Abstract  Plasma HDL levels are inversely associated with atherosclerosis. Inbred mouse strains differ in plasma HDL levels and susceptibility to atherosclerosis. Atherosclerosis-susceptible C57BL/6J mice possess plasma HDL levels 2-fold lower than atherosclerosis-resistant FVB/NJ mice. Polymorphisms have been previously identified between the two mouse strains in the major HDL apolipoproteins, ApoA-I and ApoA-II, which may affect their function on HDL. To begin to understand the HDL differences, we here report on a detailed comparison of the lipid-associated functions of the two mouse ApoA-I proteins. We demonstrate that these polymorphisms significantly alter the protein self-association properties, the ability of the proteins to clear lipid micelles from solution, and their binding affinity for mature mouse HDL. The changes in lipid binding do not appear to alter the ability of the protein to promote cholesterol efflux from cells or the formation of nascent HDL from primary hepatocytes. These apolipoprotein polymorphisms do not change the rate at which HDL protein or cholesterol are catabolized in vivo. Although the presence of the polymorphisms in ApoA-I alters important factors in HDL formation, the basis for the differences in the HDL plasma levels observed in the various mouse strains is more complex and requires additional investigation.—Sontag, T. J., R. Carnemolla, T. Vaisar, C. A. Reardon, and G. S. Getz. Naturally occurring variant of mouse apolipoprotein A-I alters the lipid and HDL association properties of the protein. J. Lipid Res. 2012. 53: 951–963.

Supplementary key words  cholesterol efflux • lipid binding • alpha helix • high density lipoprotein

Cardiovascular disease remains one of the leading causes of death worldwide (1). The progression of atherosclerosis is a major contributor to cardiovascular disease development (2). Although not fully understood, plasma lipoproteins play an important role in atherosclerosis, with plasma HDL cholesterol levels being inversely correlated with risk of cardiovascular disease (3). Much of our current knowledge of atherosclerosis comes from the use of atherosclerosis-susceptible animal models and particularly mouse models. Numerous inbred mouse strains display varying degrees of susceptibility to atherosclerotic lesion development, although the causes of these differences are unclear (4). Because wild-type (WT) mice do not develop atherosclerotic lesions, these comparisons were made using mice fed a cholate-containing diet high in fat and cholesterol in order to induce lesion formation. However, this diet also induces an inflammatory response. Since then, several studies have shown further strain differences in atherosclerosis susceptibility in apolipoprotein (Apo)E and LDL-receptor knockout mice, the most commonly used genetic models of atherosclerosis (5, 6). Most atherosclerosis studies have been performed in the C57BL/6 (C57) strain, which appears to be the most sensitive of the mouse strains. Thus, in both genetic models, the C57BL/6 mouse strain develops lesions more than 5-fold greater in size than the atherosclerosis-resistant FVB/NJ (FVB) strain. These two mouse strains also differ in lipoprotein levels. In WT, ApoE, and LDL-receptor knockout mice, the FVB strain displays 2-fold higher levels of plasma HDL cholesterol (5, 6). The same is true in numerous other atherosclerosis-resistant strains (7). The genetic basis of increased HDL cholesterol and the resulting impact on atherosclerosis susceptibility in these mouse strains is not completely understood.

Abbreviations: acetyl-CoA, cholesterol-acyltransferase; Apo, apolipoprotein; CPT-cAMP, 8-(4-chlorophenyl-thio)-cAMP; DMPC, dimyristoyl-phosphatidylcholine; IPTG, isopropyl-β-D-thiogalactopyranoside; LCAT, lecithin-cholesterol acyltransferase; LPDS, lipoprotein-deficient serum; SPR, surface plasmon resonance.

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ApoA-I and ApoA-II make up greater than 90% of the protein component of HDL, with ApoA-I being the predominant protein (~70%) (8). These two proteins exhibit several sequence differences between the C57 and the FVB strains. The 240 amino acid ApoA-I of the atherosclerosis-susceptible C57 mouse possesses two differences in amino acid sequence from the FVB ApoA-I (C57 to FVB: Q225K, V226A) (9, 10). These two sequence differences are the only known naturally occurring polymorphisms in inbred mouse ApoA-I.

ApoA-I plays a significant role in HDL formation, and its absence results in at least a 70% reduction in plasma HDL levels (11). ApoA-I interacts with the ATP-binding cassette transporter ABCA1, by which ApoA-I receives cholesterol and phospholipid that is effluxed from the cell (12). ApoA-I is composed of 10 amphipathic α-helices, with helices 9 and 10 being responsible for the initial binding of human ApoA-I to lipid and its association with the ABCA1 transporter (13, 14). The two amino acids that differ between C57 and FVB ApoA-I are adjacent to each other in the center of helix 10. Mutations in helix 10 of human ApoA-I have been shown to decrease the rate of clearance of phospholipid in solution and the efflux rate of cholesterol from cultured macrophages (14). The Nichinan variant of human ApoA-I lacks E235 in helix 10 and is associated with decreased ABCA1-mediated cholesterol efflux from macrophages and decreased plasma HDL levels (15). It has been shown that the tertiary structure of lipid-free human ApoA-I is made up of two domains: an N-terminal α-helix bundle from residues 1–187 (through helix 7) and a C-terminal less organized region (helices 8–10), which is the major lipid-binding region of the molecule (16, 17). Recent work has suggested that the mouse ApoA-I is similarly made up of the N- and C-terminal domains, but, unlike the human ApoA-I, the N-terminal domain is more effective than the C-terminal in lipid binding and cholesterol efflux from macrophages (18–20). Thus, the role of the mouse C-terminal domain in ApoA-I function remains unclear. In light of this, it is important to understand whether the naturally occurring changes in the C-terminal domain of FVB ApoA-I significantly alter the lipid interactions of the protein, thereby potentially altering the protein function.

Here, we report the first in depth study of the role of these polymorphisms on lipid binding, cholesterol efflux, hepatic HDL production, and HDL clearance. The C-terminal polymorphisms result in significant differences in lipid binding capability and HDL association. However, the sequence differences alone do not appear to alter the cholesterol efflux capabilities of the lipid-free ApoA-I or of the mature HDL particle, nor do they appear to be solely responsible for plasma HDL cholesterol levels.

**MATERIALS AND METHODS**

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. C57BL/6j or FVB/NJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were bred, and the pups were weaned at 21–28 days and maintained on chow diet #7915 from Harlan Labs (Indianapolis, IN). Female mice were used for all experiments described. All procedures performed on the mice were in accordance with National Institute of Health and institutional guidelines.

**HDL isolation**

Plasma was obtained from fasted or nonfasted anesthetized mice. HDL was isolated from plasma by FPLC (21) or density gradient centrifugation. Density gradient centrifugation was done by bringing plasma volume to 1 ml in NaBr solution adjusted to a final density of 1.300 g/ml. On top of this were layered density solutions of 1.21 g/ml (3.2 ml), 1.063 g/ml (3.6 ml), 1.019 g/ml (3.2 ml), and 1.006 g/ml (1 ml), and the samples were spun for 22 h at 178,000 g. The resulting sample was fractionated manually into 20–30 fractions of equal volume. Whole plasma FPLC fractions and density gradient fractions were analyzed for protein (Bio-Rad Laboratories Inc., Hercules, CA), total cholesterol and triglyceride (Roche Diagnostics, Indianapolis, IN), and free cholesterol and phospholipid (Wako Chemicals, Richmond, VA). Fractions containing HDL were then combined and dialyzed/concentrated into PBS using a 100 Kd cut-off Centricon concentrator (Millipore, Billerica, MA) and stored at 4°C.

HDL apolipoprotein levels were analyzed using two methods. First, FPLC HDL fractions were analyzed by SDS–PAGE, and Coomassie-stained bands were quantitated using Fluorchem v2.0 analysis software. For the second method, HDL from 10 animals of each strain was isolated by density centrifugation, and the resulting HDL fractions were combined for each mouse strain. Density gradient centrifugation was performed again on this isolated HDL for further purification. The peak fraction was determined by cholesterol analysis of each gradient fraction, and the apolipoproteins in this fraction were identified and quantitated by proteomics analysis as described below (22). 3H-cholesterol-labeled HDL was generated in nonfasted C57 or FVB mice by oral gavage of 200 µl 3H-cholesterol (Perkin-Elmer, Waltham, MA) in olive oil at a concentration of 10 µCi/ml. After 8 h, mice were terminally bled, and HDL was isolated from plasma by density gradient centrifugation as described above.

**Proteomic analysis of the HDL particles**

Isolated HDL particles (10 µg protein) were adjusted to a final concentration of 100 µg/ml with digestion buffer (0.1% Rapigest; Waters, 50 mM Tris buffer, pH 8.0). Samples were reduced, alkylated, and digested at 37°C with two aliquots of trypsin (1:50, w/w, trypsin/protein; Promega), first for 2 h and then overnight. Proteolysis was stopped by adding HCl (final concentration, 30 mM). The samples were incubated for 45 min at 37°C to hydrolyze the Rapigest detergent and then clarified by centrifugation. The supernatants were dried under vacuum and resuspended in 5% acetonitrile (0.3% acetic acid) for mass spectrometric analysis. Tryptic digests (2 µg protein) were injected onto a trap column (Paradigm Platinum Peptide Nanotrap, 0.15 × 50 mm; Michrom Bioresources, Inc.), desalted for 1 min with 0.1% formic acid (0.1% acetonitrile), 0.1% formic acid (50 µl/min), eluted onto an analytical reverse-phase column (0.15 × 150 mm, Magic C18AQ, 5 µm, 200 A; Michrom Bioresources, Inc.), and separated at a flow rate of 1 µl/min over 180 min, using a linear gradient of 5% to 55% buffer B (90% acetonitrile, 0.1% formic acid) in buffer A (5% acetonitrile, 0.1% formic acid). Mass spectra were acquired in the positive ion mode using electrospray ionization in a linear ion trap mass spectrometer (LTQ; Thermo Electron Corp., San Jose, CA) with data-dependent acquisition (one MS survey scan followed by MS/MS scans of the eight most abundant peaks in the survey scan). For protein identification, MS/MS spectra were matched against the human International Protein Index database (23), using the SEQUEST (v 2.7) search engine with fixed Cys carbamidomethylation and variable Met oxidation modifications. The mass tolerance...
for precursor ions was 2.5 kDa, and the SEQUEST default tolerance was accepted for product ions (24). SEQUEST results were further validated with PeptideProphet and ProteinProphet, using an adjusted probability of $>0.90$ for peptides and $>0.95$ for proteins (25, 26). Each charge state of a peptide was considered a unique identification. Relative abundance of the proteins was expressed in terms of spectral counts (number of identified MSMS spectra for peptides derived from a given protein) (27).

**Recombinant apolipoprotein synthesis**

Mature mouse ApoA-I cDNA was generated for each mouse strain by performing RT-PCR on total liver RNA for each mouse strain that had been isolated using Qiagen RNeasy mini kit (Qiagen, Valencia, CA). The 5’ primer used for cloning contained a BamHI site and the 3’ primer contained an XhoI site, allowing for subcloning into the pET28c+ vector (Novagen, Gibbstown, NJ). The resulting construct contained a poly-His site followed by T7 site directly upstream of the ApoA-I site and was transformed into the strain by performing RT-PCR on total liver RNA for each mouse.

**Peptide synthesis and purification**

The mouse C57 and FVB ApoA-I helix 9/10 peptides (mouse ApoA-I amino acids 206–240) were synthesized manually by solid phase methods using standard tert-butoxycarbonyl chemistry: NH$_2$-PALEDLRHSLL$_{16}$PMPLELTQK$_{20}$VQSVKAS$_{30}$-COOH (C57) or NH$_2$-PALEDLRHSLL$_{16}$PMPLELTQK$_{20}$AQSVKAS$_{30}$-COOH (FVB). Peptides were prepared using preloaded (phenylacetamido)methyl resins (AnaSpec, Freemont, CA). Amino acids were deprotected with trifluoroacetic acid and coupled for 10 min with 0.5 M 2-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (Peptides International, Louisville, KY). Peptides were cleaved from the resin with hydrogen fluoride in the presence of p-cresol and purified using a reverse-phase, C18 preparative HPLC column (Rainin, Woburn, MA). Protein expression and purification was carried out as described previously (28). This was done for C57 and FVB ApoA-I mice, yielding recombinant His6-T7-ApoA-I proteins, identified here as T7-ApoA-I, differing only in the QV to KA amino acid polymorphism found between C57 and FVB ApoA-I. Purity of the isolated apolipoproteins was assessed by SDS-PAGE with Coomassie stain. ApoA-I was found to be greater than 90% pure for both mouse strains.

**Surface plasmon resonance**

The affinity of recombinant ApoA-I proteins possessing each polymorphism for C57 or FVB HDL was measured using surface plasmon resonance (SPR) on a BIAcore 3000 biosensor. Each recombinant protein was coupled to a CM5 sensor chip via amine coupling to carboxyl moieties on the dextran surface of the chip. The HDL analyte in HBS buffer (10 mM HEPES, 0.15 M NaCl, 50 µM EDTA, pH 7.4) was injected at 25°C at concentrations ranging from 50 nM to 1000 nM at a flow rate of 20 µl/min for 3 min with a dissociation time of 3 min. The kinetics of binding were analyzed based on the response difference between the immobilized T7-ApoA-I flow cell and the control flow cell using BIAcore BlAevaluation software using a global fit of 1:1 binding with drifting baseline.

**ApoA-I dissociation from HDL**

HDL was combined in PBS with varying concentrations of the 43-amino acid ApoA-I mimetic peptide IHS to a final concentration of 50 µg/ml HDL. Samples were shaken for 1 h at 37°C and immediately loaded in native loading dye onto a 3–36% nondenaturing polyacrylamide gel and run at 200 V for 4 h. The gel was then transferred to PVDF Immobilon (Millipore, Billerica, MA). After blocking with 5% dry milk, the Immobilon was probed with rabbit anti-mouse ApoA-I antibody, followed by goat-anti-rabbit HRP-linked antibody and ECL Western detection reagent (ThermoScientific, Rockford, IL). HDL-bound and lipid-free ApoA-I bands were quantitated using FluorChem v2.0 Spot Denso software.

**J774 cholesterol efflux**

$^3$H-cholesterol efflux from J774 cells was performed as described previously (29) with the following modifications. J774 cells were loaded overnight with DMEM + 1% FBS + 25 mg/ml Acetylated LDL + 2 µg/ml Sandoz ACAT inhibitor + 1µCi/ml $^3$H-cholesterol in the presence of 0.3 mM 8-(4-chlorophenyl-thio)-cAMP (CPT-cAMP). The next day, the cells were washed 3× with

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PBS, and DMEM containing varying amounts of recombinant T7-ApoA-I, synthetic peptide, or purified HDL was added. After 4 h, the media was removed from the cells, and media and cells were extracted and counted as described previously (29).

ApoA-I binding to J774 cells

J774 cells were incubated 15 h with 0.3 mM 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP) (Sigma Aldrich, St. Louis, MO). The cells were then washed 2x with PBS and incubated for 2 h at 37°C with varying concentrations of recombinant mouse T7-ApoA-I in serum-free DMEM. The cells were washed 2x with PBS and scrapped into PBS+5% SDS, and the protein content of each sample measured using a microBCA protein kit (Pierce, Rockford, IL). Equal protein amounts of each sample were mixed with SDS-PAGE loading buffer and run on a 14% SDS-PAGE gel, transferred to Immobilon, and probed with rabbit-anti-mouse ApoA-I antibody. ApoA-I levels were quantitated using FluorChem Spot Denso software. ABCA1 levels from the same sample were run on a 4–12% gradient SDS-PAGE, transferred to Immobilon, and probed with rabbit-anti-mouse ABCA1 antibody (Novus Biologicals, Littleton, CO).

rHDL cholesterol esterification assay

rHDL was generated from C57 or FVB T7-ApoA-I using phosphatidylcholine, T7-ApoA-I, [3H]-cholesterol, and nonlabeled cholesteryl ester as internal standards. Samples were run on thin layer chromatography Silica Gel IB2-F sheets (J.T. Baker, Phillipsburg, NJ) using hexane:ethyl ether:acetic acid (70:30:1) as a running solvent, and cholesterol and cholesteryl ester spots were cut out into Instafluor scintillation fluid (Perkin Elmer, Waltham, MA) and counted for radioactivity as described previously (29).

Primary hepatocytes

Primary hepatocytes were prepared from female C57 or FVB mice. The liver was perfused first with 100 ml Krebs Ringer + 0.1 mM EGTA, followed by 100 ml Krebs Ringer + 20 mM glucose + 1.4 mM CaCl2 + 25 mg collagenase (Sigma #C5138) + 1 g BSA. The perfused liver was excised from the mouse and gently disrupted in DMEM +10% FBS using a glass pipette. The resulting cell suspension was filtered through a 70 μm cell strainer and washed once in DMEM +10% FBS by centrifugation 5 min at 50 × g. The pellet was resuspended in 6 ml DMEM + lipoprotein-deficient FBS (LPDS, 2.5 mg/ml protein), and 6 ml 100% Percoll was added to this. This resuspension was centrifuged for 20 min at 50 g to pellet only live hepatocytes. The resulting pellet was washed twice more in DMEM +LPDS. The washed cell pellet was resuspended in Williams E + 2.5 mg/ml LPDS +1% penicillin/streptomycin/glutamine (MediaTech, Herndon, VA), and cells (>80% viability by trypan blue exclusion) were plated on collagen-coated plates at 5.0 × 10^5 cells/35 mm dish.

Primary hepatocyte cholesterol/phospholipid efflux

Four hours after isolation, the primary hepatocyte media was changed to fresh Williams E media + 10% FBS + 1% penicillin/streptomycin/glutamine + 1 μg/ml [3H]-cholesterol or 1 μg/ml [3H]-choline (Perkin-Elmer, Waltham, MA). The following day, the cells were washed three times with PBS, and Williams E media + 2.5 mg/ml LPDS was added. Media and cells were harvested at various time points over 24 h. The media was used for lipid extraction (29) or density gradient centrifugation. ApoA-I-associated [3H]-cholesterol or phospholipid was immunoprecipitated from media by an overnight rotating incubation at 4°C with rabbit-anti-mouse-ApoA-I antibody followed by an overnight rotating incubation at 4°C with protein-A-Sepharose (Sigma #P391). The immunoprecipitate was washed three times with PBS and transferred to scintillation vials, and the [3H]-cholesterol/phospholipid was dissociated from the immunoprecipitate by the addition of 1 ml isopropanol. After allowing for the isopropanol to evaporate, [3H]-cholesterol or [3H]-phospholipid in each sample was counted as previously described (29).

In vivo ApoA-I and HDL clearance

In vivo clearance of recombinant T7-ApoA-I was measured in C57 and FVB mice. Recombinant C57 or FVB T7-ApoA-I in PBS was injected retro-orbitally into each mouse, and an initial bleed was taken to measure the starting T7-ApoA-I plasma concentration. Subsequent bleedings were done over the course of 30 h, and the resulting plasma was diluted 1:3 in PBS. Equal volumes of diluted plasma were analyzed by Western blot using an HRP-linked anti-T7 antibody (Invitrogen, Grand Island, NY). The T7 signal for each plasma sample was quantitated using FluorChem v2.0 Spot Denso software. Plasma samples were also run on a 4–36% nondenaturing acrylamide gel and analyzed by anti-T7 Western blot to determine the lipid association of the injected recombinant T7-ApoA-I.

In vivo clearance of C57 and FVB HDL was measured using two methods to determine ApoA-I and HDL cholesterol clearance from the plasma. ApoA-I−/−/ApoE−/− mice on a C57 background were generated previously (30). Nonfasted mice were injected via the retro-orbital sinus with unlabeled or [3H]-cholesterol-labeled HDL (generated as described above) derived from WT C57 or FVB mice and bled at various time points over the course of 36 h. At the final time point, the mice were terminally bled, and the plasma was subjected to differential centrifugation to isolate lipoproteins as described above. Plasma samples and lipoprotein fractions were analyzed for ApoA-I by Western blot (nonlabeled HDL injection) or [3H]-cholesterol ([3H]-cholesterol-labeled HDL injection) by scintillation counting.

RESULTS

HDL composition

Figure 1 shows a comparison of the properties of the HDL isolated from C57 and FVB mice. C57 and FVB HDL have equivalent ApoA-I levels, whereas ApoA-II levels are ~2.4fold greater in the FVB HDL (Fig. 1A). This observation is similar to the findings of Doolittle et al. (9, 31) when comparing the C57 HDL with Balb/c HDL, which possesses the same ApoA-I and ApoA-II polymorphisms as the FVB mouse. To further characterize the proteins found on the HDL from the two mouse strains, proteomic analysis was done on C57 and FVB HDL that had been purified 2x by density gradient centrifugation. Again, the greatest difference in apoprotein was found for ApoA-II (Fig. 1B).
Murine apolipoprotein A-I polymorphisms and HDL during the preparation of the recombinant proteins that the C57 T7-ApoA-I would not stay in solution at concentrations greater than 0.5 mg/ml, whereas the FVB T7-ApoA-I was soluble to nearly 1 mg/ml. Upon cross-linking the solubilized proteins to themselves, a significant portion of the C57 protein was found as oligomers, whereas the FVB protein was mainly still monomeric, indicating a greater degree of self-association for the C57-T7ApoA-I (Fig. 2).

Several methods were used to assess whether the sequence differences between the C57 and FVB ApoA-I altered the lipid-binding characteristics of the proteins. The ability of the recombinant FVB mouse possessed greater numbers of HDL particles, with fewer ApoA-I molecules per particle versus the C57 mouse, or a larger HDL particle, with more cholesterol and phospholipid per particle. Analysis of HDL from the two mouse strains on a native PAGE (Fig. 1C) or by FPLC (Fig. 1D) shows that the FVB HDL is indeed a larger particle than the C57 and comparable to the Balb/c HDL. This larger FVB HDL is less dense as evidenced by density gradient centrifugation (Fig. 1E).

ApoA-I properties

The recombinant C57 and FVB T7-ApoA-I proteins were compared in their degree of self-association. We had noted during the preparation of the recombinant proteins that the C57 T7-ApoA-I would not stay in solution at concentrations greater than 0.5 mg/ml, whereas the FVB T7-ApoA-I was soluble to nearly 1 mg/ml. Upon cross-linking the solubilized proteins to themselves, a significant portion of the C57 protein was found as oligomers, whereas the FVB protein was mainly still monomeric, indicating a greater degree of self-association for the C57-T7ApoA-I (Fig. 2).

Several methods were used to assess whether the sequence differences between the C57 and FVB ApoA-I altered the lipid-binding characteristics of the proteins. The ability of the recombinant ApoA-I proteins to clear multilamellar vesicles of DMPC from solution was used as a measure of lipid solubilizing capability (Fig. 3). Recombinant FVB T7-ApoA-I cleared micelles of DMPC from solution more rapidly than the C57 T7-ApoA-I. The helix 10 portion of the ApoA-I protein is the site of the two polymorphisms between C57 and FVB ApoA-I. Previous studies have shown that synthetic peptides of human ApoA-I helix 9/10 are better lipid acceptors and stabilizers of ABCA1.
were found to form lipoprotein particles that differed in size. Most notably, the FVB-T7-ApoA-I formed an HDL-sized particle larger than the C57, in accordance with what is seen in vivo (Fig. 3B).

Agarose electrophoretic separation and surface plasmon resonance (SPR) were carried out to analyze the binding affinity of C57 or FVB HDL particles for recombinant C57 or FVB T7-ApoA-I (Fig. 4). Using agarose gel separation, varying levels of HDL were coincubated with recombinant T7-ApoA-I and run on an agarose gel to distinguish lipid-free ApoA-I from that which had bound to HDL during the course of incubation. FVB T7-ApoA-I was found to bind C57 and FVB HDL better than C57 T7-ApoA-I, with C57 T7-ApoA-I proteins having a higher affinity for C57 HDL than for FVB HDL (Fig. 4A, B). SPR was also used to determine the binding kinetics of HDL to each recombinant protein. Fig. 4C–F shows representative comparisons of C57 or FVB HDL binding to each T7-ApoA-I at HDL concentrations from 50 to 1,000 nM. The SPR binding data agree with the results obtained using agarose gel separation, with C57 and FVB HDL showing a higher total binding to FVB-T7-ApoA-I. The $K_d$ values show that the FVB T7-ApoA-I has a higher affinity for HDL than the C57 T7-ApoA-I. Again, as with the agarose gel separation method, both ApoA-I isoforms display a greater affinity for the smaller C57 HDL than for the FVB HDL.

There is the possibility that the recombinant protein might interact with the HDL particle in a manner different from that of the endogenous protein. As such, the affinity of the endogenous protein for its own HDL particle was analyzed using an ApoA-I mimetic peptide (IHS) developed in our laboratory, which is able to dissociate ApoA-I from HDL in a concentration-dependent manner (29). In agreement with the results of the experiments using recombinant T7-ApoA-I, the endogenous FVB ApoA-I remained bound to its HDL at higher concentrations of the IHS peptide than C57 ApoA-I, which was easily dissociated from its HDL (Fig. 5).

**Influence of polymorphism on cholesterol efflux**

Because the polymorphisms in the ApoA-I peptides resulted in altered lipid binding, the ability of the proteins

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**Fig. 2.** Recombinant C57 T7-ApoA-I self-associates to a greater degree than FVB T7-ApoA-I. Recombinant T7-ApoA-I (0.33 mg/ml) was cross-linked with BS$_3$ (3.5 mM) for 3 h on ice and run on a 10% SDS-PAGE gel alongside noncrosslinked T7-ApoA-I. Sizes of expected cross-linked multimers are indicated.

**Fig. 3.** Recombinant FVB ApoA-I and helix 9/10 synthetic peptide clear DMPC multilamellar vesicles more rapidly than C57 ApoA-I and 9/10 peptide, with FVB-ApoA-I forming larger HDL-sized particles. A: Multilamellar vesicles of DMPC (final concentration, 0.25 mg/ml) were added to 0–0.2 mg/ml C57 or FVB T7-ApoA-I or ApoA-I-9/10 synthetic peptide, and the absorbance at 490 nm was followed over the course of 1 h and normalized to the initial absorbance reading. Representative curves at 0.05 mg/ml are shown (minimum of five samples each). Percent turbidity of DMPC remaining at 10 min was plotted against A-I or 9/10 peptide concentration and fit to Michaelis-Menten kinetics. * $P < 0.05$ compared with C57. * * $P < 0.05$ compared with corresponding T7-ApoA-I. B: Nondenaturing PAGE gel of 0.08 mg/ml T7-ApoA-I-cleared DMPC vesicles after 1 h clearance.
to efflux cholesterol from J774 macrophages was tested. Despite the greater lipid binding of the FVB T7-ApoA-I, the C57 T7-ApoA-I showed a slightly greater efflux capability when compared with FVB (Fig. 6A). The C57 9/10 peptide displayed a much greater ability to efflux cholesterol from J774 macrophages than the FVB 9/10 peptide (Fig. 6B). Additionally, the whole ApoA-I protein is a substantially more effective efflux acceptor than its 9/10 peptide counterpart, although the 9/10 peptide $V_{max}$ is probably higher. Holo-HDL isolated from each mouse strain was used in a similar cholesterol efflux study to determine whether the whole particles differ in their cholesterol efflux capabilities. The cholesterol efflux from J774 cells to HDL derived from each mouse strain is slightly greater for the C57 HDL than the FVB, similar to the results for the lipid-free T7-ApoA-I (Fig. 6C).

The binding of T7-ApoA-I to J774 macrophages was measured with and without the up-regulation of ABCA1 expression by CPT-cAMP (Fig. 7A). The binding of ApoA-I to the macrophages increases when ABCA1 expression is increased, as expected. However, although there is some indication that the C57 form of ApoA-I has slightly better binding to the macrophages, the differences are not significant ($P > 0.05$). These differences reflect the minor differences in cholesterol efflux seen for the ApoA-I proteins in Fig. 6. ApoA-I is known to stabilize the cellular ABCA1 protein.
levels by preventing degradation of ABCA1 (32, 33). The differences in the ApoA-I variants did not significantly ($P > 0.05$) affect the cellular levels of ABCA1, whereas the preincubation of the macrophages with CPT-cAMP dramatically increased ABCA1 protein levels as expected (Fig. 7B).

**Lecithin cholesterol acyltransferase activation**

The bulk of the cholesterol found in the HDL of the two mice was found in its esterified form (Fig. 1A). ApoA-I is known to activate LCAT, which converts free cholesterol to cholesteryl ester (34). When rHDL made from the two recombinant T7-ApoA-I proteins were tested for LCAT activation, C57 and FVB T7-ApoA-I activated LCAT to similar extents (Fig. 8).

**Apoprotein A-I metabolism**

The liver is the main site of ApoA-I production as well as nascent HDL formation (35). Primary hepatocytes were prepared from both mouse strains, and the endogenous ApoA-I and HDL cholesterol secretion levels were analyzed. No difference was found for ApoA-I secretion levels for all time points tested (data not shown). The difference in HDL cholesterol secretion from the FVB hepatocytes favors the C57 HDL when compared with the C57 hepatocytes.
Various inbred mouse strains demonstrate different susceptibilities to the development of atherosclerosis when fed an atherogenic diet or when genetically modified by removing ApoE or LDL receptor. These mice also have different plasma lipoprotein levels. Although the HDL of C57BL/6 mice has been extensively studied, in part because this is an atherosclerosis susceptible mouse strain, the HDL of the FVB mouse has not been as widely examined. C57 and FVB mice differ in the level and size of HDL, with FVB mice having higher plasma levels and larger-sized HDL. Even when normalized for ApoA-I content, the FVB HDL has more cholesterol, cholesteryl ester, and phospholipid than the C57 HDL; as a result, FVB HDL is larger and less dense than C57 HDL. We here demonstrate that the two amino acid sequence variations in the C-terminal helix of C57 and FVB ApoA-I, the major HDL apolipoprotein, cause this protein to display several different properties, mostly affecting lipid-binding affinities.

We here report several approaches characterizing the differences in interaction of the two ApoA-I mouse proteins with lipid and with HDL. Each of these approaches yields results that are in agreement with one another, all indicating that the FVB ApoA-I variant is greater in its lipid binding and HDL association/affinity than the C57 ApoA-I variant. The data are especially intriguing given the work of Tanaka et al. (18), in which the lipid-binding activities of the N-terminal (residues 1-186) and C-terminal (residues 267-340) helices of C57 and FVB ApoA-I were examined.
was analyzed for T7 tag by Western blot. B and C: ApoA-I were injected retro-orbitally with recombinant C57 or FVB T7-cleared from the plasma at similar rates. A: C57 or FVB WT mice.

Data points show the mean ± SD of triplicate samples.

Fig. 10. C57 and FVB HDL ApoA-I and HDL cholesterol are cleared from the plasma at similar rates. A: C57 or FVB WT mice were injected retro-orbitally with recombinant C57 or FVB T7-ApoA-I and bled at various time points. Plasma from each time point was analyzed for T7 tag by Western blot. B and C: ApoA-I−/−/ApoE−/− mice on the C57 background were injected with (B) unlabeled or (C) 3H-cholesterol-labeled HDL obtained from C57 or FVB mice. Mice were bled at various time points and the plasma from each time point was analyzed for (B) ApoA-I levels by antimouse ApoA-I Western blot, or (C) 3H-cholesterol radioactive counts/min. Data points show the mean ± SD of triplicate samples.

187–240) domains of lipid-free mouse (C57) ApoA-I are contrasted with those of human ApoA-I. In their study, most of the lipid binding of mouse A-I was found in the N-terminal region, whereas the C-terminal domain alone revealed almost no lipid binding. On the other hand, a hybrid molecule containing the human N-terminal fragment and the murine C-terminal domain exhibited improved lipid binding over the human N-terminal domain alone, suggesting that the murine C-terminal domain did afford an increment in lipid binding. They suggested that the initial lipid binding region of mouse A-I is most likely not in that C-terminal domain. It is worth noting that these are recombinant proteins, and the C-terminal domain includes helix 8 as well as 9 and 10. It is known that there are functional interactions among individual helices (13). Our results of lipid binding of the helix 9/10 peptides indicate a clear ability of these peptides to bind lipid and clear DMPC vesicles in the case of C57 and FVB 9/10 peptide, with FVB-9/10 being nearly as effective as the C57 full-length ApoA-I (Fig. 3, kinetic data). Natarajan and colleagues have observed lipid binding and cholesterol efflux, with synthetic 9/10 helices corresponding to the human sequences (13). No previous studies of the murine 9/10 helices have been done.

The mechanisms by which the polymorphisms confer greater lipid binding and HDL association on the FVB ApoA-I are not clear. The finding that the C57 apoprotein is more prone to self-association may indicate a conformational hindrance to the initial binding/association to lipid. However, the very nature of the protein when it is already in association with HDL appears to be altered, as evidenced by the ease with which the C57 protein is dissociated from its own HDL, whereas the FVB protein is difficult to dissociate from its HDL (Fig. 5). This may in part explain our HDL binding data done by SPR and the agarose separation assay, in which both variants of ApoA-I show a greater affinity for C57 HDL than for FVB. Given the limited surface area on an HDL particle, the ease with which the endogenous ApoA-I is dissociated is a key factor in the amount of recombinant A-I that can associate with the particle. In the case of the FVB HDL in which the endogenous A-I protein is tightly associated, the recombinant protein is less able to compete with it for binding to the HDL.

The functional properties of the two ApoA-I proteins that were assessed here did not reflect the differences in lipid association between the two proteins. First, the two ApoA-I proteins are not very different in promoting cholesterol efflux from cAMP-treated J774 cells, with C57 ApoA-I and HDL slightly more effective than their FVB counterparts. The fact that the efflux using the 9/10 peptides greatly accentuated this difference in favor of the C57 peptide further indicates that the increased lipid binding capabilities of the FVB ApoA-I does not result in an increase in ABCA1-mediated lipid efflux to this protein. There are numerous examples in the literature in which alterations in helix 10 of the human ApoA-I molecule results in decreased ABCA1-mediated cholesterol efflux (14, 15, 20, 36, 37). Macrophages and other cells that express ABCA1 have been found to possess two distinct ApoA-I binding sites: a low-capacity binding site on the ABCA1 protein itself, which interacts with ApoA-I at several locations on the ApoA-I molecule, and a high-capacity binding site within the membrane lipid domain generated by ABCA1 (37). The C-terminal helix 10 of human ApoA-I is largely responsible for the interaction of the protein with these lipid domains, and there is a positive correlation between the ability of different ApoA-I molecules to solubilize DMPC vesicles and their ability to promote ABCA1-mediated cholesterol efflux (37). The fact that this is not occurring in our studies is an interesting finding. Kono et al. (15) found that the human ApoA-I Nichinan E235 deletion mutant, which solubilizes DMPC vesicles less effectively than wild-type human ApoA-I, did not differ from the wild-type in its ability to promote cholesterol efflux from J774 macrophages. However, there was a significant difference in efflux from BHK overexpressing human ABCA1. They suggested that the lack of effect in the J774 cells was due to the interaction of human ApoA-I with mouse ABCA1. In our case, both ApoA-I and ABCA1 are from mouse. Whether this means the sensitivity of mouse
ABCA1 cholesterol efflux activity to helix 10 variations is overall less than that of human ABCA1 remains unknown but may reveal important clues to the mechanisms of ABCA1 function. That the binding of C57 and FVB ApoA-I to J774 differed (albeit not significantly) in favor of the C57 ApoA-I suggests the affinity of ApoA-I for DMPC or HDL does not in these assays necessarily reflect its cellular binding affinity. We expected the FVB ApoA-I protein to show greater affinity for the high-capacity lipid binding site generated by ABCA1, but this is not evident from our results, suggesting the ABCA1-mediated lipid binding of ApoA-I is not directly correlated with the general lipid affinity of the ApoA-I protein variant.

The secretion of HDL cholesterol and phospholipid from primary hepatocytes is slightly greater for the C57 hepatocytes than for FVB hepatocytes. These results are contrary to what one would expect if nascent HDL formation was driving the differences in plasma HDL cholesterol and phospholipid found in these two strains of mice. However, these results do agree with the J774 efflux experiments, particularly the efflux to the 9/10 peptide, which is more effective when the C57 variant is used. This effect is apparently not significant in vivo in terms of regulation of the two mice. Because the formation of nascent HDL by hepatocyte is ABCA1 dependent, these results with cells from the two strains seem to be in accord with the above results on the binding of ApoA-I to ABCA1 on J774 cells (i.e., that there is little distinction between the two native ApoA-I proteins). The binding to ABCA1 in J774 cells was performed with recombinant ApoA-I containing the T7 tag, whereas the hepatocyte production of HDL probably involving ABCA1 interaction is a reflection of the property of endogenous ApoA-I. In other words, with respect to interaction with ABCA1, there does not appear to be a difference between the recombinant T7-tagged protein and the natural apoprotein secreted from hepatocytes.

Given the greater affinity of the FVB ApoA-I for HDL, we expected this difference to translate into a lower rate of catabolism of the FVB HDL because the ApoA-I protein should be more rapidly catabolized in vivo once dissociated from the HDL lipid core. This was not seen in vivo as the rates of catabolism of the two ApoA-I proteins and HDLs were similar for protein and cholesterol.

Taken together, these data indicate that, despite the variations in structure and physical properties of the two apolipoproteins found in HDL, neither the formation of a more cholesterol-rich nascent HDL particle in the FVB mouse nor the level of cholesterol efflux from peripheral cells such as macrophages accounts for the differences in level and size of the two HDL particles found at steady state in the plasma. Additionally, there appear to be no differences in the catabolism of HDL from the two mice. This underlines the complexity of HDL structure and function and suggests a complex protein-protein and/or protein-lipid interaction among the components of HDL. The manner in which the structural and physical differences observed between the two ApoA-I variants in turn affect their in vivo ApoA-I/HDL function remains to be determined. That these polymorphisms are likely functionally relevant is highlighted not only by their differences in self-association and lipid binding but also by their conservation among inbred mouse strains. To our knowledge, of the more than 40 strains examined, the Q225K, V226A mutations are the only mutations found, and they are roughly equally distributed among the strains (9, 38). Additionally, these mutations are uniquely associated, with no strains examined possessing solely Q225K or solely V226A.

One potential role may be in the interaction of these ApoA-I variations with polymorphic variants of the other major apolipoprotein found on HDL, ApoA-II. Between the C57 and FVB strains, there are three major polymorphisms in ApoA-II: E18D, V24M, and V36A (C57 to FVB). ApoA-I levels are similar between the FVB and C57 mice, but ApoA-II levels are several-fold higher in the FVB mouse (31) (Fig. 1). There is strong evidence in the literature associating ApoA-II levels with HDL particle size and cholesterol levels (8, 39–41). However, the way in which the ApoA-I and ApoA-II polymorphisms interact may be important as well. The presence of ApoA-II on recombinant HDL has been recently shown to alter the conformation of ApoA-I on the HDL particle (42). Although some data suggest that ApoA-II prevents dissociation of ApoA-I from HDL, there is evidence showing that the addition of ApoA-II displaces ApoA-I from HDL (43, 44). In this case, it is therefore possible that the greater affinity of FVB ApoA-I for HDL may allow the greater levels of ApoA-II to exist on the HDL particle without resulting in dissociation of the ApoA-I protein. That the FVB ApoA-I can alone form a larger recombinant HDL particle (Fig. 3B) may be significant in determining the levels to which other apoproteins, such as ApoA-II, can interact with the HDL particle. The interaction of the two major HDL apoproteins in modulating HDL composition and size merits further study. The role of the polymorphisms in the interactions of the two apoproteins may be important in HDL formation and stability. Additionally, Cavignol et al. (45) have shown that the exchange of ApoA-I between lipid-free and lipid-bound states may provide a means by which dysfunctional HDL are generated, further emphasizing the significance of ApoA-I affinity for HDL.

Another potential role for the increased HDL affinity of the FVB mouse may be during conditions of acute inflammation. During the acute phase response, levels of serum amyloid A increase dramatically and are found in the plasma associated with HDL-sized lipoproteins, whereas ApoA-I may dissociate from HDL and be catabolized, reducing plasma levels of ApoA-I HDL (46). Suzuki et al. (47) have recently shown that HDL is protective in mice infected with Salmonella typhimurium, preventing increases in plasma levels of interferon-β. The greater HDL affinity of one ApoA-I protein over another may play a role in the response to an inflammatory challenge. This is especially interesting given the finding that when macrophages from various inbred mouse strains were tested for susceptibility to bacillus anthracis lethal factor, four of the five mice known to possess the C57 ApoA-I/ApoA-II variants were...
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