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Research Article

Receptor-mediated transport and deposition of complement component C3 into developing chicken oocytes

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Abstract. Immunological resistance of the chick embryo is dependent upon IgG present in the yolk of the layed egg. Here we show that complement factor 3 (C3), a key component of the humoral complement system, is a yolk component of chicken eggs. C3 is transported into oocytes by LR8-mediated endocytosis. LR8 also binds and transports other major yolk components such as vitellogenin, very-low-density lipoprotein, and α_2-macroglobulin. Expression studies of LR8 during chicken development and oocyte maturation, in combination with studies on the uptake of individual yolk components, suggest the following model for oocyte maturation in the chicken: all oocytes present in the ovary contain high levels of LR8 mRNA and protein long before the onset of oocyte maturation. Selected oocytes gain access to yolk precursors, and LR8 binds, internalizes, and deposits the major yolk components in the ratio of their relative abundance in the accessible pool.

Key words. Oocyte development; VLDL receptor; receptor-mediated endocytosis; complement C3.

In egg-laying species, the developing embryo depends on the egg yolk for all nutrients, vitamins, and other essential components. In the chicken, major components of the yolk mass are synthesized in the liver of the sexually mature hen, transported to the ovary and taken up by maturing oocytes by receptor-mediated endocytosis [1]. During the final 7 days of oocyte development, this cell internalizes about 5 g of lipid and protein, reaching a diameter of approximately 35 mm until it is expelled from the follicle by ovulation. The major yolk precursors are very-low-density lipoprotein (VLDL) and vitellogenin (VTG). Both macromolecules bind to LR8 [2], the chicken homologue of the mammalian VLDL receptor (VLDLR) [3] and are taken up by the oocyte via receptor-mediated endocytosis. LR8 is a close relative of the low-density lipoprotein (LDL) receptor, distinguished by the presence of an additional 8th ligand-binding repeat in its extracellular domain. The chicken LDL receptor [4] has a narrow ligand specificity and binds only apolipoprotein B (apoB); the VLDL receptor, on the other hand, has a broad spectrum of unrelated ligands such as apoE, apoB, VTG, lipoprotein lipase, urokinase-type plasminogen activator-type-1/plasminogen activator inhibitor complexes, α_2-macroglobulin, and reelin [for reviews see refs 5, 6]. The VLDLR is very similar to LRP which is a very complex member of the LDL receptor gene family and also binds a variety of diverse ligands [7]. The mammalian VLDLR not only has a broad ligand spectrum, but also plays different roles in different pathways. For example, in the Reelin pathway, the VLDLR binds reelin and mediates signaling events which orchestrate correct neuronal migration during embryonic brain development [for a review see ref. 5]. LR8 however, the avian homologue of VLDLR, functions as an endocytic receptor [8]. LR8 not only binds and transports VLDL and VTG, but also minor yolk components such as riboflavin-binding protein [9] and α_2-macroglobulin [10].

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An important function of the yolk is to provide the embryo with maternally derived immune defense components. IgG is taken up by the growing oocyte from the maternal circulation [11]; the responsible receptor, however, remains elusive. Immunological resistance of the chick embryo depends upon IgG in the yolk, which eventually becomes transferred from the yolk to the fetal circulation by IgG receptors on the embryonic yolk sac surface [12]. This fact is now widely exploited to produce polyclonal antibodies in the laying hen by recovering them from layed eggs from immunized chickens [13]. However, chickens also have a humoral complement system with complement component 3 (C3) as its key component. Chicken C3 is highly homologous to C3 proteins from other vertebrate species and belongs to the α_2-macroglobulin family [14]. The mature two-chain protein consists of a 989-residue α chain (118 kDa) and a 640-residue β chain (68 kDa) [15]. Upon complement activation, C3a, a 15-kDa fragment, and C3d/C3dg, a 34-kDa fragment, are released.

Here we show that the yolk of chicken oocytes contains C3, which is sequestered from the circulation via receptor-mediated endocytosis. The receptor mediating this process is LR8, further demonstrating the simplicity of the mechanism underlying oocyte maturation. Indeed, a single receptor not only transports the major yolk components VLDL and VTG, but also vitamins, protease inhibitors and, as shown here, part of the humoral immune system into oocytes. Uptake of C3 as well as of other yolk components appears not to be regulated at the receptor level, but most likely by processes which regulate access of yolk components to the oocyte.

Materials and methods

Purification of chicken C3 and other ligands for LR8

Chicken C3 was purified by a modification of the method described by Alsenz et al. [16]. In short, chicken plasma was precipitated with 4% PEG 4000. The supernatant was precipitated with 16% PEG 4000, the resulting pellet was dissolved in buffer (5 mM Na_2HPO_4, pH 7.6), and applied to an HR 16/10 DEAE column (Pharmacia). C3 was eluted with a NaCl gradient (0–400 mM), and identified by Western blotting using an anti-C3 antibody (see below). The fractions containing C3 were pooled and adjusted to 20 mM Na_2HPO_4, 50 mM NaCl, pH 5.6. The sample was applied to a ResourceS column (Pharmacia) and C3 was eluted with an NaCl gradient (50–400 mM) and identified by Western blotting. The purity of the protein was tested by SDS-PAGE and Coomassie Blue staining and Western blotting using antibodies against apoB100, VTG, and α_2-macroglobulin. Myc-tagged receptor-associated protein (RAP-myc) [17] and VTG [18] were prepared as described in the indicated references.

Zymosan activation of chicken C3

To generate the C3a fragment chicken serum was incubated with zymosan (5 mg/ml) for 60 min at 37 °C [14]. The fragment was eluted from the zymosan by boiling the zymosan particles for 5 min in reducing sample buffer and directly loaded for reducing SDS PAGE. The fragment was analyzed by Western blotting using an antibody against the chicken C3a fragment (see below).

Production of antibodies

A cDNA fragment encoding amino acid residues 1–76 of the chicken C3 α chain [14] (corresponding to C3a) was generated by PCR and cloned into pGEX-5x-1 (Amersham). Isolation of the corresponding fusion protein containing C3a and glutathione S-transferase was carried out as described earlier [19]. The purified fusion protein was used as antigen for the production of a polyclonal antibody against C3a as described [19].

A 21-amino-acid-peptide $C3^{741–761}$ (SEVDDAFLSDEDITSRSLFPE) representing the amino terminus of the chicken C3 α chain after liberation of C3a [14] was synthesized and used for the production of a polyclonal antibody as described elsewhere [20].

Western blotting and ligand blotting

SDS-PAGE was performed according to the method of Laemmli [21], and proteins were transferred onto nitrocellulose membranes by semidry blotting. For Western blotting, nitrocellulose membranes were blocked for 1 h in PBS, 0.1% Tween containing 5% milk. HRP-conjugated goat-anti-rabbit IgG was used as secondary antibody (1:20,000; Jackson Immuno Research) for detection with enhanced chemiluminiscence (Pierce).

For ligand blots, membranes were blocked for 1 h in TBS, 0.1% Tween, 2 mM $CaCl_2$ containing 2% BSA. Bound ligands were detected using the appropriate antibodies as described for Western blotting.

Cell culture

Human embryonic kidney cells (HEK 293) were maintained in Dulbecco's modified Eagle's medium/F-12 Nutrient Mixture (D-MEM/F-12; Gibco) supplemented with 7.5% fetal calf serum and 584 mg/l glutamine and transiently or stably transfected with a cDNA coding for full-length LR8 [2] or empty vector (pCIneo; Promega). Selection of stable clones was done by the addition of G418 (1 mg/ml).

Internalization of C3 into 293 cells expressing LR8

293 cells expressing LR8 were grown in culture slides (Becton Dickinson) to a confluency of 50–70%. After incubation with purified C3 (20 µg/ml) in the absence or presence of RAP (100 µg/ml) in serum-free medium for 30 min, the cells were fixed and permeabilized with methanol/acetone (1/1 by vol.) at –20 °C and incubated

with the relevant primary antibody followed by a secondary fluorescently labeled antibody (Alexa Fluor 488 goat anti-rabbit IgG; Molecular Probes). After a final wash, microscopy was performed on a Zeiss microscope (Axiovert 135) at ×100 magnification.

Preparation of coated vesicles

Coated vesicles were prepared from the yolk of small vitellogenic follicles (5–6 mm) using a $^1H_2O/^2H_2O$, 8% sucrose gradient as described elsewhere [10]. The protein content of the coated vesicle preparation was analyzed by Western blotting using antibodies against LR8, clathrin heavy chain, and chicken C3.

cDNA constructs and production of recombinant proteins

The expression plasmid for LR8-1-8-MBP/His (ligand-binding repeats 1–8 fused to maltose-binding protein) was constructed using a PCR fragment coding for the entire ligand-binding domain of LR8. The cDNA fragment was amplified using the following primer pair: LR8-8R-forw: 5′-TAG AAT TCG ACG GTG CAA AAG C-3′ and LR8-8R-rev2: 5′-TAG AAT TCA CAT TCC TTG AGA GGC-3′. The PCR fragment was cleaved with *Eco*RI and cloned into a modified pMAL-c2X providing a His-tag to the carboxy terminus of the protein [22]. The construct was expressed in *Escherichi coli* TOP 10 F′ cells. Cells were grown until they reached mid-log phase, and 0.5 mM isopropyl-1-thio-*β*-galactopyronoside was added to induce expression for 5 h at 37 °C. The cells were harvested, resuspended in TBS-C (TBS pH 7.4, 2 mM $CaCl_2$) with protease inhibitors (Complete) and lysed by sonication. After centrifugation, the supernatant was applied to an Ni-NTA-Sepharose column (Qiagen) at 4 °C. After washing, the column was eluted with 250 mM imidazole in TBS-C. To force the correct folding, the eluted fusion protein was dialyzed against a refolding buffer (10 mM $CaCl_2$, 3 mM reduced glutathione, 0.3 mM oxidized glutathione in TBS). Correct folding of the protein was tested by an ELISA measuring its ability to bind *Rap*-myc [23]. As a control for the solid-phase binding assay with LR8-1-8-MBP/His described below we used the equivalent fusion protein containing the ligand-binding domain of the murine VLDL receptor (mVLDLR1-8-MBP/His) [17].

Solid-phase binding assay

TBS-C (2 mM $CaCl_2$, 100 µl) containing 10 µg/ml LR8-1-8-MBP/His or mVLDLR1-8-MBP/His was incubated on a 96-well plate overnight at 4 °C. All further incubations were carried out at room temperature for 1 h, and the ligands (C3, RAP-myc) and antibodies were diluted in blocking solution (2% BSA in TBS, 2 mM $CaCl_2$, 0.05% Tween). After blocking and binding of C3 or RAP at the indicated concentrations, the appropriate antibodies (anti-C3 or anti-myc) followed by HRP-conjugated secondary antibody were used for detection of bound ligands. For the color reaction, 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine was used in 0.1 M sodium acetate, pH 6.0, containing 10 mM H_2O_2. The reaction was stopped after 5 min by addition of 0.3 M H_2SO_4, and photometrically quantified at 450 nm. Binding isotherms were analyzed and K_d values were calculated with SigmaPlot 8.0.

Preparation of extracts from oocyte membranes and 293 cells

Oocyte membrane fractions were prepared, and the membrane pellets were extracted with 1% Triton X-100 as previously described [24]. Cellular extracts (293 cells) were prepared by lysing the cells in 200 mM Tris-maleate, pH 6.5, 2 mM $CaCl_2$, 1.4% Triton X-100, and protease inhibitors (Complete) for 30 min on ice and centrifugation to remove insoluble material.

Immunofluorescence

Follicles of different sizes (3–10 mm) from an adult hen were dissected in ice-cold PBS, embedded in freezing agent (Tissue-Tec O.C.T Compound; Sakura) and immediately frozen. Cryostat sections of 16 µm thickness were prepared and transferred to Superfrost-Plus slides (Menzel). After fixing the sections with acetone, slides were washed with PBS, blocked with PBS containing 3% goat serum and incubated with the respective antibodies (anti-LR8, anti-apoB, anti-vitellogenin, anti-C3). Slides were washed and incubated with Alexa Fluor 488 goat anti-rabbit IgG. After a final wash, microscopy was performed on a Zeiss microscope (Axiovert 135).

Northern blotting

Total RNA was prepared from different immature and mature ovaries using Tri Reagent (Molecular Research Center). RNAs (20 µg) were separated by electrophoresis on a 1.5% agarose gel and transferred onto a nylon membrane. After UV crosslinking, hybridization was performed using standard methods [25]. To detect LR8 transcripts, a cDNA fragment was amplified using the following primer pair: LR8-VF: 5′-ACC CTA GTA AAC AAC CTC AAT GAT G-3′ and LR8-VR: 5′-AGG AAG AAC AGC CCA AGC TCC TGC T-3′ (nucleotide positions 2098–2382; [2]).

Results and discussion

In our quest to characterize novel yolk components and to study their uptake by growing oocytes, we concentrated on C3 for the following reasons. Chickens, like other vertebrates, have a complement system as part of their immune defense. Since maternal IgG is transported into the egg yolk, the same might apply to components of the complement system. Furthermore, C3 belongs to the α_2-

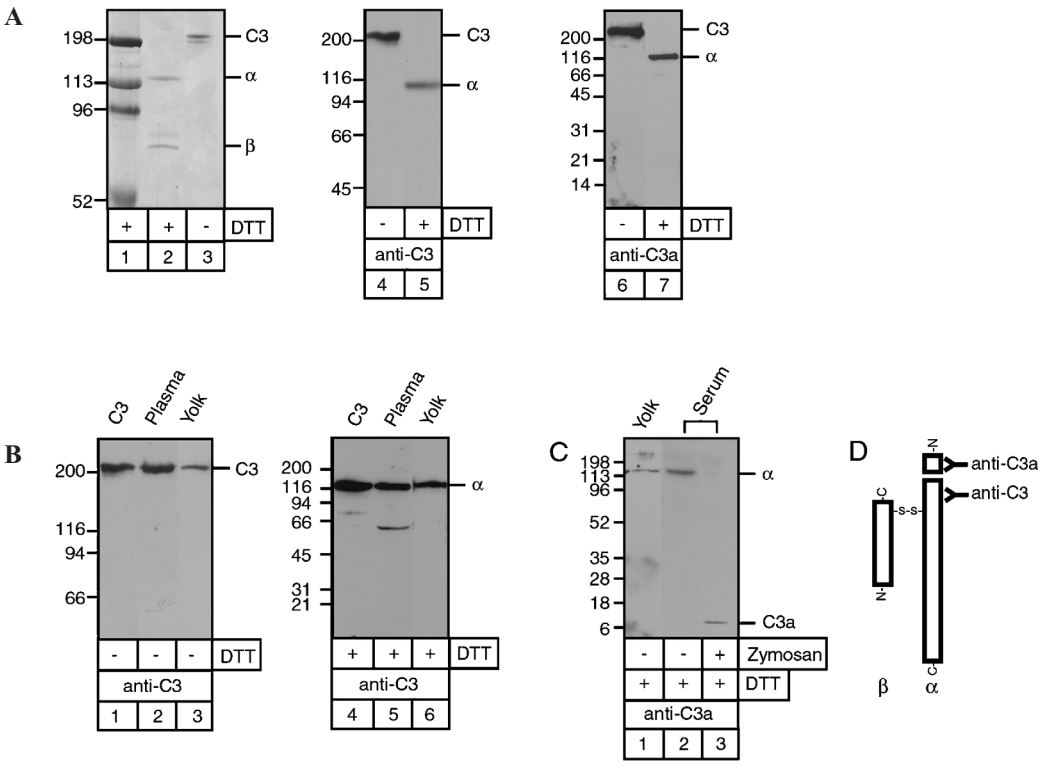

Figure 1. C3 is a component of the yolk of chicken oocytes. (*A*) Purified C3 (0.5 µg)was separated by SDS-PAGE under reducing (lane 2) or non-reducing (lane 3) conditions and stained with Coomassie blue. Purified C3 (0.1 µg) was separated by SDS-PAGE under non-reducing (lanes 4, 6) or reducing (lanes 5, 7) conditions. C3 was detected by Western blotting using anti-C3 antiserum (lanes 4, 5) or anti-C3a antiserum (lanes 6, 7). (*B*) Purified C3 (0.1 µg), 1 µl chicken plasma, or 1 µl yolk were separated by SDS-PAGE under non-reducing (lanes 1–3) or reducing (lanes 4–6) conditions. C3 was detected by Western blotting using anti-C3 antiserum. (*C*) yolk (0.5 µl), 0.5 µl chicken serum, or 0.5 µl chicken serum treated with zymosan were separated by SDS-PAGE under reducing conditions. C3 and C3a were detected by Western blotting using anti-C3a antiserum. All Western blots were incubated with HRP-goat-anti-rabbit IgG (1:10,000) and a chemiluminescence system. (*D*) Cartoon representing the structure of mature chicken C3 and indicating the positions of anti-C3 and anti-C3a epitopes.

macroglobulin family, and we have previously shown that α_2-macroglobulin is a component of chicken yolk and is sequestered from the circulation by LR8. Finally, LRP, which also belongs to the LDL receptor family binds activated C3 and clears it from the mammalian circulation [26].

First we purified C3 from chicken plasma and produced different antibodies against chicken C3 to test for the presence of C3 in egg yolk. For the purification we used a method modified from Alsenz et al. [16] which yields pure chicken C3, as tested by SDS-PAGE and Coomassie Blue staining (fig. 1A). In the gel system used, non-reduced C3 migrates with an Mr of slightly larger than 200 kDa (lane 3). Upon reduction, the protein separates into the 110 kDa α subunit and the 60-kDa β subunit (lane 2). To test whether the weak band detectable slightly below the C3 band under non-reducing conditions (lane 3) and the band detectable at about 70 kDa under reducing conditions (lane 2) represent any major yolk precursors like apoB, VTG, and $\alpha_2$macroglobulin, Western blots using appropriate antibodies were performed. These experiments revealed that the C3 preparation did not contain any detectable amounts of these proteins (data not shown). Thus, we considered that these minor impurities will not interfere with the uptake experiments (see below). Two antibodies against chicken C3 were prepared (see fig. 1D): one against a recombinant fragment of chicken C3 corresponding to residues 1–76 of the α subunit, which upon complement activation is liberated as a C3a fragment (this antibody was termed anti-C3a), and another directed against a synthetic peptide (21 amino acids) corresponding to the amino terminus of the α subunit lacking the C3a fragment (this antibody was termed anti-C3). To test the antibodies, we performed Western blotting with purified C3. As demonstrated in figure 1A, anti-C3a and anti-C3 react with the 200 kDa holoprotein (lanes 4, 6) and with the α subunit when the protein is reduced but not activated (lanes 5, 7). In this case, the C3a fragment is still part of the α subunit. Upon complement activation, C3a is cleaved off and anti-C3a no longer reacts with the holoprotein and the rest of the α subunit (see below). Next, we tested for the presence of C3 in yolk of laid eggs and used purified C3 and total chicken plasma as control (fig. 1B). Indeed, C3 is a component of egg yolk (lanes 3, 6). Anti-C3 reacts

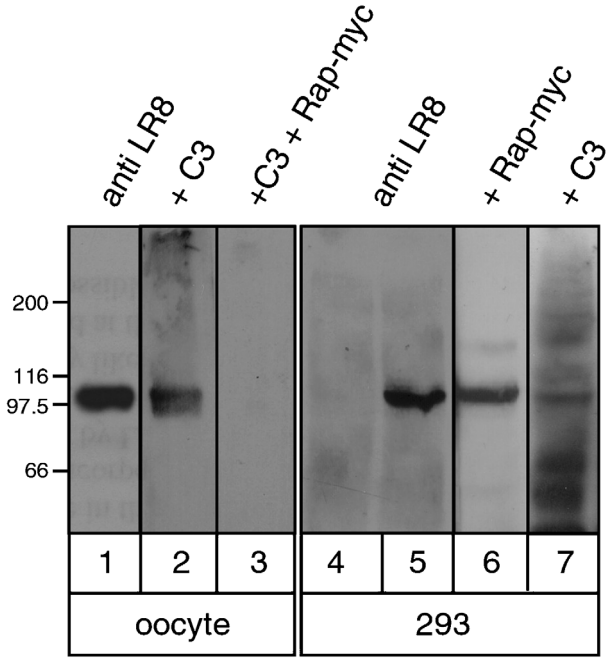

Figure 2. The oocyte receptor for C3 is LR8**.** Triton X-100 membrane extracts from chicken follicles (lanes 1–3), 293 cells expressing LR8 (lanes 5–7), or 293 cells transfected with the empty vector (lane 4) were prepared and electrophoretically separated by SDS-PAGE under non-reducing conditions. Western blotting (lanes 1, 5) was performed using an LR8-specific antiserum followed by HRP-goat-anti-rabbit IgG (1:10,000) and a chemiluminescence system. Ligand blotting was performed using purified C3 (lanes 2, 3, 7) and RAP-myc (lane 6). Bound ligands were visualized using the appropriate specific antibodies followed by HRP-goat-anti-rabbit IgG (1:10,000) and a chemiluminescence system.

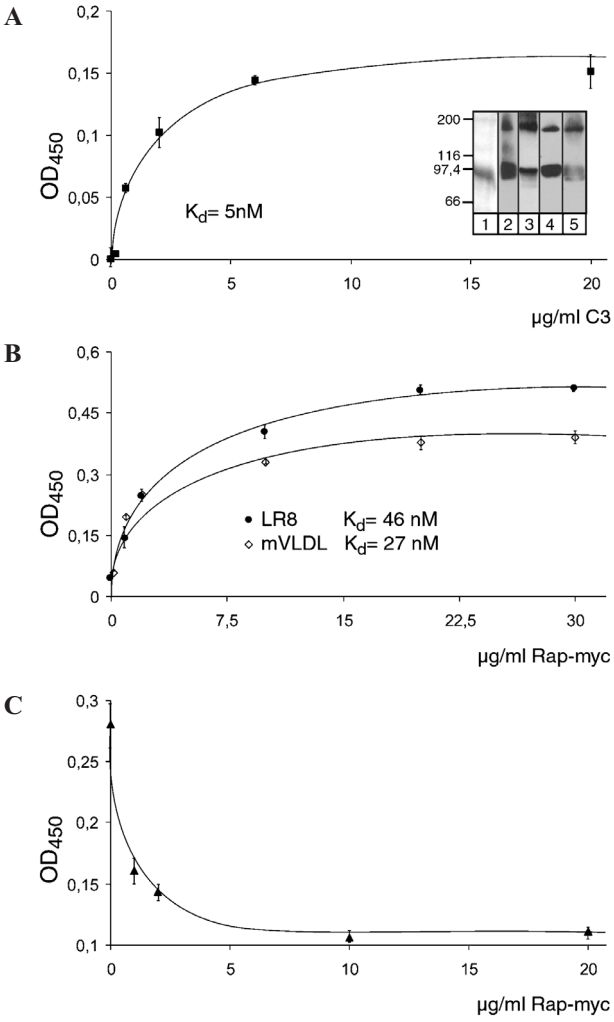

Figure 3. Ligand binding properties of C3 to LR8. (*A*) Ninety-six-well plates were coated with recombinant LR8-MBP/His. After incubation with the indicated concentrations of purified C3 in the presence of 2 mM $CaCl_2$, bound C3 was detected with anti-C3 antiserum and HRP-conjugated secondary antibody. Inset: Recombinant LR8-MBP/His was separated by SDS-PAGE under non-reducing conditions and stained with Coomassie Blue (lane 1), or Western blotted using anti-LR8 antiserum (lane 2), or ligand blotted using VTG (lane 3), *Rap*-myc (lane 4), or C3 (lane 5). Presence of the ligands was visualized using specific antibodies against VTG, RAP, or C3, and HRP-conjugated secondary antibody. (*B*) Ninety-six-well plates were coated with recombinant LR8-MBP/His or mouse VLDLR-1-8-MBP/His. After incubation with the indicated concentrations of *RAP*-myc, bound protein was detected with anti-myc and HRP-conjugated secondary antibody. (*C*) Ninety-six-well plates were coated with LR8-MBP/His and incubated with 10 µg/ml C3 in the presence of the indicated concentrations of *Rap*-myc (0–20 µg/ml). Bound C3 was detected with anti-C3 and HRP-conjugated secondary antibody.

with the same band in plasma (lane 2) and yolk (lane 3), co-migrating with the purified holoprotein (lane 1) under non-reducing conditions, and the α subunit of the purified protein under reducing conditions (lanes 4–6). This suggests that the protein present in yolk is not activated. To further support this notion, we used anti-C3a antiserum, which detects the α subunit under reducing conditions only when C3 was not activated and C3a is still part of the α subunit (fig. 1A, lane 7). This antibody reacted with the α subunit in yolk as well as in serum (fig. 1C, lanes 1, 2). In addition, no immunoreactive band corresponding to C3a was present in yolk (fig. 1C, lane 1). As control we produced the C3a fragment by zymosan activation of total serum as described previously [14] (fig. 1C, lane 3). These experiments demonstrate that C3 is present in yolk and is not activated prior to its transport into the oocyte.

Having demonstrated that C3 is a yolk component, we tested whether LR8 might be the receptor transporting it from the circulation into developing oocytes. We used the well-established procedure of ligand blotting to visualize receptors present in the oocyte membrane [20]. As demonstrated in figure 2 (lane 2), purified non-activated chicken C3 binds to a protein migrating at 97 kDa in non-reducing SDS-PAGE. This is the same position to which LR8 migrates under these conditions, as shown in lane 1 by Western blotting using an LR8-specific antibody. In addition, C3 binding can be completely blocked by the addition of RAP, a protein that binds to most members of the LDL receptor family and displaces all cognate ligands

from the receptor [27]. This set of data suggests that C3 binds to LR8. To test this possibility, we expressed LR8 in 293 cells and subjected the resulting cell extracts to Western and ligand blotting (fig. 2, lanes 4–7). LR8 expressed in 293 cells migrates with the same electrophoretic mobility as the endogenous protein (lane 5) and binds RAP (lane 6) and C3 (lane 7). The background seen in lane 7 is due to unspecific binding of C3 and/or crossreactivity of the anti-C3 antibody used to detect the bound ligand to proteins present in extracts from 293 cells, since these bands were both present when mock-transfected cells were used or when the ligand blot was performed without adding purified C3. To quantitatively evaluate binding data, we used an ELISA-based binding assay, recently developed to determine the binding characteristics for the mammalian VLDL receptor [17, 22]. For this assay, we expressed the entire ligand-binding domain of LR8 fused to maltose-binding protein in *E. coli* and refolded this protein in the presence of a redoxsystem (reduced/oxidized glutathione) and calcium ions [28]. As demonstrated in the inset of figure 3A, the fusion protein is recognized by the antibody against LR8 (lane 2) and, more importantly, is ligand-binding competent since it binds VTG (lane 3), RAP (lane 4), and C3 (lane 5). The additional band below the 200-kDa marker seen in the ligand blots (lanes 2–5; for ligand blots PAGE was carried out under non-reducing conditions) represents the dimer of the fusion protein. As shown below, dimerization of the recombinant receptor does not influence the affinity of RAP measured using the solid-phase binding assay (fig. 3B). In this assay, RAP binds to recombinant LR8 with a K_d of 46 nM, in good agreement with previous experiments using surface plasmon resonance and purified LR8 [29]. In addition, the affinity of RAP to LR8 is similar to that for the murine VLDLR (fig. 3B). Binding of C3 to LR8 was saturated at 40 nM with a K_d of 5 nM (fig. 3A). The binding site for C3 seems to be similar to the binding site for VLDL and VTG, since RAP competes for C3 binding in a similar manner as it does for the other ligands (fig. 3C) [29]. Addition of 10 mM EDTA abolished binding of C3 (data not shown), demonstrating that like all other cognate ligands of LR8, binding of C3 is calcium dependent. In summary, these experiments show that LR8 is a high-affinity receptor for non-activated C3, and that binding properties are similar to those of VLDL and VTG.

To demonstrate that LR8 is able to mediate endocytosis of non-activated C3 in a heterologous cell system, we used 293 cells expressing LR8 (see fig. 2, lanes 5–7) and visualized the uptake of C3 by fluorescence microscopy (fig. 4). Cells expressing LR8 (expression was tested by Western blotting, see fig. 2B) and incubated with purified non-activated C3 for 30 min at 37 °C show a vesicular cytosolic staining for C3, demonstrating the uptake of C3. Since we used transiently transfected cells, in this experiment only 20–30% of the cells were positive for internalized C3, giving an additional control for the experiment. Addition of RAP to the culture medium completely abolished the uptake of C3.

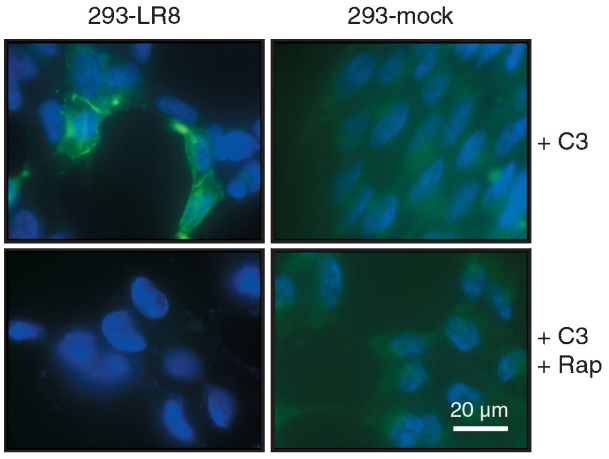

Figure 4. Internalization of purified C3 into 293 cells expressing LR8. 293 cells transiently transfected with an expression plasmid coding for LR8 or transfected with the empty plasmid were incubated with purified C3 (20 µg/ml) in the absence or presence of RAP (100 µg/ml) at 37 °C for 30 min. After fixing and permeabilizing the cells, C3 was localized by immunofluorescence as described in Materials and methods. Nuclei were visualized with DAPI.

If C3 uptake into the developing oocyte in vivo is mediated by LR8, co-localization of C3 and LR8 in coated vesicles should be possible. Therefore we prepared clathrin-coated vesicles from small vitellogenic follicles and tested by Western blotting for the presence of C3 and LR8. As demonstrated in figure 5 (lane 3), these organelles contain C3 which was detected (under reducing conditions) as the α chain migrating with an M_r of 110 kDa (see fig. 1). This preparation was characterized as clathin-coated vesicles by the presence of the clathrin heavy chain and LR8, which is highly enriched in this compartment [10]. These results demonstrate that C3 is present in LR8-containing coated vesicles in developing oocytes, further strengthening the notion that C3 is taken up by LR8-mediated endocytosis.

As mentioned above, LRP, which belongs to the same receptor family, also binds C3 [26]. However, this interaction is qualitatively different from the interaction of C3 with LR8. Whereas LRP binds activated C3, LR8 binds and transports non-activated C3. This is reminiscent of the findings on α_2-macroglobulin. LRP binds activated α_2-macroglobulin [30] thereby rapidly removing activated α_2-macroglobulin from the circulation. LR8, however, binds and internalizes native α_2-macroglobulin [10]. Apparently, binding of these components to LRP and LR8 serves different physiological functions. Whereas LRP clears activated α_2-macroglobulin and activated C3 from the circulation, LR8 transports α_2-macroglobulin and C3 into developing oocytes to supplement the embryo

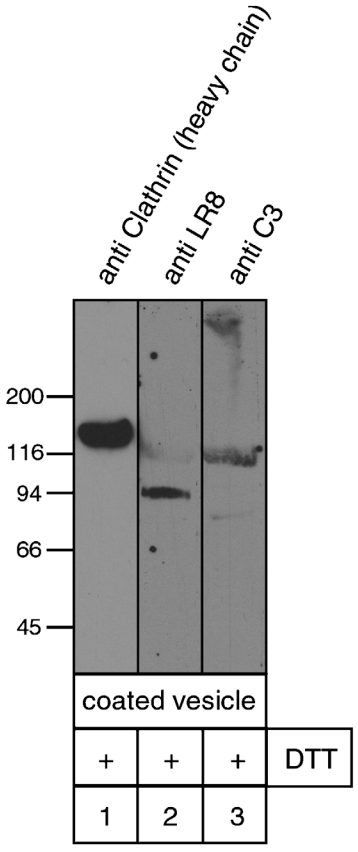

Figure 5. Western blots of clathrin-coated vesicles. Proteins from clathrin-coated vesicles prepared from vitellogenic follicles (10 µg/lane) were separated by SDS-PAGE under reducing conditions. Western blotting was performed using the indicated antibodies followed by HRP-goat-anti-rabbit IgG (1:10,000) and a chemiluminescence system.

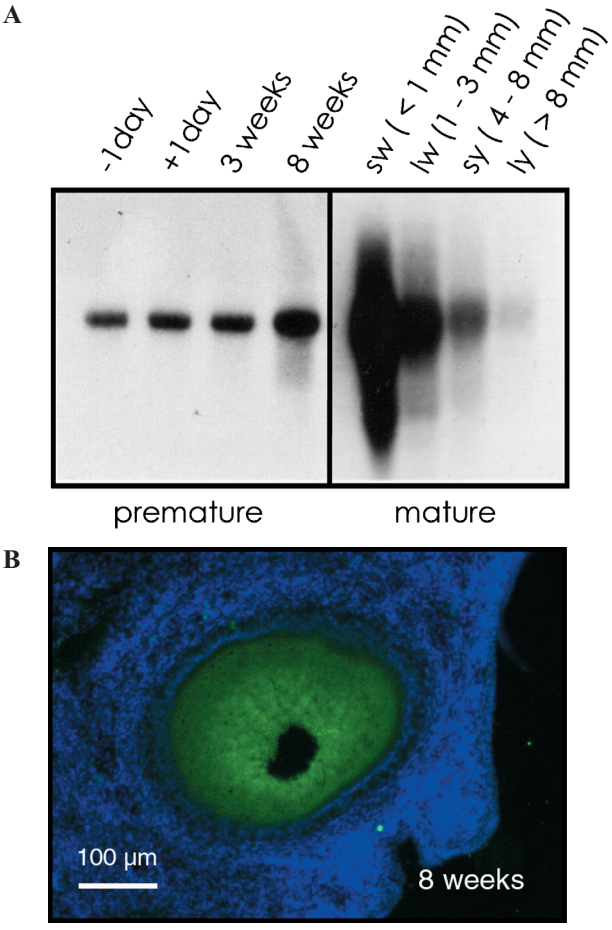

Figure 6. Expression of LR8 during chicken follicle development. (*A*) Total RNA (20 µg/lane) was isolated from premature ovaries at the indicated developmental stages and from follicles representing different growth phases of the oocyte from a mature laying hen and subjected to Northern blot analysis as described in Materials and methods. (*B*) Immunofluorescence was performed on a cryostat section of a follicle of a immature hen (8 weeks old). Presence of LR8 was tested using an anti-LR8 antibody in combination with Alexa Fluor 488 goat anti-rabbit IgG (green). Nuclei were counterstained with DAPI (blue).

with a competent protease inhibitor (α_2-macroglobulin) and with a major component of the immune system (C3). The above results can be summarized as follows: C3, a key component of the complement system, is a component of chicken egg yolk, and is transported from the circulation without activation into the developing oocyte by LR8-mediated endocytosis.

This is an interesting physiological situation. Many yolk components such as VLDL, VTG, α_2-macroglobulin, riboflavin-binding protein [8], and C3 are all transported into the oocyte using the same route and the same receptor molecule. How is the uptake of these components coordinated? To tackle this question, we started to evaluate the expression pattern of LR8 during chick development. As demonstrated in figure 6, the message for LR8 is already present in the embryonic ovary. In the mature hen, where follicles of different stages of development can be isolated, the amount of mRNA coding for LR8 dramatically decreases during oocyte maturation. These follicles can be categorized according to Johnson [31] into three major growth phases: phase 1 is characterized by a very slow growth rate where follicles reach a diameter of 1–3 mm. These follicles do not contain massive amounts of yolk, and therefore appear white (these follicles are referred to here as small and large white follicles; sw and lw). During phase 2, some of these follicles grow to a size of 4–8 mm; this phase takes about 2 months, and the follicles acquire a yellow appearance due to yolk deposition and are termed 'small yellow' (sy). During the last phase, some of these follicles are selected for a final growth spurt, which takes about 7 days. During this time, follicles take up massive amounts of yolk material and reach a diameter of about 35 mm. The largest follicles in this hierarchy are numbered from F1 to F5, where F1 will be the next to ovulate. As demonstrated in figure 6A, the mRNA for LR8 is already present in the ovary of the

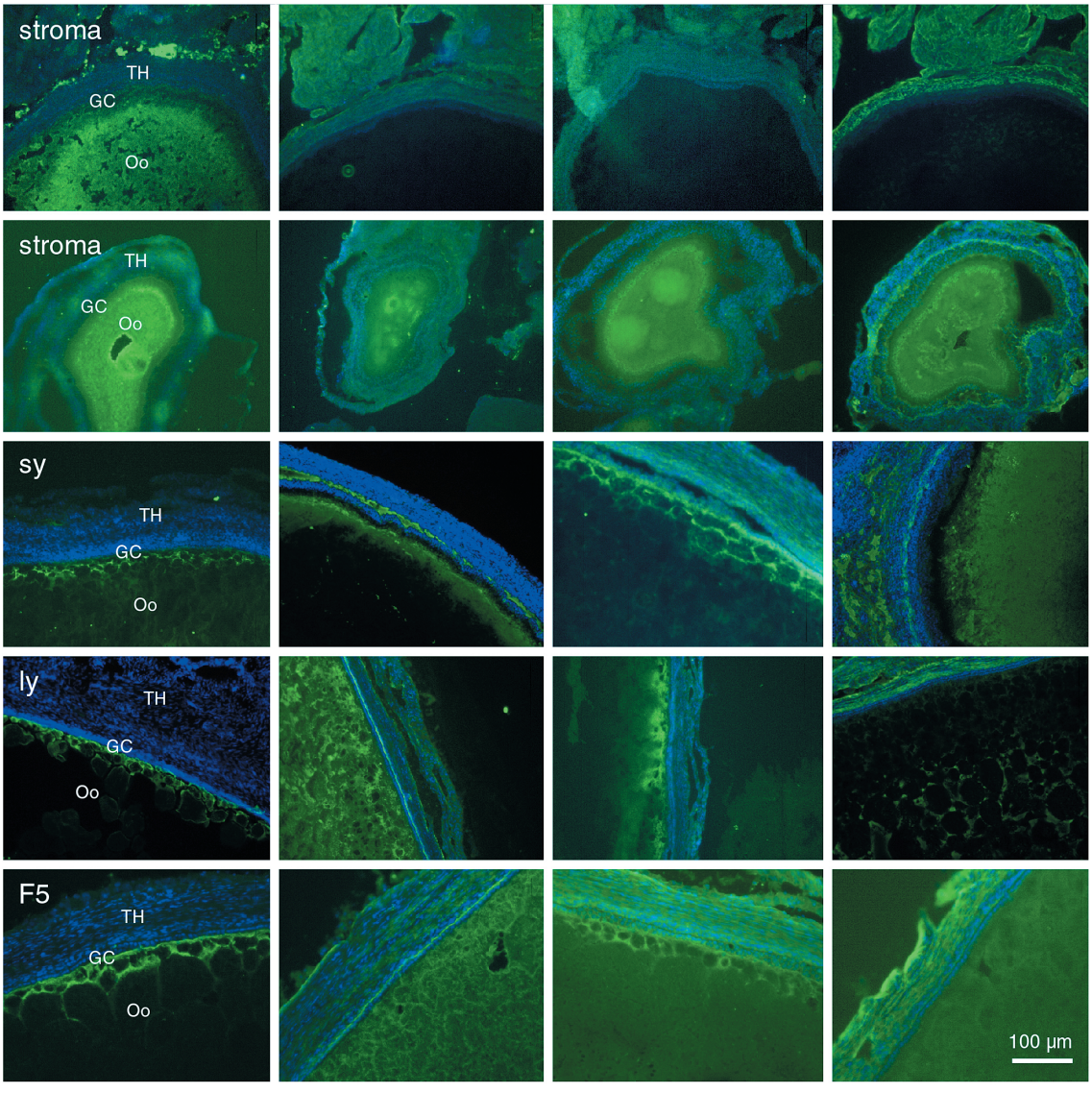

Figure 7. Expression of LR8 and incorporation of yolk components during oocyte development. Immunofluorescence was performed on cryostat sections of follicles of different developmental stages as described in Materials and methods. LR8, apoB, C3, and vitellogenin appear in green. Nuclei were counterstained with DAPI (blue). Stroma, small white follicles embedded in surrounding tissue; sy, small yellow follicles; ly, large yellow follicle; F5, follicle 5 days before ovulation; TH, theka; GC, granulosa cell layer; Oo, oocyte.

developing chick, and more importantly, LR8 protein is present in follicles of the immature hen (as an example, an immunofluorescent analysis of a follicle present in an 8-week-old hen is shown in fig. 6B). At this developmental stage, the ovary contains only follicles which have not entered any of the above-described growth phases. In addition, due to the hormonal status of the bird, no VTG is produced by the liver and the lipoprotein pattern of the female bird corresponds to that of a rooster, i.e., the prevalent lipoprotein class in the circulation is high-density lipoprotein, and very little VLDL is produced by the liver. To further evaluate the situation, we studied the expression of LR8 in parallel with the accumulation of different yolk components within the ovary of a mature hen by immunohistochemistry (fig. 7). Follicles of the third (F5 and ly), and the second (sy) growth phase were separated from the ovary; smaller follicles (2 mm and smaller) were evaluated by sectioning the stroma of the ovary. As expected, follicles that started to accumulate yolk (sy, ly, F5) contain LR8 and all the yolk components tested (apoB for VLDL, VTG, and C3). All small follicles present in the stroma of a mature hen contain large amounts of LR8, consistent with the observation that in the immature ovary, follicles already contain the receptor. However, as demonstrated in figure 7 (top row), many follicles, despite the presence of LR8, do not contain any of the yolk components. If they contain yolk components (row 2) all of the components tested are present at the same time. This suggests that independent of the presence of LR8, a yet undefined mechanism selects certain follicles to accumulate yolk. As demonstrated in the cartoon of figure 7, within the follicle, the oocyte is surrounded by an epithelial-like cell layer called granulosa cells. Macromolecules transported into the oocyte most likely pass the granulosa cell layer by paracellular routes [1]. Recent studies on the dynamics of tight junctions present in the granulosa cell layer show that the expression of occludin, a major component of the tight junction, is reduced in follicles which have started to accumulate yolk [32]. This process seems to be regulated by follicle-stimulating hormone and activin. Taken together, these results suggest the following mechanism: as early as during embryonic development, oocyte precursors start to accumulate mRNA for LR8. In the immature hen, weeks before the bird starts to produce major yolk precursors like VTG and VLDL in the liver and to lay eggs, oocytes start to produce large amounts of LR8 protein. At onset of egg laying, a subset of follicles is selected to accumulate yolk, likely facilitated by breakdown of tight junctions in their granulosa cell layer. Thus, macromolecules derived from the vascularization of the theca layer may gain access to the oocyte membrane bypassing the granulosa cells. Once present at the surface of the developing oocyte, they are endocytosed by LR8. Similar K_ds of VLDL, VTG, α_2-macroglobulin, and C3 for the receptor suggest that LR8 does not discriminate between these molecules, and their uptake more or less reflects their relative abundance in the extraoocytic space. As outlined in this report, at least in birds, nature has apparently chosen a rather simple mechanism to ensure oocyte development and reproduction. However, the mechanism regulating the selection of follicles to establish the strict hierarchy of developing oocytes resulting in egg deposition every 25 h remains to be elucidated.

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