

Avian apolipoprotein A-V binds to LDL receptor gene family members

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Abstract Apolipoprotein A-V (apoA-V) affects plasma triglyceride (TG) levels; however, the properties of apoA-V that mediate its action(s) are still incompletely understood. It is unclear how apoA-V, whose plasma concentration is extremely low, can affect the pronounced TG differences observed in individuals with various apoA-V dysfunctions. To gain novel insights into apoA-V biology, we expanded our previous studies in the chicken to this apolipoprotein. First, we characterized the first avian apoA-V, revealing its expression not only in liver and small intestine but also in brain, kidney, and ovarian follicles and showing its presence in the circulation. Second, we demonstrate directly that galline apoA-V binds to the major LDL receptor family member (LR) of the laying hen and that this interaction does not depend on the association of the apolipoprotein with lipid or lipoproteins. **■** We propose that a direct interaction with LRs may represent a novel, additional mechanism for the modulation of TG levels by apoA-V.—Dichlberger, A., L. A. Cogburn, J. Nimpf, and W. J. Schneider. Avian apolipoprotein A-V binds to LDL receptor gene family members. *J. Lipid Res.* 2007. 48: 1451–1456.

Supplementary key words triglycerides • low density lipoprotein receptor • chicken

Apolipoprotein A-V (apoA-V) was discovered by comparative genomic analysis of human and murine DNA and by a cDNA subtraction approach in the rat (1). In murine models and human subjects, strong correlations of apoA-V expression levels and DNA sequence polymorphisms, respectively, with plasma triglyceride (TG) levels have been observed (2; reviewed in Ref. 3). In the rat, hepatic apoA-V expression is induced significantly after partial hepatectomy (1). The effects of genetically manipulating murine apoA-V underscore the importance of this exchangeable apolipoprotein (4) in the maintenance of normal TG levels: overexpressing mice are hypotriglyceridemic, and knockout mice are hypertriglyceridemic (2). However, in contrast to results in animal studies, human

plasma apoA-V positively correlates with plasma TG levels (5), and APOAV may thus define a TG-modifier gene (6).

Despite many recent studies, the apoA-V activity responsible for the effects on TG levels and metabolic events remains unclear. As discussed previously (7), possible functions include proteoglycan-dependent direct modulation of LPL activity, interference with the secretion of nascent TG-rich lipoproteins, and/or indirect effects on lipolysis via apoA-V binding to heparan sulfate proteoglycans. Another possibility for the action of apoA-V has been indicated by the finding (8) that VLDL particles from *apoA5*^{-/-} mice are poorer competitors for binding to the LDL receptor than those from normal mice, implying a possible role for apoA-V in mediating or modulating lipoprotein receptor binding. This mode of action is supported by a report (9) that appeared during the revision of this article. By surface plasmon resonance measurements, the authors demonstrated the interaction of human recombinant apoA-V with two membrane receptors, the LDL receptor-related protein (10) and the mosaic receptor SorLA/LR11 (11).

Studies in nonmammalian species can reveal novel aspects of apoA-V biology. We and others are exploiting the laying hen to answer questions concerning key components of lipoprotein metabolism. Studies have focused on the molecular genetics of endocytic receptors belonging to the low density lipoprotein receptor gene family in oocytes and somatic cells (12), lipoprotein-modifying enzymes (13), follicular lipoprotein transport (14), and apolipoproteins (15, 16). For instance, a study in the chicken indicated that the enzymatic activity of LCAT was not dependent on cysteine residues at positions 31 and 184, as the active avian enzyme contains phenylalanine and asparagine, respectively, in the corresponding positions (13). Another example of the model's power was the demonstration of a mutation in ABCA1 as the cause for severe HDL deficiency in the Wisconsin hypoalpha mutant chicken strain (17), which is the only known naturally occurring animal model for Tangier disease. Finally, as

Manuscript received 21 December 2006 and in revised form 15 March 2007 and in re-revised form 26 March 2007.

Published, JLR Papers in Press, March 30, 2007.
DOI 10.1194/jlr.C600026.JLR200

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for the delineation of apolipoprotein function, chicken apoVLDLII has been identified as a prototype LPL inhibitor (18).

The chicken *APOA1* and *APOA4* genes are located in a cluster on chicken chromosome 24. To our knowledge, the exact primary structure of galline apoA-V has not yet been determined, nor has an *APOC3* locus been unambiguously identified in the chicken to date. Here, we show that *i*) chickens do synthesize the protein encoded by the avian *APOA5* gene (i.e., ggapoA-V); *ii*) ggapoA-V is detectable in the circulation; and *iii*) chicken apoA-V interacts with a prominent member of the avian LDL receptor gene family, as determined by direct ligand blotting methodology.

MATERIALS AND METHODS

Animals

Mature Dercu-Brown hens (30–40 weeks old) were purchased from Heindl Co. (Vienna, Austria). All animals were maintained

on layer's mash with free access to water and feed and with a daily light period of 14 h.

Protein expression and antibodies

A 984 bp cDNA fragment encoding full-length chicken apoA-V (ggapoA-V) lacking the 23 residue signal peptide (Fig. 1) was cloned into the pET25b+ expression vector (Novagen), providing a C-terminal His₆ tag. The primers were as follows: forward, 5'-ATCCATGGATCGGAGTGGCTTCTGGGAG-3' (*NcoI* site in boldface); and reverse, 5'-AAGAATTCCGGCGGTCCCCGCCGCG-3' (*EcoRI* site in boldface). Recombinant ggapoA-V was expressed in BL21 cells (Invitrogen) and purified under native conditions using nickel-nitrilotriacetic acid agarose (Qiagen). Expression and purification were performed according to the protocol from Qiagen. Antiserum against recombinant ggapoA-V was raised in adult female New Zealand White rabbits by injections of 250 µg each of antigen as described previously (14). Murine monoclonal Penta-His antibody (Qiagen; used at 1:500) and mouse anti-glutathione *S*-transferase (GST) antibody (BD Pharming; used at 1:2,000) were purchased from the indicated sources. Rabbit anti-LR8 IgG was prepared as described previously (19). Recombinant GST-receptor-associated protein (RAP) fusion protein was produced in DH5 bac-

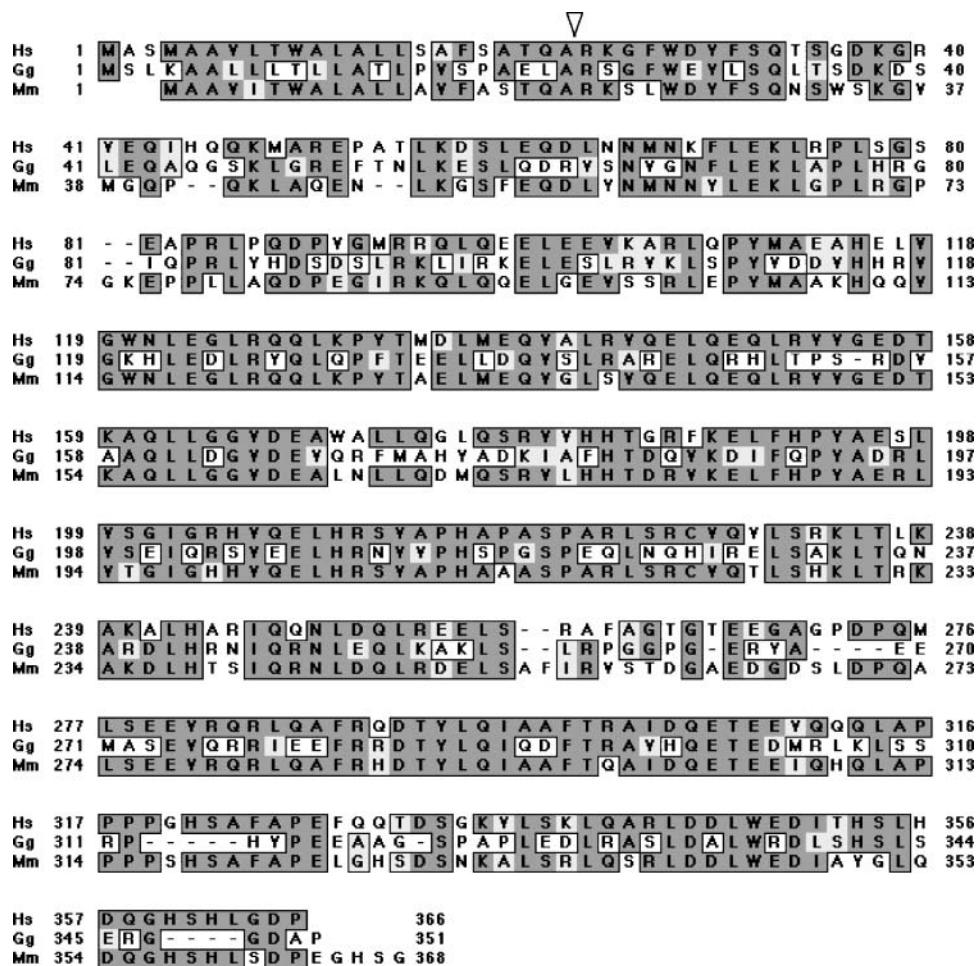


Fig. 1. Alignment of the amino acid sequences of apolipoprotein A-V (apoA-V) from human (Hs), chicken (Gg), and mouse (Mm). Identical residues are boxed in dark gray, and conservative substitutions are boxed in light gray. The predicted signal cleavage site in the apoA-V precursor molecules is indicated by an open arrowhead. Numbering starts with the initiator methionine, 23 residues upstream of the signal sequence cleavage site.

teria using a PGEX 2T-derived (Pharmacia) expression plasmid (20).

Preparation of tissue and membrane protein extracts

All operations were performed at 4°C. For total protein extracts, fresh chicken tissues were homogenized (5 ml/g wet tissue) with an Ultra-Turrax T25 homogenizer in buffer containing 20 mM HEPES (pH 7.4), 300 mM sucrose, 150 mM NaCl, and complete protease inhibitor cocktail (Roche; catalog No. 11836170001). Homogenates were spun at 620 *g* for 10 min, and Triton X-100 was added to the supernatant to a final concentration of 1%. After incubation for 30 min, a supernatant (designated the total tissue extract) was obtained by centrifugation at 300,000 *g* for 60 min, quickly frozen in liquid N₂, and stored at -70°C until use. Membrane fractions and extracts were prepared from fresh chicken tissues as described (21), except that the extraction buffer contained 1% Triton X-100. The clear supernatant, designated the membrane extract, was treated as described above.

Blotting procedures

For Western blotting of apoA-V and, where indicated, of LRS, extracts were analyzed by one-dimensional 12% SDS-PAGE under reducing conditions and electrophoretically transferred to nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences). Nonspecific binding sites were blocked with TBS (20 mM Tris-HCl, pH 7.4, and 137 mM NaCl) containing 5% (w/v) nonfat dry milk and 0.1% Tween-20 (blocking buffer) for 1 h at 23°C. ggapoA-V was detected with rabbit anti-ggapoA-V antiserum (1:2,000 dilution) followed by incubation with HRP-conjugated goat anti-rabbit IgG (1:40,000) and development with the enhanced chemiluminescence protocol (Pierce). The sizes of proteins were estimated with a broad-range molecular mass standard (20–250 kDa) from Bio-Rad.

For ligand blotting, nitrocellulose strips containing SDS-PAGE-separated ovarian follicle membrane extract were incubated for 1 h at 23°C with blocking buffer containing 2 mM CaCl₂. The strips were then incubated with Triton X-100 extracts of BL21 cells expressing recombinant ggapoA-V, or with ggapoA-V purified as described above (designated as crude or purified, respectively, in Figs. 4, 5 below) for 18 h at 4°C. Subsequently, the strips were washed three times for 20 min each with TBS containing 2 mM CaCl₂ and 0.1% Tween-20 and then incubated in blocking buffer with anti-ggapoA-V antiserum (1:2,000). After washing three times for 10 min each, the primary antibody was detected with HRP-conjugated goat anti-rabbit IgG (1:40,000) and enhanced chemiluminescence as described above.

Preparation of chicken lipoproteins

Blood of laying hens was collected in a tube containing EDTA (final concentration, 1 mg/ml) and placed on ice, and plasma was obtained by centrifugation at 4°C. For the preparation of chicken VLDL (*d* < 1.006 g/ml), the plasma was subjected to ultracentrifugation at 200,000 *g* for 24 h, and chicken VLDL was recovered from the top. To obtain the total lipoprotein fraction, plasma was subjected to centrifugation at a density of 1,210 g/ml (22). The lipoproteins were dialyzed against 10 mM Tris-HCl, 140 mM NaCl, and 1 mM EDTA, pH 7.4, sterile-filtered, and stored at 4°C. For electrophoretic analysis, aliquots were delipidated in chloroform-methanol (3:1, v/v) and diethylether-ethanol (2:1, v/v) at -20°C.

RESULTS

The sequence of XM_417939.2 (National Center for Biotechnology Information; predicted: *Gallus gallus*, similar

to apoA-V, gi:118101988) was used to produce a 1,053 bp cDNA fragment by RT-PCR using RNA from laying hen liver and the primer pair 5'-ATGTCGCTGAAGGCTGCGCTTCTG-3' (forward) and 5'-TCAGGGCGCGTCCCCGCC-3' (reverse). Comparative genomic sequence analysis confirmed that the chicken *APOA5* locus is localized in a gene cluster on chromosome 24 that also contains *APOA1* and *APOA4*. The DNA sequences determined from five independent amplicons were identical, and the predicted precursor protein sequence of 351 amino acids is shown in Fig. 1. The alignment with human (366 residues) and murine (368 residues) apoA-V proteins revealed a high degree of conservation of this gene product from chicken to mammals: identities and similarities (considering conservative replacements) are 42% and 61%, respectively, between chicken and human proteins and 39% and 59%, respectively, between chicken and murine apoA-V; for comparison, human and murine apoA-V are 72% identical and 82% similar. On the other hand, galline apoA-I and apoA-V are only 18% identical and have a similarity of 35%. The ggapoA-V precursor protein is predicted to be cleaved between alanine-23 and arginine-24, a position corresponding exactly to alanine/arginine pairs in the human and murine homologs (Fig. 1). In the center of ggapoA-V, a region rich in positively charged and hydrophobic residues reminiscent of motifs in mammalian apoE and apoB is particularly well conserved. Consensus *N*-glycosylation sites are absent from all apoA-V proteins analyzed.

Next, the mature protein (328 residues) was expressed as a His₆-tagged fusion protein in BL21 cells, purified, and used to generate rabbit antiserum. The antiserum recognized an ~40 kDa plasma protein in hens and roosters; preimmune serum showed no reactivity (Fig. 2). Western blotting revealed the presence of ggapoA-V in the VLDL fraction obtained by ultracentrifugation (*d* < 1,006 g/ml)

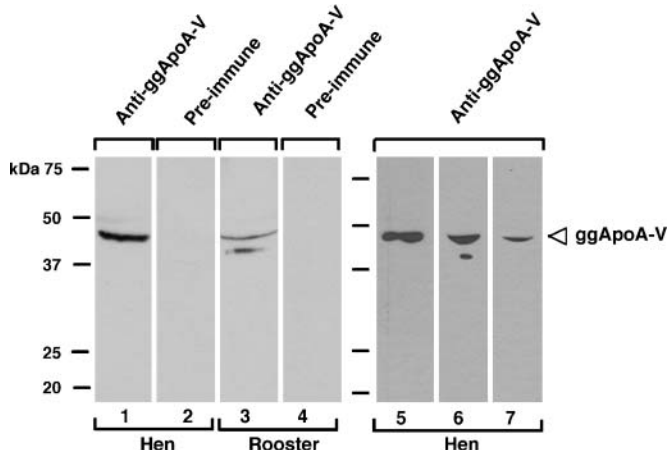


Fig. 2. Western blot analysis of apoA-V in the circulation of the chicken. Plasma proteins from hens (lanes 1, 2, 7) and roosters (lanes 3, 4) as well as VLDL (lane 5) and the *d* > 1.21 fraction (lane 6) from hen plasma were separated by 12% SDS-PAGE under reducing conditions and probed with either anti-ggapoA-V antiserum or preimmune serum, as described in Materials and Methods. The positions of migration (kDa) of marker proteins are indicated.

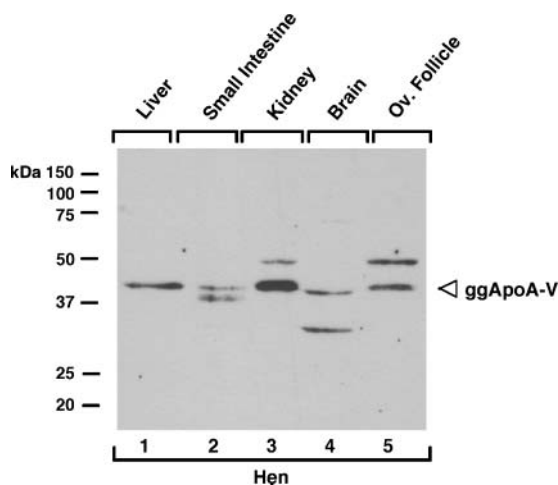


Fig. 3. Detection of ggapoA-V protein in peripheral tissues. Total protein extracts (50 μ g per lane) from mature hen liver (lane 1), small intestine (lane 2), kidney (lane 3), brain (lane 4), and ovarian small white follicles (diameter, 3 mm; lane 5) were separated by 12% SDS-PAGE under reducing conditions and analyzed by Western blotting with rabbit anti-ggapoA-V antiserum, as described in Materials and Methods. The positions of migration (kDa) of marker proteins are indicated.

as well as in the resulting bottom fraction (Fig. 2, lane 6); also, the total lipoprotein fraction (floated at $d < 1,210$ g/ml) contained the protein in significant amounts (data not shown). In addition, ggapoA-V was detected in total protein extracts of liver, small intestine, kidney, brain, and ovarian small white follicles (Fig. 3). Interestingly, the level of immunoreactive protein in the kidney, a very active site of apolipoprotein synthesis in the chicken (23), was among the highest observed (Fig. 3, lane 3).

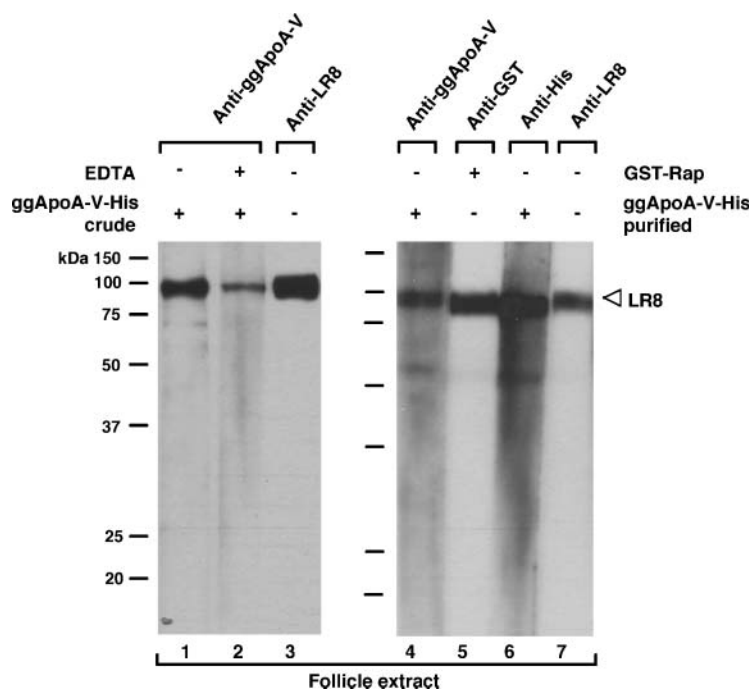


Fig. 4. Recombinant ggapoA-V binds to chicken lipoprotein receptor. Nitrocellulose strips containing SDS-PAGE-separated proteins from ovarian follicle membrane extract were incubated with Triton X-100 extracts obtained from BL21 cells expressing His₆-tagged ggapoA-V (lanes 1, 2) or with purified His₆-tagged ggapoA-V (lanes 4, 6) in the absence (lane 1) or presence (lane 2) of EDTA or with glutathione *S*-transferase-receptor-associated protein (GST-RAP; lane 5). The strips were washed and incubated with anti-ggapoA-V antiserum (lanes 1, 2, 4), anti-His antibody (lane 6), or anti-GST antibody (lane 5). LDL receptor relative 8 (LR8) in the ovarian follicle membrane extract was identified by Western blotting with antibody against LR8 (lanes 3, 7). The bound primary antibodies were detected either with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG and the chemiluminescence detection method, as described in Materials and Methods. The positions of migration (kDa) of marker proteins are indicated.

Next, in our efforts to gain insight into apoA-V action, we directly tested for the binding of chicken apoA-V to LDL receptor relatives (LRs) by ligand blotting (24). As a source of receptor, we used membrane extracts of chicken ovarian follicles, which are enriched in the major oocyte receptor for yolk precursors, termed LR8 (25). The ligand blotting incubation buffer contained a crude extract of BL21 expressing His₆-tagged ggapoA-V (Fig. 4, lanes 1–3) or purified ggapoA-V (Fig. 4, lanes 4, 6), and the position of bound ggapoA-V was visualized by immunoblotting with anti-His (lane 6) or anti-ggapoA-V (lanes 1, 2, 4) antibodies. Clearly, both ggapoA-V from the crude bacterial extract and the purified ggapoA-V bound to a 95 kDa protein in the follicle extract that comigrated with, and was immunologically demonstrated to represent, LR8 (Fig. 4, lanes 3, 7). When the incubation contained ggapoA-V plus EDTA, which chelates divalent cations required for LR-ligand interactions, a greatly diminished signal was observed (Fig. 4, lane 2). Figure 4, lane 5 directly demonstrates the binding of RAP, a known pan-inhibitor of LR-ligand interactions, to LR8, confirming the ligand binding competence of LR8 in the follicle extract.

Accordingly, Fig. 5 shows that GST-RAP very efficiently competes with ggapoA-V for binding sites on LR8 (lane 1 vs. lane 2). Furthermore, when the follicle extract had been subjected to SDS-PAGE under reducing conditions, the binding of ggapoA-V was abolished, demonstrating that the interaction with LR8 required intact disulfide bonds in the receptor, as shown previously for the binding of apoB, apoE, RAP, and clusterin (reviewed in Ref. 12). These experiments confirm that the interaction of ggapoA-V, either in a crude bacterial detergent lysate or in purified form, is specific for a prototype member of the LR gene family and that this binding displays all of the properties

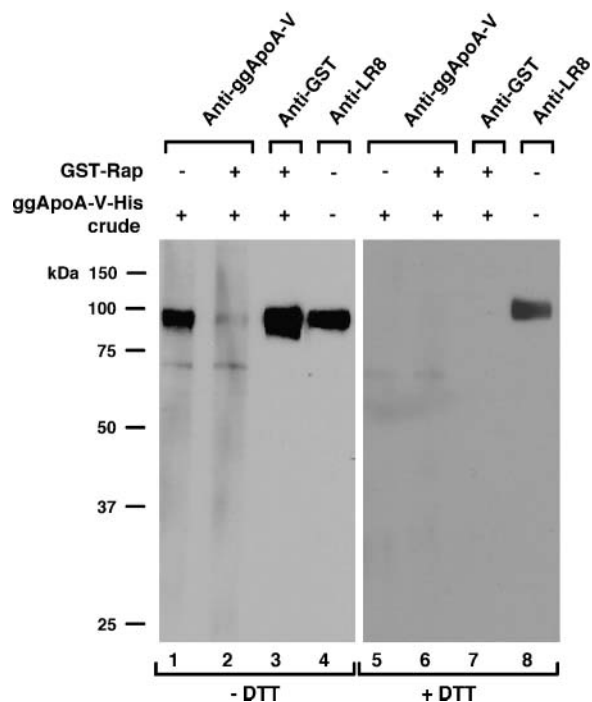


Fig. 5. Receptor-associated protein competes with ggapoA-V for receptor binding sites. Nitrocellulose strips containing SDS-PAGE-separated proteins under nonreducing (lanes 1–4) or reducing (lanes 5–8) conditions with ovarian follicle membrane extract were incubated either with crude protein extract obtained from BL21 cells expressing His₆-tagged ggapoA-V (lanes 1, 5) alone or together with purified chicken GST-RAP (lanes 2, 3, 6, 7). The washed strips were incubated with anti-ggapoA-V antiserum (lanes 1, 2, 5, 6) or anti-GST antibody (lanes 3, 7). Anti-LR8 antibody (lanes 4, 8) was used to visualize LR8 in the ovarian follicle membrane extract. The positions of migration (kDa) of marker proteins are indicated.

that characterize the interactions of bona fide ligands with the cysteine-rich ligand binding domains of LRs.

DISCUSSION

This study has revealed a novel property of apoA-V, which suggests an additional mechanism for the action of apoA-V in regulating plasma TG levels. As indicated previously (6, 7), apoA-V must be a potent agent despite its low plasma concentration. In addition to the proposed apoA-V-mediated activation of lipolysis by bridging TG-rich lipoproteins and LPL-proteoglycan complexes (26), the possibility that apoA-V may be a ligand for lipoprotein receptors has been raised (7, 8). If such an interaction occurred, it would likely influence directly or indirectly the extent of receptor-mediated clearance of particles generated in lipolytic pathways. In this context, it is of interest that the proportion of lipoprotein-free ggapoA-V appears to be significant (Fig. 2; data not shown) and that this form binds to LRs (Figs. 4, 5). If this holds true for humans, free apoA-V, possibly represented by the exchangeable pool (27), might be a potent TG modulator despite the low

concentration of total apoA-V (28, 29). Nilsson et al. (9) reported an interaction of human apoA-V, in both free and lipid-bound form, with LRP and SorLA/LR11, as determined by surface plasmon resonance. They suggested that circulating levels of apoA-V may be low because free apoA-V remains receptor-bound during recycling after endocytosis. Finally, the possibility that lipid-free human wild-type apoA-V exists was demonstrated in a heterozygote whose mutant allele produced truncated apoA-V [Q139X; (30)] and is compatible with the reported solubility of apoA-V [insoluble at >0.1 mg/ml, which is, however, 100- to 1,000-fold greater than even total apoA-V concentrations (31)].

We propose that the regulation of the receptor-modulating activity of apoA-V occurs at the level of its distribution in serum and at the site(s) of clearance, parameters that are related to LR levels, to liver status (1), to apoC-II/C-III ratios, to the apolipoprotein content at the hepatocyte surface (6, 8), and/or to expression levels of proteoglycans (7). Fine-tuned binding of apoA-V to LRs appears to be compatible with the positive correlation between apoA-V and TG levels in human (29) (via competing for binding sites for TG-rich LPs) and, on the other hand, conforms with the severe hypertriglyceridemia and reduced LPL mass and activity of patients with truncated forms of apoA-V (30, 32) (via dysfunction of apoA-V as a ligand for LRs, LPL, and proteoglycans).

These studies in the chicken, which have revealed a novel property of apoA-V, also have new implications and raise new questions. These are concerned with whether or not receptor binding affinity is influenced by the degree of lipid association of ggapoA-V and whether binding is invariably coupled to internalization. Also, because the chicken does not express apoE (33, 34), ggapoA-V may compensate functionally for the absence of this important LR ligand. Finally, delineation of the relevance and role(s) of apoA-V in the kidney, brain, and ovarian follicles may reveal further general insights into apoA-V biology. ■

This study was supported by the Austrian Science Foundation and the Herzfeldersche Endowment (to W.J.S.).

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