Molecular characterization of the first avian LDL receptor: role in sterol metabolism of ovarian follicular cells

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Abstract **Low levels of expression and sluggish sterol-mediated regulation have been likely reasons for the failure to molecularly characterize a bona fide LDL receptor (LDLR) in egg-laying species to date. The overall structure of the chicken LDLR, delineated here by cDNA cloning, has been conserved in evolution, since hallmark properties of mammalian LDLRs are already present in the avian protein. The chicken receptor appears to prefer LDL over VLDL as ligand, in compliance with its main role in providing lipoprotein-derived cholesterol for steroid production in ovarian follicular cells. This is also compatible with the fact that estrogen administration increased hepatic LDLR expression in roosters despite dramatically stimulated VLDL production. In cultured chicken embryo fibroblasts, expression of the receptor was induced by incubation with cholesterol synthesis inhibitors such as a statin. Furthermore, preincubation of induced cells with a specific anti-receptor antibody blocks LDL endocytosis, demonstrating that the receptor is ligand-endocytosis competent. Finally, the distribution of LDLRs among the extraoocytic cell populations lends support to a three-cell model for estrogen production within the ovarian follicle.** **In summary, the molecular characterization of the first avian LDLR reveals novel information about evolutionary, structural, and functional aspects of members of the supergene family of LDLR-related proteins.**—Hummel, S., E. G. Lynn, A. Osanger, S. Hirayama, J. Nimpf, and W. J. Schneider. **Molecular characterization of the first avian LDL receptor: role in sterol metabolism of ovarian follicular cells.** ***J. Lipid Res.*** **2003.** 44: **1633–1642.**

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One of the main tasks of females of oviparous (egg-laying) species is to coordinate synthetic and metabolic pathways for the production of eggs, i.e., their reproductive effort. In the domesticated chicken (*Gallus gallus*), lipoprotein metabolism in the laying hen (LH) is under stringent hormonal control, which assures that the massive lipoprotein flow required for oocyte growth can be met without compromising systemic lipid homeostasis (1, 2). Chickens lack apolipoprotein B-48 (apoB-48) and apoE; furthermore, only apoA-I, but not apoA-II, is synthesized by this species. Our past studies in the avian system have shown that hepatically derived triglyceride-rich VLDL particles harboring apoB-100 and the unique protein apoVLDL-II (3) as main functional surface components are directed toward two sets of receptors belonging to the supergene family of the LDL receptor (LDLR) and LDLR relatives (LRs). One set of LRs is expressed almost exclusively and at high levels in oocytes; these receptors recognize, in addition to apoB-100 of VLDL, the lipophosphoglycoprotein vitellogenin, which together with VLDL constitutes the vast majority of yolk. The other set of LRs is synthesized in cells and tissues other than the oocytes; receptors of this group bind apoB, but not vitellogenin, and are generally expressed at low levels compared with receptor genes directly involved in oocyte growth (2, 4, 5).

The yolk precursor receptors thus far identified and characterized are the avian homolog of the mammalian so-called VLDLR (6), in chicken termed LR8, (1, 2) and an avian LR originally named oocyte-specific LDLR-related protein (LRP) (7), and more recently designated LRP380 (8). In concert, these receptors are responsible for uptake into rapidly growing oocytes of up to 95% of the yolk mass (2, 9). The nonoocytic LRs of the chicken identified to date are the so-called somatic cell-specific 600 kDa LRP (10), a brain-specific LR termed LR8B, (11), and a hitherto uncharacterized 130 kDa LDL binding protein expressed by fibroblasts (12) assumed to represent a bona fide LDLR, i.e., the focus of the present study.

The human LDLR is known to bind apoB-100 (but not apoB-48) and apoE (13). While chickens do not express

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apoE, we have previously shown that the avian oocyte's vitellogenin binding receptors interact with mammalian apoE, likely due to the presence of cluster(s) containing at least eight ligand binding repeats (LA) in the receptors and certain structural similarities between the two animal kingdom-specific ligands (14, 15), respectively. On the other hand, the above-described 130 kDa protein of chicken fibroblasts has been shown to bind apoB, but not apoE, indicating that the LA domain of this receptor might have a different structure from that of the other chicken LRs (12). Thus, different ligand binding properties may direct vitellogenin and VLDL to oocytes via multiligand receptors, and target LDL to somatic cells via a bona fide LDLR.

In addition to these systemic aspects of LDL metabolism in the chicken, the LH model has elicited specific questions related to oocyte growth and ovarian follicle development (2). In this context, steroid hormones, especially estrogen, are produced in follicles by a coordinated effort of follicular cells, i.e., the granulosa and thecal cells (16–19). Inasmuch as LDL-derived cholesterol is the main substrate for steroid synthesis in mammals, we have aimed at gaining insight into the possible role of an LDL-specific receptor in this process. However, such a receptor has to date resisted efforts to be characterized at the molecular level, possibly due to generally low levels of expression, sluggish lipoprotein-mediated regulation in the classical fibroblast model system (12), and limited sequence information about the chicken genome. Here, we describe the cDNA cloning of the first avian LDLR and its expression pattern and regulation by a powerful statin in vitro and by estrogen in vivo, and reveal a correlation between the receptor's expression levels in follicular cells and the developmental status of ovarian follicles.

MATERIALS AND METHODS

Animals

Derco Brown LHs (30–40 weeks old) were purchased from Heindl Co. (Vienna, Austria), and the restricted ovulator (R/O) breeding colony was reared from fertilized eggs kindly provided by Dr. R. G. Elkin (Pennsylvania State University, State College, PA). All animals were maintained on layer's mash with free access to water and feed under a daily light period of 14 h. Fertilized chicken eggs were obtained from Schropper Co. (Schottwien, Austria) and incubated at 38°C and 70% humidity. After 10 days incubation, the eggs were used for the isolation of chicken embryo fibroblasts (CEFs), as described below.

Cell culture

CEFs were isolated (12) and propagated in DMEM supplemented with 10% FCS, 1% penicillin-streptomycin, and 2 mM L-glutamine at 39°C in an atmosphere of 95% air and 5% CO_2. Granulosa cells (GCs) were prepared by collagenase dispersion of GC sheets isolated from preovulatory follicles (F1–F4) as described (8). Cells were plated at a density of 1×10^5 cells/ml in DMEM with 5% FCS, 1% penicillin-streptomycin, 2 mM L-glutamine, 50 ng/ml FSH (Calbiochem), and 25 ng/ml human recombinant activin A (R and D Systems), and propagated at 37°C in 5% CO_2. The chicken hepatoma cell line, LMH, was maintained as described previously (20). For experiments, cells (at 60–70% confluency) were cultured in medium containing 10% FCS or 10% lipoprotein-deficient serum (LPDS) in the absence or presence of 1 μM NK104 (also named pitavastatin; a kind gift from KOWA Co. Ltd., Tokyo, Japan). After 24 h incubation, cells were harvested and prepared as described below.

Library screening

An LH liver λ ZAPII cDNA library (Takara) was screened under high stringency conditions with a 148 bp cDNA probe (5′-ACCAAGGTGCCTGGCATGCCCTGCGGCCCCGACCAGTTCA-GCTGCGTGGTCAGCGGCGCCTGCATCCCCGCCAGCTACCA-CTGTGACGAGGAGCCCGACTGCCCCGACCGCTCCGACGAG-GTCGGCTGCATGCCGCCGCAGGTGGTGA-3′), corresponding to nucleotides 1,045–1,192 of full-length chicken perlecan (unpublished observations); this region specifies the 4th LDLR LA repeat found in all reported perlecan sequences. Positive clones were identified, and the generated pBluescript SK(−) clones were sequenced and subjected to a homology search at the amino acid level using BLAST software (NCBI). A clone with a ~4.5 kb insert, designated pggLDLR, showed the highest degree of homology to LDLRs from seven species.

Cloning of the LDLR LA domain (LDLR-LA1-7) into the pMAL-vector

PCR of the ggLDLR LA domain (LA1-7) was performed using the forward primer 5′-CTGCTGGAATTCGCCACAGACGTGTG-3′ (*Eco*RI site underlined) corresponding to nucleotides 43–56, and the reverse primer 5′-TCTAGAGTCCACGTCGCAGTGCT-3′ with an *Xba*I site (underlined) corresponding to nucleotides 974-990. The PCR product was cloned into the pMAL vector (New England Biolabs), which had been modified to provide a His-tag at the C-terminal end of the resulting fusion protein (a kind gift of Dr. D. Blaas). All constructs were confirmed by sequencing.

Expression of pMAL-LDLR-LA1-7 and GST-RAP-myc, GST-RAP purification and refolding

The vectors pMAL-LDLR-LA1-7 and pGEX-myc-receptor-associated protein (RAP) were expressed in the *Escherichia coli* strain Top10F' (Invitrogen). The proteins were isolated from lysates of isopropyl β-thiogalactopyranoside-induced bacteria and purified by Ni-Agarose chromatography (Qiagen); elution buffer contained 250 mM imidazol. The purified proteins were dialyzed overnight against TBS (2 mM $CaCl_2$, pH 7.4) at 4°C. To generate RAP-Sepharose, purified myc-RAP was coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instructions and stored in TBS with 0.002% sodium azide. The LDLR-LA1-7 was refolded by dialysis against TBS in the presence of RAP-Sepharose with or without 2 mM reduced glutathione and 1 mM oxidized glutathione. After 72 h dialysis at 4°C, the glutathione was gradually removed as described in the QIAexpressionist handbook. The bound and refolded protein was then eluted from the RAP–Sepharose with 0.5 × TBS, 1 M NH_4OH. The eluted proteins were immediately adjusted to pH 8 with 0.1 M HCl and dialyzed against TBS for 24 h.

Preparation of antibodies and lipoproteins

Polyclonal antibodies directed against the bacterially expressed LA domain (LDLR-LA1-7) were raised in adult female New Zealand White rabbits (9) and are designated anti-LA1-7. Antisera were tested by Western blotting. Polyclonal rabbit antibodies against maltose binding protein (MBP) were obtained from New England Biolabs. Lipoproteins were isolated from the serum of estrogen-treated roosters (ERs) by differential floatation as described previously (12). ^{125}I-labeled LDL was prepared

by the iodine monochloride method as described (21). All lipoproteins were dialyzed extensively against 50 mM NaCl, 0.2 mM EDTA (pH 7.4) before use and stored at 4°C. Indicated concentrations represent the protein content.

Preparation of cell and tissue protein extracts

CEFs, GCs, and LMH cell monolayers were collected and solubilized with Buffer B [200 mM Tris-maleate (pH 6.5), 2 mM $CaCl_2$, 1.4% Triton X-100, 1 mM PMSF, and 2 μM leupeptin]. Insoluble components were removed by centrifugation for 40 min at 4°C at 300,000 *g*. The supernatant was quickly frozen in liquid N_2 and stored at −70°C. Thecal cell layers were prepared from follicles (F5 and larger) by first removing the surrounding adhering tissue. The follicles were punctured, and yolk and GCs extruded. The remaining thecae were washed in PBS to remove the yolk. Theca and other tissues were collected and homogenized with an Ultra Turrax T25 homogenizer in Buffer A (4 ml/g wet weight) containing 20 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 150 mM NaCl, 1 mM PMSF, and 2 μM leupeptin for 1 min on ice. After homogenization, cellular debris was removed by centrifugation at 5,000 *g* for 10 min at 4°C and the supernatant was spun at 100,000 *g* for 1 h at 4°C. The pellet was resuspended in Buffer B and spun at 300,000 *g* for 40 min at 4°C. The supernatant was quick frozen in liquid N_2 and stored at −70°C. Protein concentrations were determined by a modified Lowry procedure (22).

Electrophoresis, transfer to nitrocellulose, ligand, and Western blotting

Protein extracts were separated by one-dimensional 7.5% SDS-PAGE under reducing or nonreducing conditions according to Laemmli (23). The molecular weights of proteins were estimated with a broad-range molecular mass standard (6.5–200 kDa) from BioRad. Proteins were either visualized with Coomassie brilliant blue or transferred to Hybond-enhanced chemiluminescence (ECL) nitrocellulose (Amersham Pharmacia Biotech) in 20 mM Tris-HCl, 0.15 M glycine (pH 8.4) on a semidry trans-blot SD transfer cell (BioRad). For Western blot analyses, the membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20. Bound polyclonal antibodies were detected with HRP-labeled goat anti-rabbit IgG (1:50,000; Sigma) and an ECL system (Pierce). For ligand blotting, membranes were blocked with 5% BSA (fatty acid-free, Sigma) in TBS. Following incubation with 10 μg/ml RAP-myc, bound ligand was detected with monoclonal anti-myc antibodies (1:500), HRP-goat-anti-mouse IgG (1:10,000; Sigma), and ECL. For blots with ^{125}I-LDL (specific radioactivity, 460 cpm/ng), we used 1.5×10^6 cpm/ml in 50 mM Tris-HCl, 90 mM NaCl, and 2 mM $CaCl_2$ (pH 8.0), as previously described (12). For competition experiments, ^{125}I–LDL was coincubated with 20- or 100-fold excess of unlabeled lipoproteins or with 5 μg/ml myc-RAP.

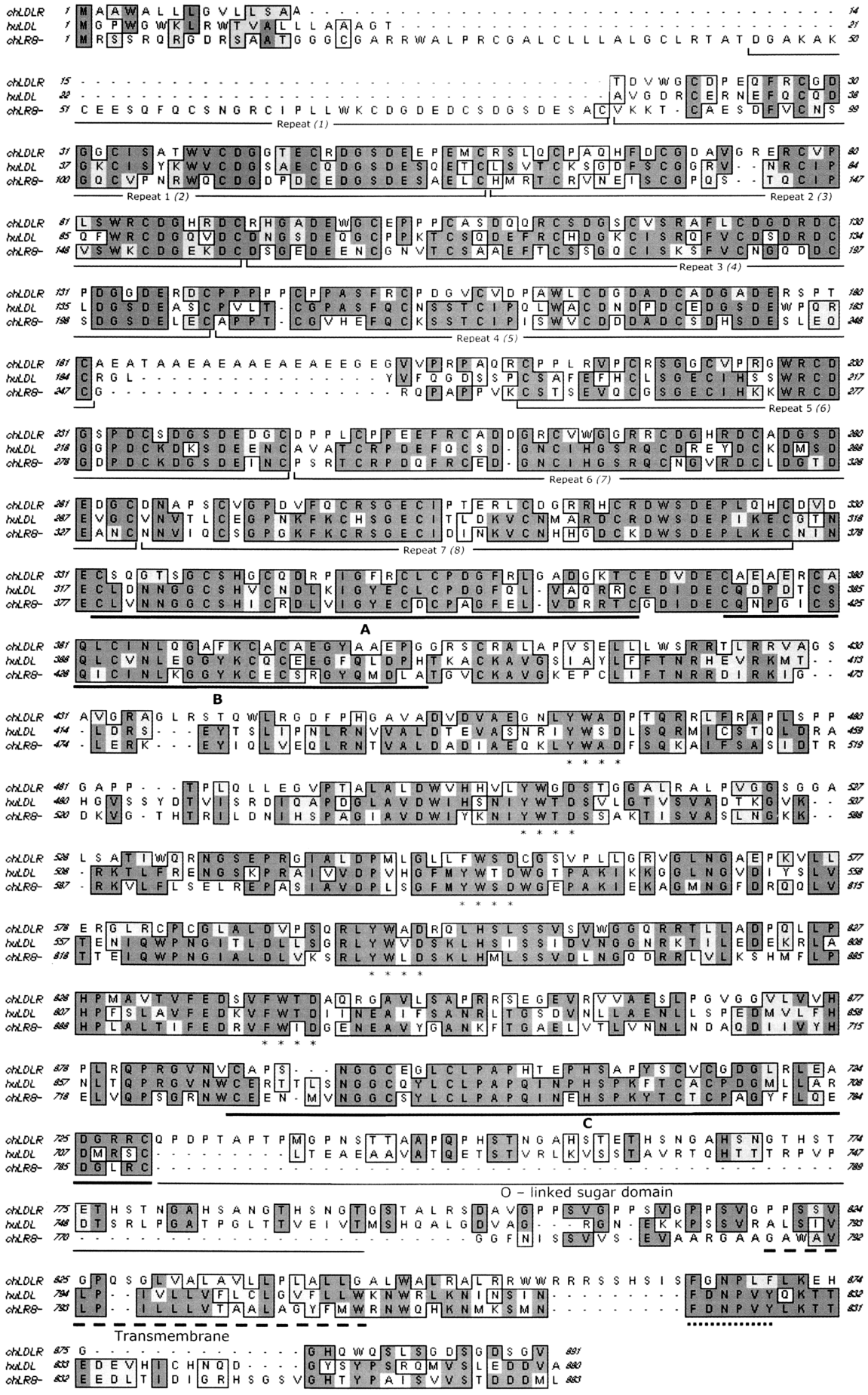
Immunocytochemistry and immunofluorescent uptake studies

For immunocytochemistry, the third-largest follicle (19 mm diameter) from a mature hen was dissected, embedded in freezing agent (Microm, Austria), and immediately frozen. Cryostat sections of 20 μm thickness were prepared and fixed on Superfrost-Plus slides with acetone-methanol (1:1, v/v). The slides were blocked with 3% goat serum, 1% milk powder in PBS (pH 7.4) for 1 h at room temperature, and then incubated with preimmune or anti-LA1-7 antiserum (1:100) for 1 h, followed by rinsing in PBS and incubation with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). Microscopy was performed on a Zeiss Axiovert 135 microscope. For receptor localization and LDL-uptake studies in cultured embryo fibroblasts, the cells were grown on culture slides coated with poly-D-lysine to 60% confluency and then cultured in FCS-containing medium for an additional 24 h in the absence or presence of 1 μM NK104 to induce LDLR expression. Slides were then transferred onto ice, washed with PBS, and incubated for 1 h with preimmune or anti-LA1-7 antiserum (1:100). The cells were washed three times with ice-cold PBS and incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes). Microscopy was performed on a Zeiss Axiovert 135 microscope. For uptake studies, we used chicken LDL labeled with rhodamine (Molecular Probes) according to the manufacturer's instructions. CEFs were cultured on poly-D-lysine-coated slides and treated with NK104 as described above. Slides were transferred onto ice, washed once with PBS, and incubated with preimmune or anti-LA1-7 antiserum (1:100) for 1 h. Cells were washed three times with ice-cold PBS and then incubated at 4°C for 1 h with PBS containing 10 μg/ml rhodamine LDL. The cells were either fixed immediately or were incubated at 37°C for 30 min and then fixed with methanol-acetone (1:1, v/v) for 15 min at −20°C. The cells were counterstained with 4′,6′-diamidino-2′-phenylindole, and microscopy was performed on a Zeiss Axiovert 135 microscope.

RESULTS

Molecular characterization of the chicken LDLR

The cDNA clone pggLDLR had a size of ~4.5 kb, with an open reading frame of 2,676 bp and a 3′-untranslated region of ~1.6 kb. The nucleotide sequence has been deposited in the EMBL data bank, accession No. AJ515243. Translation of the open reading frame of the cDNA obtained from the LH liver library revealed an 891-residue precursor protein with high homology to the LDLR from man (**Fig. 1**) and from several other species, such as mouse, rabbit, rat, pig, hamster, and xenopus (not shown). Sequence comparison with its homologs and application of the von Heijne algorithm identified Thr-16 as the N-terminal residue of the mature receptor. Alignment of the primary protein sequence of ggLDLR with that of the human LDLR and of chicken LR8 (Fig. 1), the major mediator of oocyte growth (14, 24), demonstrates that ggLDLR shares significant structural properties with the human LDLR, and that its overall domain architecture is different from LR8's. In particular, ggLDLR and mammalian LDLRs contain seven LA repeats and a so-called *O*-linked sugar domain, whereas the major VLDLR variants (LR8 in Fig. 1) of avian and mammalian origin contain an extra N-terminal LA repeat, but lack the *O*-linked sugar domain. The differences in some of the structural elements between mammalian and chicken LDLRs are subtle; e.g., *i*) the insertion between the sixth cysteine in LA repeat 4 and the 1st cysteine of LA repeat 5, ranging in length from 11 to 13 residues in all LDLRs studied thus far, is comprised of 28 residues with a 19-residue Ala/Glu-rich stretch in ggLDLR; *ii*) the arrangement of negatively charged residues in the C-terminal region of LA repeats, particularly in LA1 and LA5, differ; *iii*) in the intracellular domain, the sequence



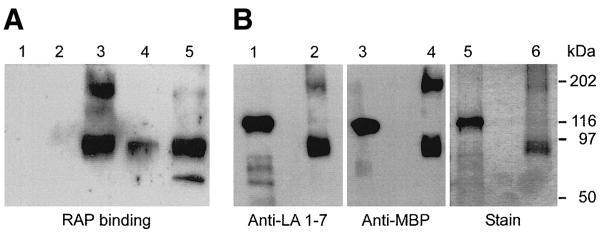


Fig. 2. Analysis of LA function of recombinant LDLR LA1-7. LDLR-LA1-7 was bacterially expressed as a His-tagged fusion protein with maltose binding protein (MBP), and purified, and refolded as described in Materials and Methods. A: Protein (2 μg) of each of the preparations was analyzed by ligand blotting with receptor-associated protein (RAP)-myc following electrophoresis under nonreducing conditions on 7.5% SDS gels as described in Materials and Methods. A: Original Ni-Agarose purified material; lanes 2–5, following dialysis with RAP-Sepharose in the absence (lanes 2, 3) or the presence (lanes 4, 5) of 2 mM reduced glutathione and 1 mM oxidized glutathione. The material bound to and eluted from the RAP-Sepharose (lanes 3, 5) and the unbound fractions (lanes 2, 4) were analyzed. B, Lanes 1, 3, 5: Reduced LDLR-LA1-7 (2 μg); and lanes 2, 4, 6, nonreduced LDLR-LA1-7 (2 μg) were subjected to immunoblotting with anti-LA1-7 (1:1,500; lanes 1, 2) or anti-MBP (1:1000; lanes 3, 4), or stained with Coomassie blue (lanes 5, 6). Molecular weight markers are indicated.

of the internalization signal Phe-Asp-Asn-Pro-Val-Tyr (FDNPVY) in mammalian LDLRs is different from that of ggLDLR, in which FDNPVY is replaced by FGNPLF; and *iv*) the single cysteine present in the cytoplasmic tails of the LDLRs from mammals is absent from ggLDLR. We have previously observed by ligand blotting with radiolabeled chicken LDL that the sterol-sensitive LDL binding protein in chicken fibroblasts migrated slower on SDS gels (~130 kDa) than did the corresponding oocytic protein LR8 (~95 kDa) (25). Thus, the structural insight gained from the cloning of ggLDLR strongly suggests that this apparent size difference is due to the presence of the *O*-linked sugar domain in ggLDLR, which is known to disproportionately decrease the electrophoretic mobility of, e.g., the human LDLR (26), overriding the opposing effect of ggLDLR harboring one less LA repeat than LR8.

Expression and sterol regulation of ggLDLR

Attempts to obtain useful anti-peptide antibodies for the study of ggLDLR failed due to multiple cross-reactivities or poor antigenic response. However, for antigen production as well as for LA studies, the generation of the entire LA domain of ggLDLR (LA repeats 1–7) inserted into His-tagged MBP provided a valuable tool. In agreement with previous results (27), LA activity of the recombinant protein (~90 kDa, where the contribution of MBP is 42 kDa) was gained by refolding/binding onto immobilized RAP (**Fig. 2A**, lanes 3–5). Binding was analyzed by ligand blotting with myc-RAP, which decorated the same 90 kDa band as the antibody against MBP (Fig. 2B, lanes 3 and 4). Furthermore, rabbit antiserum against the functionally folded recombinant LA domain (Anti-LA1-7) recognized the expressed protein even before functional refolding (Fig. 2B, lanes 1 and 2). A band approximately twice the size of the major RAP binding protein was visible only under nonreducing conditions, most notably with anti-MBP antibody (Fig. 2B, lane 4), and likely represents a dimeric form of the recombinant fusion protein.

Previous results on the expression of lipoprotein receptors in the LH and related studies by others (28, 29) in pigeons revealed low levels of LDLR expression in cultured avian cells, even when grown under sterol-depleted conditions (12). Indeed, given the overall hyperlipidemic status of the LH, low levels of ggLDLR expression in somatic cells in vivo would be expected. Thus, when a tissue survey was performed by Northern blot analysis using 20 μg total RNA and a full-length cDNA probe (not shown), transcript was undetectable in heart, kidney, lung, spleen, liver, and muscle, despite testing a wide range of conditions. However, with 5 μg poly(A)$^+$ RNA, a 4.5 kb transcript was detected in the adrenal gland, in agreement with its steroidogenic function. In cultured primary CEFs, LDLR transcripts were induced to detectable levels by treatment with 1 μM NK104 (pitavastatin), a powerful blocker of sterol synthesis via inhibition of HMG-CoA reductase (30) (not shown, but see below).

Immunological studies

These findings were corroborated by immunoblot analysis with our anti-ggLDLR antibody, anti-LA1-7. Indeed, the antibody reacted with a single protein in extracts of adrenal glands (**Fig. 3A**) having an apparent M_r of 130 kDa under nonreducing conditions, in agreement with previous ligand blotting results (12), and demonstrating the monospecificity of the antibody. To gain insight into the regulation of ggLDLR protein in cultured cells, we analyzed receptor levels in CEFs and the chicken hepatoma cell line LMH (20) under various conditions. Previously, sluggish regulation of LDL binding activity in CEFs has been reported, in that the lipoprotein content of the culture medium only had a small effect on high-affinity LDL uptake compared with the dramatic lipoprotein-mediated effects on LDLR levels in human fibroblasts (31). Here, we observed that over a 2-fold range in confluency (35% to 70%) and lipoprotein depletion for 24 h retarded the growth of CEFs as well as LMH (not shown). Possibly re-

Fig. 1. Sequence comparison of chicken LDL receptor (LDLR), the oocyte yolk precursor receptor LR8 (14), and human LDLR (39). Numbering of the amino acid sequences starts at the methionine residue corresponding to the initiation codon. Gaps (-) have been introduced to optimize the alignment. Identical and conserved residues are boxed and shaded. The ~40 amino acid ligand binding (LA) repeats 1–7 (or *1–8*, in LR8); the cysteine-rich repeats A, B, and C in the epidermal growth factor precursor homology domain, and the *O*-linked sugar domain are underlined. The transmembrane domain is indicated by a dashed line. The internalization sequence required for clustering of the LDLR in coated-pits is indicated by a dotted underline.

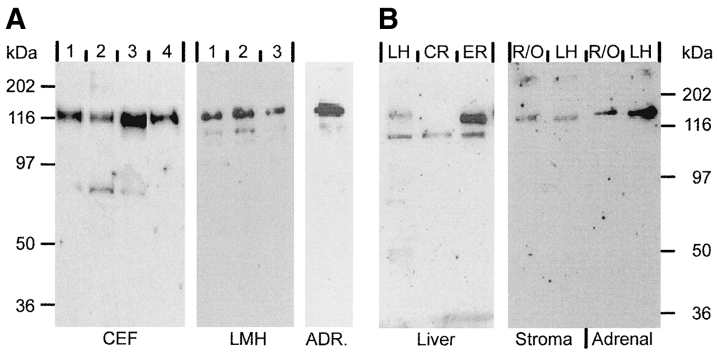


Fig. 3. A: Immunoblot analysis of LDLR protein in chicken embryo fibroblasts (CEFs) and LMH cells in the absence or presence of NK104. CEFs were cultured for the last 24 h before the experiment in 10% FCS (lanes 1, 3) or 10% lipoprotein-deficient serum (LPDS) (2, 4) plus (3, 4) or minus (1, 2) 1 μM NK104. LMH cell culture was in FCS (1), LPDS (3), or LPDS plus 1 μM NK104. Protein extracts were prepared and then electrophoretically separated (10 μg each) under nonreducing conditions and probed with anti-LA1-7 antibody as described in Materials and Methods. Protein extract (10 μg) from laying hen (LH) adrenal gland (ADR) was used as a positive control. Molecular weight markers are indicated. B: Immunoblot analysis of LDLR protein in tissues of hen and rooster. Protein extracts (10 μg) from the livers, stroma, and adrenal glands of LHs, restricted ovulator hens (R/O), control roosters (CRs), and estrogen-treated roosters (ER) were electrophoretically separated under nonreducing conditions, transferred to a nitrocellulose membrane, and then probed with anti-LA1-7 antibody as described in Materials and Methods. Molecular weight markers are indicated.

lated to this finding, ggLDLR protein levels slightly decreased, rather than increased, upon culture in LPDS (lane 1 vs. lane 2 in Fig. 3A), and when cellular sterol synthesis was suppressed by pitavastatin, ggLDLR was induced in CEFs (again, to a larger extent in FCS-grown cells than in LPDS, lanes 3 and 4 in Fig. 3A) and to a lesser degree in the LMH cells.

In the LH, an important parameter is the level of LDLR expression in the liver, the major site of synthesis of VLDL destined for oocyte uptake (2). At onset of egg-laying and throughout the reproductive phase, estrogens chronically stimulate the hepatic synthesis of the major yolk precursors, VLDL and vitellogenin, as well as that of the unique apolipoprotein, apoVLDL-II (32); due to their rapid uptake into oocytes, yolk precursor steady state levels remain rather low. In roosters on the other hand, a single administration of estrogen leads to dramatic hypertriglyceridemia and hypercholesterolemia of hepatic origin. Figure 3B demonstrates that the chronic levels of estrogen in the LH are accompanied by lower levels of hepatic LDLR protein than in acutely estrogen-treated rooster, while in the livers of untreated roosters, ggLDLR was very low to undetectable. A faster migrating cross-reacting band (about 110 kDa) was present in immunoblots of hepatic and hepatoma cell samples, but did not appear to be regulated in the same fashion as ggLDLR.

The physiological role of the LDLR in the chicken ovary

Another organ of particular interest in the LH is the ovary, in which follicles harbor oocytes in progressive developmental stages, and which is a source of steroid hormones. Thus, we investigated the expression of ggLDLR in whole ovary and ovarian stroma of normal LHs and of the dramatically hyperlipidemic mutant R/O hens that lack functional LR8. While whole ovary did not contain sufficient amounts of ggLDLR mRNA or protein for detection in either strain (not shown), ovarian stroma from both normal LHs and the nonlaying R/O hens displayed comparable levels of the 130 kDa immunoreactive band (Fig. 3B, stroma). As a control, the two animals' adrenal glands showed significantly higher levels than did ovarian stroma.

If LDL is the main source of cholesterol for steroidogenesis, as is the case in mammals, we would anticipate that the chicken receptor would show preference for LDL versus VLDL, which is directed to oocytes. To test for ligand specificity, we performed competitive ligand blotting using ggLDLR-LA1-7 with ^{125}I-labeled chicken LDL (**Fig. 4**).

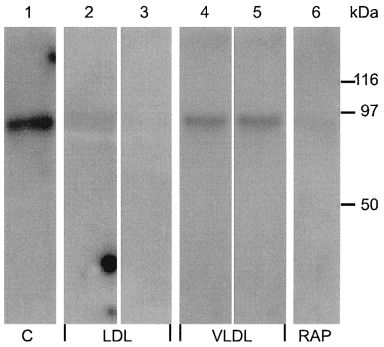


Fig. 4. Inhibition of ^{125}I-LDL binding to LDLR-LA1-7 with VLDL, LDL, and RAP-myc. LDLR-LA1-7 (3 μg each) was subjected to SDS-PAGE under nonreducing conditions followed by transfer to nitrocellulose as described in Materials and Methods. Nitrocellulose strips were incubated in buffer containing the following lipoproteins: lanes 1–6, ^{125}I-LDL (3.3 μg/ml; specific activity 460 cpm/ng); lanes 2–5, plus excess of unlabeled lipoproteins (based on protein concentration) as follows: lane 2, 20× LDL; lane 3, 100× LDL; lane 4, 20× VLDL; lane 5, 100× VLDL; lane 6, the incubation contained RAP-myc (5 μg/ml). Autoradiography was performed by exposure to Kodak X-omat Blue XB-1 film. Molecular weight markers are indicated.

When the binding of radiolabeled LDL was competed for by excess unlabeled LDL (lanes 2 and 3) or VLDL (lanes 4 and 5), LDL displaced the radiolabeled ligand more efficiently than did VLDL. As a control, RAP (5 μg/ml) abolished ^{125}I-LDL binding (lane 6). These data are compatible with a preference of ggLDLR for LDL versus VLDL.

In order to demonstrate the endocytotic competence of the chicken receptor, we performed uptake experiments with fluorescently labeled chicken LDL (**Fig. 5**). First, the localization of ggLDLR on the surface of cultured CEFs was analyzed by immunofluorescence using anti-LA1-7; as shown in Fig. 5A–C, ggLDLR could clearly be localized to the cell surface in nonpermeablized cells. Furthermore, the experiment confirmed the induction of ggLDLR by prior growth of the cells in the presence of pitavastatin (compare Fig. 5B and C). When rhodamine-labeled LDL was prebound at 4°C to pitavastatin-induced cells previously incubated with preimmune serum (Fig. 5D), subsequent warming of the cells to 37°C for 30 min led to efficient internalization of the bound LDL (Fig. 5E). However, when the cells had been preincubated with the monospecific anti-LA1-7 antiserum and then with LDL, no internalization of the ligand could be visualized (Fig. 5F). We conclude that ggLDLR mediates the efficient uptake of LDL by cells.

To shed light on the possible biological role as provider of substrate for steroid synthesis in the follicle, we analyzed ggLDLR expression in terms of cell type, protein level, and developmental stage of the follicle, as shown in **Fig. 6**. GC layers from the third-largest (F3) to the largest (F1) follicle (this is the follicle to ovulate next) as well as the thecal layers from each of these follicles were isolated and ggLDLR protein levels determined. We found that

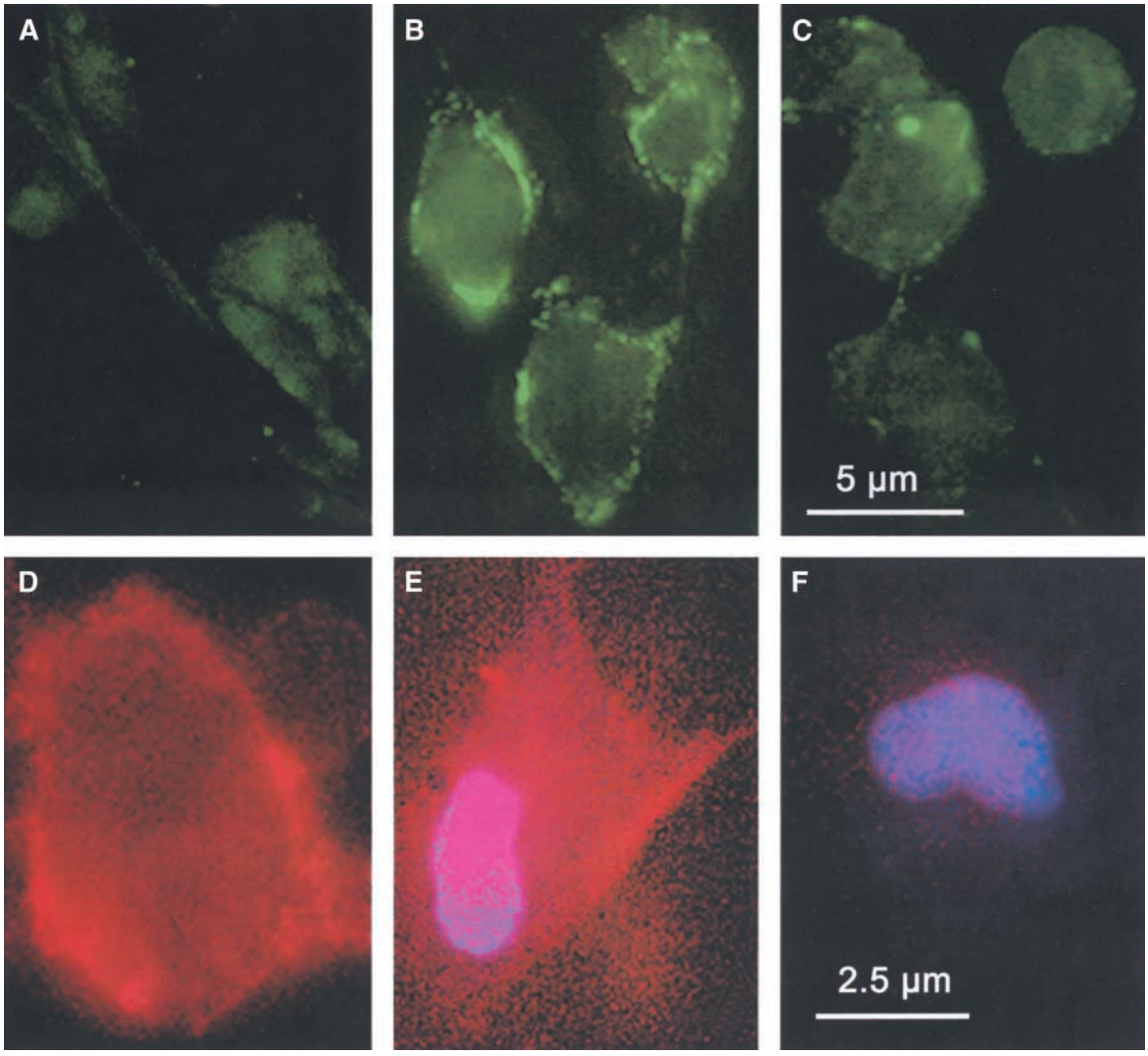


Fig. 5. Immunofluorescence analysis of ggLDLR and rhodamine-labeled LDL binding and uptake. CEFs treated (A, B, D, E, F) or not treated (C) with 1 μM NK104 were grown on culture slides and stained using preimmune (A) or anti-LA1-7 antiserum (1:100; B, C) as described in Materials and Methods. Primary antibodies were visualized with Alexa Fluor 594 goat anti-rabbit IgG. For binding and uptake of rhodamine-labeled LDL, cells were incubated (1 h at 4°C) with preimmune (D, E) or anti-LA1-7 antiserum (F; 1:100) and then with rhodamine-labeled LDL for 1 h at 4°C (D, E, F) as described in Materials and Methods. The cells in D were processed immediately, and the cells in E and F were subsequently warmed up to 37°C for 30 min. The cell nuclei were counterstained with 4′,6′-diamidino-2′-phenylindole (DAPI) (E, F). Bar: 5 μm (A–C); 2.5 μm (D–F).

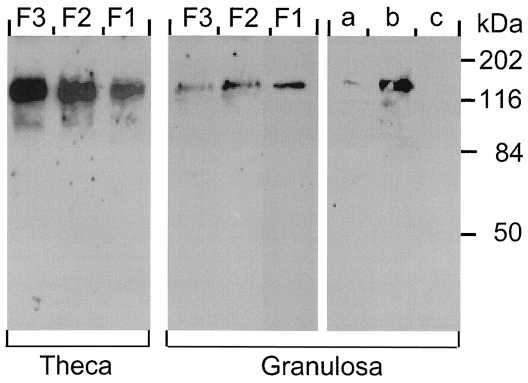


Fig. 6. Immunoblot analysis of LDLR during follicular growth and NK104 induction of LDLR protein in granulosa cells (GCs). Theca and granulosa protein extracts (10 μg each) from F3, F2, and F1 follicles of the LH were electrophoretically separated under nonreducing conditions and subjected to immunoblotting with anti-LA1-7 antibody as described in Materials and Methods. Cultured GCs were incubated as follows: lane a, 10% FCS; lane b, 10% LPDS and 1 μM NK104; lane c, 10% LPDS. Molecular weight markers are as indicated.

with increasing follicle size, ggLDLR levels in the theca decrease, while those in GCs appear to remain constant or increase slightly. As discussed below, this finding may be related to the sterol requirements of the two cell populations and their different roles in ovarian steroidogenesis. Compared with the action of pitavastatin in fibroblasts and LMH cells (Fig. 3A), the compound was the most effective in raising ggLDLR levels in cultured GCs (Fig. 6, lanes a–c). Finally, to shed light on the localization of ggLDLR in the follicle using a different approach, we performed immunohistochemistry with anti-ggLDLR on follicle sections (**Fig. 7**). The highest level of immunoreactivity was observed in the epithelial cells of the follicle wall, and considerable levels were localized in the theca externa, while the GC layer displayed weak but discernible immunoreactivity and the theca interna was devoid of receptor.

DISCUSSION

The current studies provide a definitive answer to the long-standing question of whether birds do express a bona fide LDLR. First, the structure of the novel protein entirely conforms to that of mammalian LDLRs (Fig. 1). Besides containing all of the common structural elements of the LR family, the hallmarks of bona fide LDLRs are *i*) seven LA repeats in the N-terminal domain, where the first four are separated from repeats five through seven by a spacer region of variable length; *ii*) an *O*-linked sugar domain just outside the plasma membrane; and *iii*) a short (~50 residues) cytoplasmic domain with a signature sequence that is important for endocytic activity. All of these elements are present in ggLDLR. Other members of the LR family contain different numbers of LA repeats, may or may not display an *O*-linked sugar domain, and often harbor longer cytoplasmic tails with one to three putative endocytosis consensus sequences. Second, ggLDLR appears to interact preferentially with chicken LDL, and less so with chicken VLDL (Fig. 4). This is significant, since avian LDL and VLDL particles differ in lipid composition, but not in their content of apolipoproteins interacting with LA repeats. Chicken LDL and VLDL contain apoB-100 and lack apoE, which is not expressed in birds. ApoVLDL-II, present on VLDL particles of LHs or ERs, is not involved in binding to LR8 (32); whether it prevents VLDL binding to ggLDLR remains be investigated. The physiological implications of the binding properties of ggLDLR are discussed below. Third, the receptor is induced in cultured cells by blocking cellular cholesterol synthesis with statins. Here we used the first totally synthetic statin, pitavastatin (CAS 147526-32-7, NK104; KOWA Co., Japan), and demonstrated induction of ggLDLR in CEFs, LMH hepatoma cells, and ovarian GCs.

Previous efforts to molecularly characterize any avian LDLR homolog likely have been hampered by the extremely low expression levels in cells and tissues commonly used for LDLR studies (fibroblasts, liver, and even adrenals). Thus, the possibility of testing for the sterol sensitivity of the receptor's expression, the generation of a functionally folded LA domain, and the production of a monospecific inhibitory antibody were key steps in obtaining the current results. Ronacher et al. (27) had observed that when bacterially expressed fragments of human VLDLR (LA1-3, 1–6, and 1–8) were incubated in the presence of immobilized RAP, the yield of LA-competent protein was substantially increased. We obtained the same result with the LA domain of ggLDLR (Figs. 2, 4). Furthermore, the soluble LA domain of ggLDLR fused to MBP provided a strong antigen. Using the antibody raised against this recombinant protein revealed for the first time that in addition to adrenal glands and estrogen-induced liver (mature hens and ERs) (Fig. 3B), the somatic cells of the ovarian follicle express considerable levels of the receptor (Fig. 6).

CEFs, LMH hepatoma cells (Fig. 3A), and GCs (Fig. 6) were more sensitive to growth in LPDS than is generally observed for mammalian cells in culture (12, 33). Transfer to lipoprotein-deficient conditions slowed the growth of subconfluent chicken cell cultures, and per se did not lead to up-regulation of LDLR as observed in human fibroblasts (31). Receptor induction required the inhibition of cellular cholesterol synthesis (Figs. 3A, 6). Previously, we reported that following culture in sterol-free medium, the addition of sterols, but not of lipoproteins, led to down-regulation of LDL binding sites in chicken fibroblasts (12, 33). All of these observations are compatible with limited LDL endocytosis due to low numbers of ggLDLR, and with a high basal activity of cholesterol synthesis. These properties, in turn, support the needs of the oocytes as main targets of lipoprotein uptake for yolk formation. Future studies will address the molecular mechanisms of the regulation of sterol-sensitive genes in the chicken. In this context, differential expression of the transcription factor ADD-1/SREBP-1 appears to be a major determinant of the lipogenic activity (i.e., fatty acid

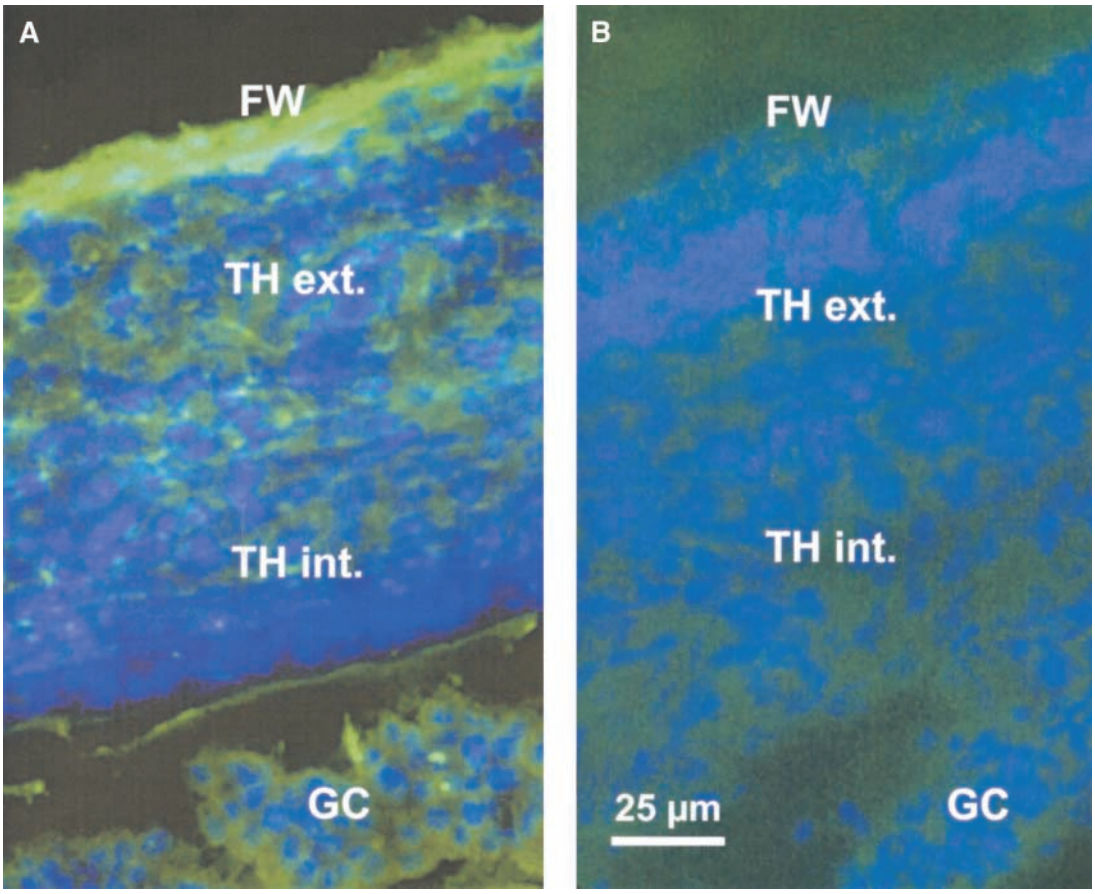


Fig. 7. Immunohistochemical localization of ggLDLR in chicken follicle. Immunohistochemistry was performed on cryostat sections of a chicken F3 follicle (diameter ~2 cm) as described in Materials and Methods using the indicated antibodies. A: Incubation with anti-LA1-7 (1:100); ggLDLR appears in green. B: Incubation with preimmune serum (1:100). Nuclei were counterstained with DAPI and appear in blue. Anatomically distinct parts of the follicle are indicated (TH int., theca interna; TH ext., theca externa; FW, follicular wall).

synthase expression) of the liver in chickens and adipose in mammals, respectively (34).

We have previously characterized the receptor defect in a hyperlipidemic, nonlaying mutant strain of chickens, the R/O strain (35, 36). In short, due to a point mutation in the gene for the receptor that mediates yolk precursor incorporation into oocytes, termed LR8, these animals fail to develop normal oocytes. Thus, LR8 function is absent from R/O oocytes, but ggLDLR levels in the extraoocytic cells of the follicles are not affected, in agreement with an autonomous function of ggLDLR, i.e., to provide LDL-derived cholesterol to the follicle cells. Current models for steroid production by avian follicle cells hold that GCs and theca cells divide the task of steroidogenesis (16, 37, 38). In this context, the expression of ggLDLR in GCs and theca externa cells (Figs. 6, 7) indicates that these cells utilize lipoprotein-cholesterol. Based on the presence of steroid-synthesizing and -converting enzymes and their products (38), cholesterol is converted mainly to progestins in GCs. Theca interna cells (which have low to undetectable ggLDLR levels) have the ability to convert GC-derived progestins to androgens. Finally, the role of theca externa cells is estrogen production, which may occur by aromatizing androgens derived either from the theca interna or by complete synthesis from cholesterol. The latter possibility is suggested by our finding of ggLDLR expression in the theca externa cell layers, thus adding novel details to the multiple-cell model of estrogen production in the avian follicle.

In summary, these studies shed first light on the potential roles of bona fide LDLRs in birds and possibly other egg-laying species. As demonstrated here, compared with the function of the LDLR in regulation of mammalian metabolism and sterol homeostasis, in the LH the delivery of LDL-derived cholesterol appears to be of importance almost exclusively in tissues involved in steroid synthesis, such as the adrenal and the ovary. Thus, in ovarian follicles, there is an economical cooperation between members of the LR family: LDLR plays a key role in steroidogenesis by somatic cells, whereas LR8 is indespensible for yolk precursor uptake by oocytes. Future studies will focus on the mechanisms for coordinated control of the genes involved in these processes.

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REFERENCES

1. Nimpf, J., and W. J. Schneider. 2000. From cholesterol transport to signal transduction: low density lipoprotein receptor, very low density lipoprotein receptor, and apolipoprotein E receptor-2. *Biochim. Biophys. Acta.* **1529:** 287–298.
2. Schneider, W. J., A. Osanger, M. Waclawek, and J. Nimpf. 1998. Oocyte growth in the chicken: receptors and more. *Biol. Chem.* **379:** 965–971.
3. Schneider, W. J., R. Carroll, D. L. Severson, and J. Nimpf. 1990. Apolipoprotein VLDL-II inhibits lipolysis of triglyceride-rich lipoproteins in the laying hen. *J. Lipid Res.* **31:** 507–513.
4. Nimpf, J., and W. J. Schneider. 1994. The chicken LDL receptor-related protein/alpha 2-macroglobulin receptor family. *Ann. N. Y. Acad. Sci.* **737:** 145–153.
5. Schneider, W. J., J. Nimpf, C. Brandes, and M. Drexler. 1999. The low-density lipoprotein receptor family: genetics, function, and evolution. *Curr. Atheroscler. Rep.* **1:** 115–122.
6. Takahashi, S., Y. Kawarabayasi, T. Nakai, J. Sakai, and T. Yamamoto. 1992. Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity. *Proc. Natl. Acad. Sci. USA.* **89:** 9252–9256.
7. Stifani, S., D. L. Barber, R. Aebersold, E. Steyrer, X. Shen, J. Nimpf, and W. J. Schneider. 1991. The laying hen expresses two different low density lipoprotein receptor-related proteins. *J. Biol. Chem.* **266:** 19079–19087.
8. Mahon, M. G., K. A. Lindstedt, M. Hermann, J. Nimpf, and W. J. Schneider. 1999. Multiple involvement of clusterin in chicken ovarian follicle development. Binding to two oocyte-specific members of the low density lipoprotein receptor gene family. *J. Biol. Chem.* **274:** 4036–4044.
9. Barber, D. L., E. J. Sanders, R. Aebersold, and W. J. Schneider. 1991. The receptor for yolk lipoprotein deposition in the chicken oocyte. *J. Biol. Chem.* **266:** 18761–18770.
10. Nimpf, J., S. Stifani, P. T. Bilous, and W. J. Schneider. 1994. The somatic cell-specific low density lipoprotein receptor-related protein of the chicken. Close kinship to mammalian low density lipoprotein receptor gene family members. *J. Biol. Chem.* **269:** 212–219.
11. Novak, S., T. Hiesberger, W. J. Schneider, and J. Nimpf. 1996. A new low density lipoprotein receptor homologue with 8 ligand binding repeats in brain of chicken and mouse. *J. Biol. Chem.* **271:** 11732–11736.
12. Hayashi, K., J. Nimpf, and W. J. Schneider. 1989. Chicken oocytes and fibroblasts express different apolipoproteins-B- specific receptors. *J. Biol. Chem.* **264:** 3131–3139.
13. Schneider, W. J., U. Beisiegel, J. L. Goldstein, and M. S. Brown. 1982. Purification of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. *J. Biol. Chem.* **257:** 2664–2673.
14. Bujo, H., M. Hermann, M. O. Kaderli, L. Jacobsen, S. Sugawara, J. Nimpf, T. Yamamoto, and W. J. Schneider. 1994. Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family. *EMBO J.* **13:** 5165–5175.
15. Steyrer, E., D. L. Barber, and W. J. Schneider. 1990. Evolution of lipoprotein receptors. The chicken oocyte receptor for very low density lipoprotein and vitellogenin binds the mammalian ligand apolipoprotein E. *J. Biol. Chem.* **265:** 19575–19581.
16. Gomez, Y., P. N. Velazquez, M. A. Juarez-Oropeza, and E. Pedernera. 1998. Steroid metabolism in granulosa and theca interna cells from preovulatory follicles of domestic hen (Gallus domesticus). *Anim. Reprod. Sci.* **52:** 81–91.
17. Huang, E. S., K. J. Kao, and A. V. Nalbandov. 1979. Synthesis of sex steroids by cellular components of chicken follicles. *Biol. Reprod.* **20:** 454–461.
18. Huang, E. S., and A. V. Nalbandov. 1979. Testosterone synthesis by chicken follicular cells. *Adv. Exp. Med. Biol.* **112:** 197–202.
19. Kowalski, K. I., J. L. Tilly, and A. L. Johnson. 1991. Cytochrome P450 side-chain cleavage (P450scc) in the hen ovary. I. Regulation of P450scc messenger RNA levels and steroidogenesis in theca cells of developing follicles. *Biol. Reprod.* **45:** 955–966.
20. Hermann, M., F. Seif, W. J. Schneider, and N. E. Ivessa. 1997. Estrogen dependence of synthesis and secretion of apolipoprotein B-containing lipoproteins in the chicken hepatoma cell line, LMH-2A. *J. Lipid Res.* **38:** 1308–1317.
21. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
22. Schneider, W. J., J. L. Goldstein, and M. S. Brown. 1980. Partial purification and characterization of the low density lipoprotein receptor from bovine adrenal cortex. *J. Biol. Chem.* **255:** 11442–11447.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
24. Bujo, H., and T. Yamamoto. 1996. VLDL receptor in health and disease: interview with a receptor in avian oocytes and mammalian muscle and fat cells. *J. Atheroscler. Thromb.* **2:** 71–75.
25. Hayashi, K., S. Ando, S. Stifani, and W. J. Schneider. 1989. A novel sterol-regulated surface protein on chicken fibroblasts. *J. Lipid Res.* **30:** 1421–1428.
26. Tolleshaug, H., K. K. Hobgood, M. S. Brown, and J. L. Goldstein. 1983. The LDL receptor locus in familial hypercholesterolemia: multiple mutations disrupt transport and processing of a membrane receptor. *Cell.* **32:** 941–951.
27. Ronacher, B., T. C. Marlovits, R. Moser, and D. Blaas. 2000. Expression and folding of human very-low-density lipoprotein receptor fragments: neutralization capacity toward human rhinovirus HRV2. *Virology.* **278:** 541–550.
28. Reagan, J. W., Jr., L. R. Miller, and R. W. St Clair. 1990. In vivo clearance of low density lipoprotein in pigeons occurs by a receptor-like mechanism that is not down-regulated by cholesterol feeding. *J. Biol. Chem.* **265:** 9381–9391.
29. Sulistiyani, and R. W. St Clair. 1991. The method of isolation of primary cells and their subculture influences the expression of LDL receptors on pigeon and chicken embryo cells in culture. *Atherosclerosis.* **91:** 123–35.
30. Aoki, T., H. Nishimura, S. Nakagawa, J. Kojima, H. Suzuki, T. Tamaki, Y. Wada, N. Yokoo, F. Sato, H. Kimata, M. Kitahara, K. Toyoda, M. Sakashita, and Y. Saito. 1997. Pharmacological profile of a novel synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Arzneimittelforschung.* **47:** 904–909.
31. Goldstein, J. L., and M. S. Brown. 1976. The LDL pathway in human fibroblasts: a receptor-mediated mechanism for the regulation of cholesterol metabolism. *Curr. Top. Cell. Regul.* **11:** 147–181.
32. Nimpf, J., R. George, and W. J. Schneider. 1988. Apolipoprotein specificity of the chicken oocyte receptor for low and very low density lipoproteins: lack of recognition of apolipoprotein VLDL-II. *J. Lipid Res.* **29:** 657–667.
33. Brown, M. S., and J. L. Goldstein. 1979. Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc. Natl. Acad. Sci. USA.* **76:** 3330–3337.
34. Gondret, F., P. Ferre, and I. Dugail. 2001. ADD-1/SREBP-1 is a major determinant of tissue differential lipogenic capacity in mammalian and avian species. *J. Lipid Res.* **42:** 106–113.
35. Bujo, H., T. Yamamoto, K. Hayashi, M. Hermann, J. Nimpf, and W. J. Schneider. 1995. Mutant oocytic low density lipoprotein receptor gene family member causes atherosclerosis and female sterility. *Proc. Natl. Acad. Sci. USA.* **92:** 9905–9909.
36. Nimpf, J., M. J. Radosavljevic, and W. J. Schneider. 1989. Oocytes from the mutant restricted ovulator hen lack receptor for very low density lipoprotein. *J. Biol. Chem.* **264:** 1393–1398.
37. Gomez, Y., P. N. Velazquez, I. Peralta-Delgado, M. C. Mendez, F. Vilchis, M. A. Juarez-Oropeza, and E. Pedernera. 2001. Follicle-stimulating hormone regulates steroidogenic enzymes in cultured cells of the chick embryo ovary. *Gen. Comp. Endocrinol.* **121:** 305–315.
38. Velazquez, P., Y. Gomez, M. Gonzalez del Pliego, and E. Pedernera. 1991. Steroidogenic cell subpopulations obtained from the theca of preovulatory follicles in the ovary of the domestic fowl. *Gen. Comp. Endocrinol.* **83:** 243–248.
39. Yamamoto, T., C. G. Davis, M. S. Brown, W. J. Schneider, M. L. Casey, J. L. Goldstein, and D. W. Russell. 1984. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell.* **39:** 27–38.