; SY, small yellow; LY, large yellow; F7–F1, growing follicles (7 (smallest) to 1 (preovulatory); for diameters, see text); Atretic, pool of three atretic follicles of 4–5 mm diameter; Pov. sac, postovulatory sac from a recently ovulated follicle. Hybridization was performed with a 180-nt chicken clusterin fragment (nucleotides 333–512 in Fig. 1).  $\lambda$  DNA digested with *Hind*III was used as a size marker (in kilobase pairs). The blot was standardized by methylene blue staining of 28S rRNA (bottom panel).

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

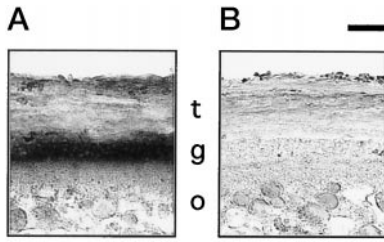


FIG. 6. **Localization of clusterin transcripts in chicken follicles.** Cryostat sections (8  $\mu$ m) of a phase II follicle (small yellow, 5 mm diameter) were subjected to *in situ* hybridization with digoxigenin-labeled clusterin RNA probes as described under "Experimental Procedures" (A, antisense; B, sense). g, granulosa cell layer; t, thecal cell layers; o, oocyte proper. Bar represents 50  $\mu$ m; magnification is  $\times 200$ .

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  $\sim 43$  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  $\alpha_2$ -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 chicken 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  $M_r$  of  $\sim 380,000$  (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 antibodies (Fig. 8). In addition to endogenous 70-kDa clusterin and the 43-kDa fragment (cf. Fig. 7), three clusterin-binding proteins of 95, 180, and 380 kDa were visualized (Fig. 8, lane 3). Binding of clusterin to the 95- and 380-kDa bands, but not to the 180-kDa band, was inhibited by EDTA (lane 4), and all bands were abolished when the membrane extract had been subjected to electrophoresis under reducing conditions (lane 5). The nature of the 180-kDa band is unknown. However, the 95- and 380-kDa proteins were identified with specific antibodies (17) as LR8 and LRP380, respectively (lane 6). Thus, these two LDLR

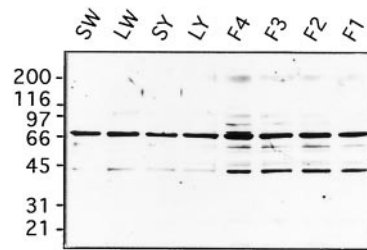


FIG. 7. **Immunoblot analysis of chicken clusterin in different stages of follicle development.** Triton X-100 extracts (30  $\mu$ g protein/lane) prepared from follicles of various sizes (for designations, see Fig. 5) were subjected to SDS-PAGE under nonreducing conditions and blotted onto a nitrocellulose filter. The blot was processed for immunoblotting with rabbit antiserum (1:250) raised against a synthetic chicken clusterin peptide (antibody A; see under "Experimental Procedures").

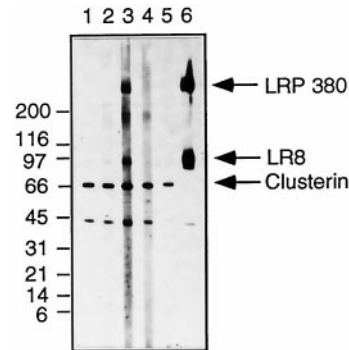


FIG. 8. **Identification of clusterin receptors by ligand blotting.** Nitrocellulose strips containing SDS-PAGE-separated oocyte membrane proteins ( $\sim 25$   $\mu$ g/lane) were incubated in the presence (lane 2, 10  $\mu$ g/ml; lanes 3–5, 40  $\mu$ g/ml) or absence (lanes 1 and 6) of chicken GST-clusterin fusion protein. In lanes 1–4 and 6, SDS-PAGE was performed in the absence, and in lane 5, in the presence of 10 mM dithiothreitol. The incubation mixture in lane 4 contained 50 mM EDTA. Lanes 1–5 were subsequently incubated with 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 follicle.

Finally, in order to obtain further evidence for the possibility that LR8 and LRP380 mediate clusterin uptake from the extraoocytic 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 intraoocytic (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.<sup>2</sup> This finding, if confirmed, is in

<sup>2</sup> L. E. French, personal communication.

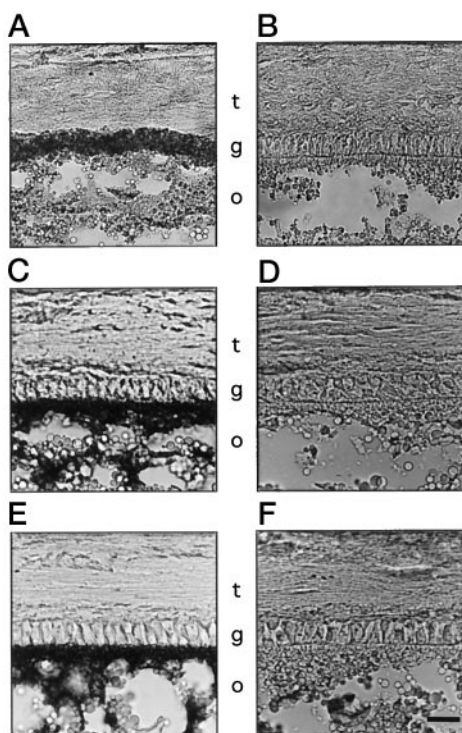


**FIG. 9. Immunohistochemical localization of clusterin, LR8, and LRP380 in chicken follicles.** Microtome sections (6  $\mu$ m) of paraffin-embedded follicles (small yellow, 5–6 mm) were subjected to immunohistochemistry as described under "Experimental Procedures." The antisera and their dilutions were as follows: A, anti-clusterin antibody B (1:400); C, anti-LR8 (1:500) (28); E, anti-LRP380 (1:500) (28). The sections in panels B, D, and F were incubated with the respective preimmune sera. Thecal cell layers (*t*), the granulosa cell monolayer (*g*), and the oocyte proper (*o*) are indicated for each pair of panels. Bar represents 20  $\mu$ m.

support of the suggested role of clusterin in lipid metabolism, but unfortunately it does not provide functional insight. In a situation where a protein is in search of its function(s), new knowledge gained from a different experimental system can often provide important clues. Thus, the facts that (i) clusterin is expressed in the genital tract of mammals and (ii) a member of the LDLR gene family, megalin, binds clusterin (12), combined with our ongoing studies on gonadal development and oocyte growth, lipid metabolism, and the LDLR gene family in the chicken, prompted us to embark on studies of the biology of clusterin in the bird.

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  $\mu$ m 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-x* long 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 (RIVR-cleavage-SLM) but identical in quail and chicken clusterin (RLSRELH). Whereas both sequences appear to conform to cleavage site requirements for furin (RXXR) (54), neither the identity nor the subcellular localization of the protease(s) that processes human clusterin has been demonstrated. In fact, these findings suggest evolutionary changes from the production of a single-chain protein in chicken to that of a heterodimeric, constitutively secreted form in mammals. Data on human clusterin imply that the heterodimeric form of clusterin is predominantly secreted via the classical secretory pathway (53). On the other hand, the absence of immunoreactive material in chicken serum points to a lack of secretion of the single-chain chicken clusterin, at least into systemic fluids. The presence of a truncated form of clusterin in the oocyte, which expresses clusterin receptors, may indicate that granulosa cells, which have known high secretory capacity (55, 56), are able to secrete clusterin by a specialized and/or inducible mechanism. Studies on the biosynthetic pathway of clusterin in granulosa cells are under way in the laboratory; to date, these have shown that granulosa cells produce and secrete apolipoprotein AI (a component of high density lipoprotein, the major clusterin-carrying lipoprotein fraction in mammals) (55) and are the source for at least one of the proteins involved in sperm binding, termed zona pellucida 3 (56).

One of the most significant findings of the current investigation is the identification of two novel receptors for clusterin, *i.e.* the oocyte-specific members of the LDL receptor gene family, LR8 and LRP380 (Figs. 8 and 9). Up to now, the only receptor known to interact with clusterin is mammalian megalin, a large member of the LDLR gene family (12). LR8, a well characterized receptor present on the chicken oocyte plasma membrane (17, 19, 22) has been known thus far to bind and internalize VLDL, VTG,  $\alpha_2$ -macroglobulin, and riboflavin-binding protein/VTG complexes (57), *i.e.* molecules essential for the normal growth and development of the chicken embryo. In that it is the smallest LDLR family member with a ligand spectrum resembling that of mammalian LRP, its exquisite multifunctionality is underscored by the present identification of an additional ligand, clusterin. LRP380, also present on the plasma membrane of the chicken oocyte (Fig. 9), has been

demonstrated so far only to bind VTG and  $\alpha_2$ -macroglobulin (28). Although the complete structure of LRP380 remains to be determined, our observation of clusterin binding to this receptor suggests that it has key characteristics resembling those of mammalian megalin, rather than those of LRP.

The interaction of clusterin with the two oocyte-specific receptors and the accumulation of a truncated form of clusterin is of interest for several reasons and gives rise to several speculations. First, it provides further evidence for the previously suggested synergy of LR8 and LRP380 in mediating oocyte development (28). Second, the apparent absence of clusterin transcripts in the oocyte but accumulation of the immunoreactive 43-kDa protein may indicate that a receptor-mediated endocytotic transport mechanism(s) becomes active during the vitellogenic phase of oocyte growth. Third, if the 43-kDa form indeed arises from postendocytic cleavage, the responsible protease(s) needs to be identified. Previously, we have observed that cathepsin D single-handedly catalyzes the cleavage of the apolipoprotein B moiety of VLDL (38), VTG (38), and possibly riboflavin-binding protein (57), as well as  $\alpha_2$ -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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