The deletion mutation Δ6 apolipoprotein A-I lacks residues 143–164 or repeat 6 in the mature apoA-I protein. In vitro studies show this mutation dramatically reduces the rate of lecithin:cholesterol acyltransferase (LCAT) catalyzed cholesterol esterification. The present study was initiated to investigate the effect of this mutation on in vivo high density lipoprotein (HDL) cholesterol esterification and metabolism. Transgenic mice expressing human Δ6 apoA-I (TgΔ6+/+) were created and then crossed with apoA-I knockout mice (−/−) to generate mice expressing only human Δ6 apoA-I (TgΔ6−/−). Human Δ6 apoA-I was associated with homogeneous sized α-HDL, when wild-type mouse apoA-I was present (in TgΔ6+/+ and +/+ mice). However, in the absence of endogenous mouse apoA-I, Δ6 apoA-I was found exclusively in cholesterol ester-poor HDL and lipid-free HDL fractions. This observation coincides with the 6-fold lower cholesterol ester mass in TgΔ6−/− mouse plasma compared with control. Structural studies show that despite the structural perturbation of a domain extending from repeat 5 to repeat 8 (137–178), Δ6 apoA-I binds to spherical unilamellar vesicles with only 2-fold less binding affinity. In summary, these data show a domain corresponding to apoA-I repeat 6 is responsible for providing an essential conformation for LCAT catalyzed generation of cholesterol esters. Deletion of apoA-I repeat 6 not only blocks normal levels of cholesterol esterification but also exerts a dominant inhibition on the ability of wild-type apoA-I to activate LCAT in vivo.

The potent antiatherosclerotic properties associated with elevated plasma HDL1 levels in humans (1) and in animal models (2–5) are generally ascribed to apoA-I and its role in co-activating the conversion of cholesterol to cholesterol ester by lecithin:cholesterol acyltransferase (LCAT) (6, 7). Conversion of cholesterol to its ester stimulates the efflux of peripheral tissue cholesterol to apoA-I containing particles (8–10). Together efflux and esterification act synergistically to provide net cholesterol removal from peripheral tissues and deliver them to the liver where they are selectively removed by SR-B1 (11).

Cellular efflux may occur by a number of different pathways (9, 10, 12–14), but it is commonly believed that the first functional acceptor is a small lipid-poor apoA-I containing particle called preβ-HDL (7, 15, 16). Several types of preβ-HDL are excellent substrates for plasma LCAT and are rapidly converted to spherical HDL particles or α-HDL (16, 17). If these particles are not converted to α-HDL, it has been shown that the lipid-poor apoA-I are rapidly cleared from circulation by the kidney (18, 19).

The antiatherogenic role of plasma HDL apoA-I in cholesterol removal has been recently questioned because of reports describing mutations within the apoA-I coding sequence, which lead to low concentrations of HDL apoA-I and for which little or no association to coronary heart disease has been demonstrated in affected individuals. Clinical studies of low apoA-I concentrations have been restricted by the limited number of subjects identified with this disorder and the high incidence of confounding differences in lipoprotein metabolism among subjects (20, 21). Animal models of apoA-I deficiency have shown that the apoA-I knockout mice are not predisposed to diet-induced atherogenesis (22, 23). The lack of plasma apoA-I does not by itself lead to the development of atherosclerosis but instead, its absence leads to an increase in atherosclerosis susceptibility especially when accompanied by other risk factors, such as elevated low density lipoprotein concentrations (4). Humans unlike mice have greater concentrations of “proatherogenic lipoproteins” such as very low density lipoprotein and low density lipoprotein in their plasma. Therefore, it appears that HDL apoA-I serves a protective role by minimizing the effects of pro-atherogenic factors (4) consistent with the hypotheses deduced from a large number of human epidemiological studies (1, 25).

More than 40 naturally occurring human mutations within the apoA-I coding sequence have been documented. In only 14 cases are these mutations linked to reduced plasma HDL apoA-I concentrations (26–29). Three of the 14 mutations occur at the N terminus (between residues 1–60) and are associated with hereditary amylodosis (30), whereas 8 of the 14 are located within a domain between residues 143–164 or repeat 6 (29, 31–39). The remaining apoA-I mutations that appear to lower HDL apoA-I levels are located within repeats adjacent to repeat six, namely repeats four, five, and seven. Of the human...
mutations associated with low HDL concentrations, five show dominant negative effects on plasma HDL apoA-I concentrations (34, 36–39). Because most of these mutations occur within repeat 6 or adjacent to repeat 6 we have focused mainly on this region. In particular, one of the naturally occurring human mutations, apoA-I Seattle (36) is caused by an in-frame deletion of 15 amino acids that leads to re-orientation of repeat six’s hydrophobic face. Our laboratory has studied a similar mutation, apoA-I Δ6, and has hypothesized that the placement and depth of penetration of repeat six’s hydrophobic face into the phospholipid bilayer is one critical determinant of LCAT activation and catalysis (40–42). In this manuscript we report the metabolic consequences of expressing Δ6 apoA-I in transgenic mice in the presence and absence of endogenous mouse apoA-I. Results from this study show that deletion of apoA-I repeat 6 sharply increases HDL catabolism, dramatically lowers plasma cholesterol esterification, and blocks the maturation of lipid-poor HDL to cholesterol ester-rich HDL even in the presence of wild-type mouse apoA-I.

**EXPERIMENTAL PROCEDURES**

**Creation of ApoA-I Δ6 Transgenic Mice**—The apoA-I Δ6 mutation was prepared by polymerase chain reaction megaprimer mutagenesis using a 2.2-kilobase PstI DNA fragment of the human apoA-I gene as previously reported (40, 43). The production of human Δ6 apoA-I transgenic mice was carried out by DNA microinjection into C57BL/6J × SJL/J F2 zygotes using standard techniques (44, 45). Following weaning of founder mice, a mouse tail biopsy of approximately 1 cm in length was used for DNA analysis. Tail DNA was analyzed by polymerase chain reaction for the presence of the mutant apoA-I transgene. All transgenic founders were further genotyped by Southern blot (46), Western blot, and enzyme-linked immunosorbent assay (47). All mice were maintained in specific pathogen-free barrier facilities in Microisolator TM caging (Lab Products, Maywood, NJ). All experiments and animal procedures conformed to protocols approved by the University of Alabama at Birmingham and Wake Forest University School of Medicine Institutional Animal Care and Use Committee. Founder animals carrying the transgene were bred to either C57BL/6J or apoA-I knock-out (apoA-I−/−) mice (22) and the appropriate transgenic lines were established. Transgenic mice for human wild-type apoA-I were obtained from Charles River Laboratories (48) and were bred with apoA-I−/− mice (22) obtained from the Jackson Laboratory.

**Quantification of Lipids and Apoproteins and Fractionation of Whole Plasma**—Total plasma-free and esterified cholesterol was determined by enzymatic assay (Roche Molecular Biochemicals) (49), and HDL cholesterol was determined in supernatants after dextran-sulfate precipitation of plasma (50). An enzyme-linked immunosorbent assay was developed for the quantification of human Δ6 apoA-I, similar to that described for wild-type human apoA-I (51). Esterified cholesterol in the HDL peak were identified by cholesterol assay, combined, and then separated by a thin-layer chromatography. Radioactivity decay curves, using a multieponential computer curve-fitting program (52). Plasma volumes were estimated as 5.77 ml/100 g of body weight (56).

**Determination of Intrinsic Disassociation Constant (K_D) — Small unilamellar phospholipid vesicles (SUV) were prepared from sn-1-palmitoyl-sn-2-oleoyl-phosphatidylcholine containing 0.5 μCi of 1,2-[3H(N)]cholesterol (50 Ci/mmol) (58 Ci/mmol) following established procedures (57).** The lipoprotein was dialyzed against a buffer containing 0.9% NaCl containing 0.05% EDTA, pH 7.4, 140 mM NaCl, and 0.02% NaN3 (51). Approximately 36,500–46,000 cpm were added to the mixture which was then sonicated for 1 h at 4 °C. The total percent apoA-I recovery was calculated for each column from unbound labeled apoA-I using a 35-cm ACA34 spectra gel column and fractionated. Each fraction was counted to determine the [3H]cholesterol distribution. Fractions were combined to obtain homogeneous sized SUV. The purified SUV were then assayed for phospholipid mass (58).

**Radiolabeled apoA-I was prepared for use in the SUV binding assay by reacting 0.5 μCi of [125I] (NEN Life Science Products) with 500 μg of either purified recombinant (wild-type or mutant Δ6) apoA-I or purified plasma apoA-I in the presence of Iodo-Beads (Pierce) according to standard procedures (54). All traces of unincorporated label were removed by passage through a G-25 Sepharose (15-ml bed volume) column and then extensive dialysis against SUV buffer. Lowry assay (59) was conducted on the purified radiolabeled protein to determine the specific activity of each labeled preparation. The specific activity was defined as the radioactivity content/unit of protein mass (cpm/μg). The apoA-I binding to the phospholipid vesicles (K_D) was determined by adding varying amounts of radiolabeled apoA-I (0–25 μg) to 100 μg of SUV phospholipid and incubating at room temperature for 15 min (60). Fractions containing the SUV-bound apoA-I were separated from unbound labeled apoA-I using an 35-cm ACA34 spectra gel column. The total percent apoA-I recovery was calculated for each column run and ranged between 80–90%. The mass of labeled apoA-I was determined in the pooled bound and unbound apoA-I fractions by the use of each preparations’ specific activity. Scatchard plots (61) were used to derive the binding constant for each apoprotein studied. For each reported K_D, six different mass amounts of apoprotein were studied in triplicate using at least two different preparations of radiolabeled protein.

**Enzymatic LCAT Assay**—The reconstituted LCAT for the endogenous LCAT assays was prepared at a molar ratio of 80:4 sn-1-palmitoyl-sn-2-oleoyl-phosphatidylcholine:cholesterol:apoA-I protein and contained a trace amount of [3H]cholesterol, as described previously (62). The apoA-I used for preparing the labeled reconstituted HDL substrate was purified from human plasma and was provided by Dr. John Parks (63). Assays were carried out in duplicate using ~1.2 μg of substrate cholesterol (0.6 mM sodium palmitate concentration) in a final concentration of 10 mM Tris, pH 7.4, 140 mM NaCl, 0.25 mM EDTA, and 0.15 mM sodium azide, 0.6% fatty acid-free bovine serum albumin, 2 μM β-mercaptoethanol, and 4 μl of fresh mouse plasma as the source of LCAT. The reactions were carried out for 30 min at 37 °C, and the conversion of [3H]cholesterol to [3H]cholesterol ester was determined by lipid extraction followed by thin layer chromatography (49). Background values were determined by comparison to protein standards of known Stokes’ diameter. To ascertain the apoprotein distribution after FPLC fractionation of whole plasma 16-μl aliquots were analyzed after separation on 4–30% SDS gradient gel electrophoresis. The gels were electroblotted to nitrocellulose according to standard procedures and then subjected to Western blot analysis (55).

**In Vivo ApoA-I Turnover Studies**—Purified mouse wild-type apoA-I (Biodesign Inc.) and Δ6 apoA-I protein (40, 43) were radiolabeled with either 5 μCi of [3H] or [123I] (NEN Life Science Products), respectively, using Iodo-Beads (Pierce) according to standard procedures (54). Both radiolabeled proteins (~7 × 10⁶ cpm) were incubated at 4 °C with the appropriate mouse plasma for 4–1 h. The plasma was then added to a Superose 6 column (Amersham Pharmacia Biotech) and the labeled mouse HDL was isolated by FPLC. Fractions containing the HDL peak were identified by cholesterol assay, combined, and then filtered through a Spin-X filter (0.22 μm) unit before injection. Transgenic mice (3–8-month-old males) were injected with approximately 2.0 × 10⁶ cpm of [3H]wild-type mouse HDL and 6.0 × 10⁶ cpm of Δ6 mouse HDL through a jugular vein catheter. Approximately 40 μl of blood were collected at each time point by retrobulbar plexus bleeding following isoflurane administration. Blood samples were collected at 5 and 30 min, 1, 6, and 24 h into tubes containing 1 μl of 0.5 × EDTA then spun at 14,000 × g for 5 min, and 20 μl of plasma was removed for radioactivity measurements using a Beckman 4000 γ counter. Fractions eluted from the column were then used for radioactivity decay curves, using a multieponential computer curve-fitting program (55). Plasma volumes were estimated as 5.77 ml/100 g of body weight (56).

To ascertain the apoprotein distribution after FPLC fractionation of whole plasma 16-μl aliquots were analyzed after separation on 4–30% SDS gradient gel electrophoresis. The gels were electroblotted to nitrocellulose according to standard procedures (54). All traces of unincorporated label were removed by passage through a G-25 Sepharose (15-ml bed volume) column and then extensive dialysis against SUV buffer. Lowry assay (59) was conducted on the purified radiolabeled protein to determine the specific activity of each labeled preparation. The specific activity was defined as the radioactivity content/unit of protein mass (cpm/μg). The apoA-I binding to the phospholipid vesicles (K_D) was determined by adding varying amounts of radiolabeled apoA-I (0–25 μg) to 100 μg of SUV phospholipid and incubating at room temperature for 15 min (60). Fractions containing the SUV-bound apoA-I were separated from unbound labeled apoA-I using an 35-cm ACA34 spectra gel column. The total percent apoA-I recovery was calculated for each column run and ranged between 80–90%. The mass of labeled apoA-I was determined in the pooled bound and unbound apoA-I fractions by the use of each preparations’ specific activity. Scatchard plots (61) were used to derive the binding constant for each apoprotein studied. For each reported K_D, six different mass amounts of apoprotein were studied in triplicate using at least two different preparations of radiolabeled protein.

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determined by omitting plasma from the reaction tube. The fractional cholesteryl esterification rate was multiplied by the nmol of substrate cholesterol in the assay tube, corrected for the background, and converted to nmol cholesterol ester formed/h/ml of LCAT.

Epitope Mapping Studies—Competitive solid phase immunosassays were used to assess the binding of monoclonal antibodies to either lipid-free mutant apoA-I and rHDL sn-1-palmitoyl-sn-2-oleoyl-phosphatidylcholine substrate complexes containing mutant apoA-I, as described previously (40, 42, 64).

Data Analysis—Values are given as the mean ± standard deviation (SD) or ± standard error of the mean (S.E.). Statistical comparisons were made using analysis of variance (ANOVA).

RESULTS

To study the effect of deleting apoA-I repeat 6 on HDL metabolism, transgenic mice producing human Δ6 apoA-I were created and then crossed into apoA-I knockout mice. A series of mice having the Δ6 mutation and three levels of mouse apoA-I expression, TgΔ6 ++/+ , ++/− , −/− , were then generated. Table I shows that both the production rate and the rate of HDL particle formation decreased proportionally with the decrease in mouse apoA-I plasma levels. Enzyme-linked immunosorbent assay values also showed plasma Δ6 apoA-I levels dropped proportionally to mouse plasma apoA-I concentrations. The dependence of plasma Δ6 apoA-I concentration on the presence of mouse apoA-I was specific because no change was observed when human wild-type apoA-I transgenic mice (48) were crossed with apoA-I knockout mice (data not shown). Overall, these data suggest that plasma concentrations of Δ6 apoA-I were dependent on the presence of plasma wild-type mouse apoA-I containing HDL.

The fractional clearance rate for human Δ6 apoA-I was compared with that for mouse wild-type apoA-I in both TgΔ6 apoA-I ++/+ and ++/− mice. Mouse HDL was doubly labeled with [125I]human apoA-I and [131I]mouse wild-type apoA-I in TgΔ6 ++/+ mice (0.086 ± 0.022 pools/h) than in TgΔ6 ++/− mice (0.055 ± 0.008 pools/h) (Table II). If one compares these values to the fractional clearance rate for mouse wild-type apoA-I in nontransgenic C57BL/6 control mice (0.061 ± 0.012 pools/day), the fractional clearance rate in these mice was similar to that measured in TgΔ6 ++/− mice. Another difference between TgΔ6 ++/+ and TgΔ6 ++/− mice was the Δ6 apoA-I transport rate observed in TgΔ6 ++/− mice compared with TgΔ6 ++/+ mice. Overall, these results suggest that both the production rate and the rate of Δ6 apoA-I catabolism contribute to the reduced concentration of Δ6 apoA-I in the plasma of TgΔ6 mice. Furthermore, the presence of mutant Δ6 apoA-I has a negative effect on wild-type mouse apoA-I concentrations when only one copy of the mouse apoA-I allele is present (e.g. TgΔ6 ++/−).

To assess the binding affinity of Δ6 apoA-I for spherical lipoprotein particles, the intrinsic dissociation constant, Kd, was determined. Table III shows that the Kd for Δ6 apoA-I bound to phospholipid vesicles was approximately 1.8-fold higher than for either wild-type or plasma apoA-I. These results demonstrate that Δ6 apoA-I binds less tightly than wild-type apoA-I; however, the decreased lipid binding affinity was not large enough to explain the dependence of Δ6 apoA-I concentrations on plasma wild-type mouse apoA-I levels.

To characterize the distribution of Δ6 and wild-type mouse apoA-I among plasma lipoproteins, TgΔ6 apoA-I was segregated on a 4–30% NDGGE, transferred to nitrocellulose, and then probed with antibodies specific to either mouse or human apoA-I, as shown in Fig. 1. Probing with the anti-mouse antibody (left panel), TgΔ6 ++/+ and TgΔ6 ++/− plasma showed HDL sized particles ranging between 90–94 Å in diameter and migrating at the same position on NDGGE as HDL from control mouse plasma (C57Bl/6), in agreement with previous reports (65).

Probing with the anti-human antibody (right panel), plasma from TgΔ6 ++/+ and TgΔ6 ++/−, but not TgΔ6 −/− mice, showed HDL particles with an average diameter of 90–94 Å, Fig. 1. In contrast, control human plasma showed HDL ranging in size from 80–93 Å in diameter. The absence of a detectable band in the C57Bl/6 plasma lane demonstrated little cross-reactivity between mouse plasma and the anti-human antibody. Thus, the HDL size distribution for Δ6 apoA-I was similar to that reported for mouse HDL and distinctly different from the HDL distribution found in human plasma. Overall, these results suggest that in TgΔ6 ++/+ and Δ6 ++/− mice, human Δ6 apoA-I and wild-type mouse apoA-I reside on particles of similar diameters.

To retain smaller lipid-poor and lipid-free apoA-I containing particles on the nitrocellulose filter it was necessary to modify the electrophoretic procedure to conduct a more sensitive characterization of the Δ6 apoA-I distribution in TgΔ6 −/− mouse plasma. As shown in Fig. 2, plasma from individual TgΔ6 apoA-I −/− mice contained Δ6 apoA-I in lipid-poor and lipid-free HDL subfractions but very little in the lipid-rich HDL size range. Whereas all three genotypes of mice contained plasma Δ6 apoA-I on particles between 77–84 Å in diameter, these results suggest that Δ6 apoA-I, in the absence of wild-type mouse apoA-I does not reside on mature lipid-rich HDL.

To determine whether the TgΔ6 −/− plasma contained li-
TABLE II
Fractional catabolic rates for wild-type and Δ6 apoA-I-labeled plasma HDL

| Tg mouse genotypea | Pool Size | Wild-type apoA-I | Δ6 apoA-I | 125I-ΔΔ apoA-I | 131I- wild-type apoA-I | Transport rateb |
|-------------------|-----------|------------------|----------|---------------|-----------------------|----------------|
|                   | mg/dl     |                  |          |               |                       | µg/h           |
| TgΔ6 +/+          | 50.1 ± 2.1c| 131.0 ± 6.0c     |          | 0.146 ± 0.039c| 0.055 ± 0.008c        | 91.7 ± 13.7c   |
| TgΔ6 +/–          | 21.5 ± 2.5d| 67.5 ± 10.5d     |          | 0.160 ± 0.045c| 0.086 ± 0.022c        | 76.5 ± 13.4c   |
| C57Bl/6           | ND        | 128.3 ± 9.5c     |          | 0.061 ± 0.012c| ND                    | 117.1 ± 23.3c  |

a See legend to Table I for mouse genotype identification.

b Mouse HDL was doubly labeled with 125I-human Δ6 apo A-I and 131I-mouse wild-type apoA-I as described under “Experimental Procedures” and was injected into the jugular vein of n = 3 male mice/genotypic group.

c One-way ANOVA; values with unlike superscripts are statistically different at p < 0.05. ND, not detected.

d All values represent the mean ± SD.

TABLE III
Intrinsic disassociation constant for apoA-I bound to small unilamellar vesicles

| Apoprotein          | Kd (µM) |
|---------------------|---------|
| Plasma apoA-I       | 1.63 ± 0.12b |
| Wild-type apoA-I    | 1.45 ± 0.17c |
| Δ6 apoA-I           | 2.77 ± 0.58d |

a SUEs were prepared and purified to size homogeneity as described under “Experimental Procedures.” The indicated 125I-apoA-I was added to 100 µg of SUV and incubated at 22 °C for 15 min. The mixture was separated on a ACA34 spectra gel column and the bound and unbound fractions pooled and then analyzed for 125I-content. Mass of bound and unbound apoA-I was determined using the specific activity of each 125I-apoA-I preparation. Scatchard plots were used to determine the Kd. For each Kd reported six different mass amounts of apoA-I were bound (0.5–25 µg) to a standard amount of SUV for at least two different radiolabeled protein preparations.

b,c One-way ANOVA; values with unlike superscripts are statistically different at p < 0.05.

FIG. 1. Western blot of TgΔ6 mouse plasma separated on 4–30% nondenaturing polyacrylamide gradient gel. Tg mice producing human Δ6 apoA-I were crossed into apoA-I knockout mice (−/−). A series of mice having the Δ6 mutation and three levels of mouse apoA-I expression, TgΔ6 +/+ , Δ6 +/– , Δ6 −/− , were then generated. After electrophoresis gels were blotted onto nitrocellulose and probed using antibodies to either anti-mouse (left) or anti-human (right) apoA-I, as described under “Experimental Procedures.” Each lane contains approximately 1 µl of fasted plasma from chow fed male mice. Hu, human plasma; C57, C57Bl/6 mouse plasma. Δ6 apoA-I lacks the proline-punctuated repeat 6 or residues 143–164 of human apoA-I. HDL size was determined by the use of calibrating high molecular weight standards and their corresponding Stokes’ diameter (nm): thyroglobulin 17.0 nm, ferritin 12.4 nm, catalase 9.8 nm, lactate dehydrogenase 8.6 nm, and albumin 7.0 nm.

proteins that migrated with preβ-HDL mobility, agarose gel electrophoresis followed by Western blot analysis was carried out. These blots showed that the lipid-poor Δ6 apoA-I from TgΔ6 −/− mice migrated as preβ-HDL (data not shown).

To further characterize the lipoproteins from TgΔ6 mice, whole mouse plasma was separated by FPLC and each individual fraction analyzed for cholesterol and apoprotein mass (Fig. 3). The predominant lipoprotein peak from the FPLC separation of TgΔ6 +/+ plasma eluted in fractions 24–32 as indicated by the total cholesterol mass (Fig. 3A top panel). SDS gradient gel electrophoresis was then carried out on individual FPLC fractions and analyzed by Western blot analysis for apoprotein content. Fig. 3A (middle and bottom panels) shows mouse wild-type apoA-I, human Δ6 apoA-I, and mouse apoE were all present in cholesterol-rich particles of similar size, respectively. A much smaller peak of cholesterol mass (fractions 26–31) was observed when TgΔ6 −/− plasma was separated by FPLC, as shown in Fig. 3B (top panel). Here SDS gradient gel electrophoresis shows when mouse wild-type apoA-I was absent, the human Δ6 apoA-I eluted in fractions corresponding to lipid-poor HDL (fractions 30–34). The mouse apoE containing fractions (fractions 26–33) also eluted differently than in TgΔ6 +/+ mice.

Whole mouse plasma FPLC fractions were also separated by NDGGE and probed for human apoA-I and mouse apoE. The size distribution of human Δ6 apoA-I and mouse apoE containing particles in TgΔ6 +/+ plasma, Fig. 4A, were very similar. This pattern was similar to that seen for mouse wild-type apoA-I containing particles (data not shown) and similar to Fig. 1. However, FPLC fractions from TgΔ6 −/− mouse plasma showed that Δ6 apoA-I and apoE containing lipoproteins only colocalize on larger lipid-rich particles. These results suggested that few Δ6 apoA-I-containing particles mature into 94Å particles in the absence of wild-type mouse apoA-I. Furthermore,
in the TgΔ6−/− mice it appears that plasma apoE-containing particles are the preferred substrate for LCAT-generated cholesterol esters.

We next measured the total plasma content of free and esterified cholesterol in transgenic and control mice to determine the effects of Δ6 apoA-I on their mass concentrations. Table IV shows the values for free and ester cholesterol mass, as well as the ratio of ester cholesterol to total cholesterol (EC/TC), for C57BL/6 and apoA-I−/− mice, and each of the three TgΔ6 mouse genotypes expressing different levels of the mouse wild-type apoA-I gene. In both C57BL/6 and TgΔ6−/+ mice the EC/TC ratio was found to be approximately 0.83 ± 0.03. However, in TgΔ6+/− mice the EC/TC ratio was reduced about 22%, to 0.67 ± 0.03 (n = 7, p < 0.05). This decrease in the EC/TC ratio was due entirely to a decreased cholesterol ester mass (Table IV). The EC/TC ratio in TgΔ6-/− mice was further reduced to 42% of control levels, (C57BL/6 or Tg Δ6+/+). This dramatic reduction in ester cholesterol mass in the absence of wild-type mouse apoA-I demonstrates that other plasma apoproteins such as apoA-II, apoA-IV, or apoE are not able to support normal mass levels of cholesterol ester by activating LCAT-catalyzed cholesterol esterification.

Fig. 5 illustrates another finding from the analysis of Table IV that transgenic mice expressing Δ6 apoA-I have a significantly lower EC/TC ratio than mice without the mutant transgene given the same number of mouse wild-type apoA-I gene alleles. Shown in this figure the EC/TC ratio in TgΔ6+− mice was found to be significantly reduced compared with the EC/TC ratio in mice lacking the transgene and expressing only one half the gene dose of mouse wild-type apoA-I (designated apoA-I+−). These results suggest a negative role for plasma Δ6 apoA-I on LCAT-catalyzed cholesterol esterification. Similarly, the presence of Δ6 apoA-I reduced the EC/TC ratio in TgΔ6−/− mice (0.48 ± 0.04), which was significantly lower (p < 0.05, n = 7) from the EC/TC ratio in apoA-I−/− (0.58 ± 0.04) mice. The EC/TC ratio obtained from our apoA-I−/− mice agree well with previously reported values by other investigators (66). Thus, these data suggest that a dominant inhibition of a mutant apoA-I allele over the wild-type allele occurs when LCAT activator levels are limiting such as in the heterozygous state, and LCAT activation is dramatically inhibited by mutant apoA-I in plasma.

The level of exogenous LCAT activity was determined for all genotypic groups using a standard rHDL as the standard substrate to determine the effects of low HDL concentration on the level of circulating LCAT. As shown in Fig. 6, exogenous cholesterol esterification was unchanged in plasma from TgΔ6+/+ and TgΔ6+/− mice. In contrast, plasma from TgΔ6−/− and −/− mice showed a similar 30% reduction (n = 5, p < 0.05) in exogenous cholesterol esterified compared with both types of control mice (TgΔ6+/+ or C57BL/6) with the percent reduction in cholesterol esterification by −/− mice similar to that previously reported (66).

Monoclonal antibody mapping was employed to assess the conformational alterations arising from the deletion of apoA-I repeat six. First, the binding capacities of each antibody for lipid-free and lipid-bound wild-type, Δ6 (deletion of residues 143–164), and Δ5/6 apoA-I (deletion of residues 121–164) were compared using competitive immunoassays (64). Of the 13 antibodies tested five N-terminal epitopes between residues 1–115 and two that identify C-terminal epitopes between residues 187–243 were found to bind well to all apoproteins (data not shown). Because the lipid-free and lipid-bound competition curves were similar (40, 42), only the lipid-bound competition data are described in detail. Most antibodies were expressed equally well among all the apoproteins tested and thus only minimal differences in the extent of epitope expression were noted. In contrast, three antibodies that identify epitopes between 115 and 144 (115.1, 119.1, and 119.8) (repeats four and five) on wild-type apoA-I either did not bind to Δ5/6 apoA-I or bound very poorly. These results were anticipated because the
the production and catabolism of plasma HDL. Transgenic mice expressing only Δ6 apoA-I (TgΔ6 Δ/Δ) contain predominately lipopoor and lipid-free Δ6 apoA-I particles in their plasma. We hypothesize that Δ6 apoA-I only particles are poor activators of LCAT, are not readily converted to mature cholesterol ester-rich HDL in vivo, and are more rapidly catabolized than particles containing wild-type mouse apoA-I. This hypothesis is supported by the smaller size and lower cholesterol ester content of Δ6 apoA-I-containing particles and by in vitro studies showing the poor LCAT activation properties of this (40, 41) and similar apoA-I repeat 6 mutants (67–72).

Plasma Δ6 apoA-I levels were highest in TgΔ6 Δ/+ mice because of their association and circulation on HDL particles containing wild-type mouse apoA-I. This is supported by the co-localization of Δ6 and wild-type apoA-I on cholesterol-rich particles of similar size. In both TgΔ6 Δ/+ and TgΔ6 Δ/Δ mice NDGGE and Western analysis revealed HDL particles of 90–94 Å diameter that contained both mouse and Δ6 apoA-I. These two proteins are not likely to exist on different particles because Δ6 apoA-I in the absence of mouse wild-type apoA-I (TgΔ6 Δ/Δ mice) was found almost exclusively in the lipid-poor and lipid-free subfractions. In additional, preliminary studies of HDL binding to an anti-mouse apoA-I affinity column suggests both proteins reside on the same HDL particle.

We hypothesize that nascent or lipid-poor HDL particles containing both wild-type mouse and Δ6 apoA-I mature into lipid-rich HDL because of the activation of LCAT by mouse wild-type apoA-I. This hypothesis is supported by the demonstrated reduction in cholesterol ester mass as a function of wild-type mouse apoA-I gene dose. When two copies of the mouse wild-type apoA-I allele are present, the EC/TC ratio is similar to that in control mice, and Δ6 apoA-I concentrations are at their maximum. However, with only one gene dose of wild-type mouse apoA-I, the EC/TC ratio and cholesterol ester mass was reduced along with the Δ6 apoA-I concentrations. Finally, in the absence of wild-type mouse apoA-I, the Δ6 apoA-I concentrations were lowest, and the EC/TC ratio and cholesterol ester mass were further reduced compared with TgΔ6 apoA-I Δ/Δ mice. This gene dose-dependent drop in cholesterol ester mass and EC/TC ratio could not be explained by the reduction in plasma LCAT levels (Fig. 6). Nor could the decrease in cholesterol ester mass and EC/TC ratio be explained by the lower lipid binding affinity of Δ6 apoA-I for spherical unilamellar vesicles. A lower lipid binding affinity may contribute to the rapid catabolism of Δ6 apoA-I in vivo, but the presence of Δ6 apoA-I on lipid-rich HDL particles from TgΔ6 Δ/+ and TgΔ6 Δ/Δ mice suggests that it does not prevent its association with cholesterol ester-rich HDL particles.

The presence of Δ6 apoA-I on mouse wild-type apoA-I particles appears to have a negative effect on LCAT-catalyzed cholesterol esterification. These studies show that the EC/TC ratio for the TgΔ6 Δ/+ and TgΔ6 Δ/Δ mice are lower than for the corresponding apoA-I Δ/– and apoA-I Δ/Δ mice. Previous studies (66) demonstrated that the EC/TC ratio in mice with either one or two copies of wild-type mouse apoA-I are similar (Δ/+ = 0.82 ± 0.02 versus Δ/– = 0.78 ± 0.03). These data strongly suggested that one single gene copy of mouse apoA-I is sufficient to activate the normal esterification of cholesterol in vivo. Therefore, we would expect that both TgΔ6 Δ/+ and apoA-I Δ/Δ mice, each of which contain one copy of wild-type mouse apoA-I, to have similar EC/TC ratios. However, this is not the case, and the EC/TC ratio for TgΔ6 Δ/+ equals 0.67 ± 0.03 compared with Δ/+ = 0.78 ± 0.03. Thus, Δ6 apoA-I appears to weakly inhibit the conversion of cholesterol to cholesterol ester by

**TABLE IV**

| Tg genotype  | Total cholesterol a | Esterified cholesterol a | Free cholesterol a | EC/TC a |
|--------------|---------------------|--------------------------|--------------------|--------|
| C57BL/6      | 102 ± 9             | 83 ± 6                   | 21 ± 2             | 0.81 ± 0.03 |
| TgΔ6 Δ/+     | 118 ± 11            | 100 ± 7                  | 20 ± 3             | 0.85 ± 0.03 |
| TgΔ6 Δ/Δ     | 61 ± 7              | 45 ± 1                   | 20 ± 2             | 0.67 ± 0.03 |
| TgΔ6 Δ/Δ Δ/Δ | 33 ± 2             | 16 ± 2                   | 17 ± 2             | 0.48 ± 0.04 |

a Mean ± S.E.M. on n = 7 fasted, chow fed male mice.

b The EC/TC value in this study agree with earlier published reports of 0.58 ± 0.03 (66).

c Unlike superscripts show significant differences at p < 0.05 betw
between values as derived by ANOVA. All assays were as described under “Experimental Procedures.”

121–164 region was absent from the Δ5/6 protein. Two antibodies (137.1 and 17) that identify epitopes between residues 137–165 (repeats 5 and 6) did not bind to either Δ6 or Δ5/6 (data not shown). Again these results were anticipated because Δ6 apoA-I does not contain this region of the protein. However, an antibody that identifies epitopes corresponding to residues 178–200 (178.1) was either absent or bound very poorly to Δ6 apoA-I (data not shown). These results suggest that a conformational domain extending from residue 178–187, a region beyond the deleted region of 143–164, was also altered by the removal of repeat 6 of apoA-I. This was not seen for Δ5/6 apoA-I and strongly suggests that the deletion of repeat 6 has a greater impact on the global conformation of apoA-I than does the deletion of 2–22-mers as in Δ5/6 apoA-I.

**DISCUSSION**

These studies show that the deletion of a single 22-mer repeat within the central domain of apoA-I interferes with both

**FIG. 4.** Nondenaturing PAGE of FPLC fractionated plasma from TgΔ6 Δ/+ (A) and TgΔ6 Δ/Δ mice (B). Aliquots (16 μl) were removed from each fraction and run on a 4–20% nondenaturing PAGE and then electroblotted onto nitrocellulose. Blots were treated with the indicated anti-human or anti-mouse antibody as described under "Experimental Procedures."
Repeat 6 ApoA-I Deletion Blocks Presβ-HDL Maturation

LCAT. In this case, one gene dose of mouse wild-type apoA-I is not sufficient to maintain the normal EC/TC ratio. We hypothesize that only after the concentration of wild-type mouse apoA-I drops below a certain level does Δ6 apoA-I inhibit HDL maturation by LCAT. Thus, the presence of plasma Δ6 apoA-I appears to inhibit the maturation of nascent HDL only after the concentration of mouse wild-type apoA-I containing substrate becomes limiting. The inhibition of LCAT activation by Δ6 apoA-I may take place through several mechanisms. For example, Δ6 apoA-I could act to “dilute” the amount of activating apoA-I particle. Whereas it is also possible that LCAT may bind directly to Δ6 apoA-I in lipid-poor particles (73) and by so doing reduce the rate of LCAT-catalyzed cholesterol esterification.

Consistent with the later mechanism, our studies show that Δ6 apoA-I inhibits cholesterol esterification even in the absence of endogenous mouse apoA-I. We demonstrated that the EC/TC ratio in TgΔ6 −/− mice was significantly lower than in apoA-I −/− mice. In previous studies (66), apoA-I −/− mice were shown to have a 75% reduction in plasma cholesterol ester mass, very similar to the reduction in cholesterol ester mass seen in our group of −/− mice. Given that apoA-I −/− mice do not express any plasma apoA-I, the low cholesterol ester mass suggests that other mouse plasma apoproteins, such as apoE, apoA-II, and apoA-IV at their physiological concentrations, cannot maintain normal levels of cholesterol esterification in vivo. However, residual cholesterol esterification does occur and based on in vitro studies, apoE most likely serves to activate and carry circulating plasma cholesterol esters in the absence of apoA-I (74). In our TgΔ6 −/− mice, cholesterol ester-rich apoE-containing particles were detected by FPLC-NDGGE/Western analysis lacking any Δ6 apoA-I. Thus, although apoE may activate LCAT in TgΔ6 −/− mice, Δ6 apoA-I appears to reduce the rate of this process as well, supporting the idea that interaction between LCAT and Δ6 apoA-I on lipid-poor particles reduces or inhibits plasma esterification, when substrate is limiting.

The importance of the central helices (repeats 5–7) within apoA-I for LCAT activation and HDL maturation has been suggested by LCAT activation studies on both spontaneous (29, 34, 36–38, 72, 75–77) and nonsporontaneous (41, 67–71) mutations within the apoA-I coding sequence. Of the 40 known apoA-I-coding sequence mutations so far identified only 14 significantly reduce HDL apoA-I concentrations in humans. Of these 14 mutations that alter HDL metabolism, 8 are located in repeat 6. In the past our studies have focused on this region and we have shown that removal (41, 67), substitution (68), or re-orientation (69) of repeat 6 causes conformational alterations to adjacent repeats, suggesting that physical interaction between this region and LCAT may be necessary for activation and catalysis. Of particular relevance, is the human mutation, apoA-I Seattle (36), that lacks 15 of the 22 residues within the proline-punctuated repeat 6. This in-frame deletion (146Δ160) within repeat 6 causes a dominant negative reduction in apoA-I HDL concentration in the heterozygous state. In this case, as in several other examples of apoA-I mutants displaying a dominant negative phenotype in the heterozygous state, this apoA-I mutant may not be “esterification neutral,” but may weakly inhibit LCAT-catalyzed cholesterol esterification. In support of this hypothesis, recent in vitro studies show that apoA-I Seattle inhibits LCAT-catalyzed cholesterol esterification in particles isolated from transfected Chinese hamster ovary cells (72).

It is not clear how alteration in apoA-I structure can have such a extreme effect on plasma HDL apoA-I metabolism without invoking the concept of a direct LCAT and apoA-I interaction. Monoclonal antibody mapping indicate that conformational alterations induced by the Δ6 apoA-I deletion have a more profound effect on global conformation than those alterations induced by the Δ5Δ6 apoA-I deletion. However, despite the disruption of the domain extending from repeat 5 to repeat 8 (137–178), Δ6 apoA-I binds to spherical unilamellar vesicles with only 2-fold less affinity. These results are consistent with...
on the ability of wild-type apoA-I to activate LCAT formation for LCAT-catalyzed generation of cholesterol esters.

In summary, these data show a domain corresponding to the idea that an apoA-I central domain (repeats 5–8) is not implicated in stimulating LCAT activity. Deletion of apoA-I repeat 6 not only blocks normal levels of cholesterol esterification but also exerts a dominant inhibition on the ability of wild-type apoA-I to activate LCAT in vivo.

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Single Repeat Deletion in ApoA-I Blocks Cholesterol Esterification and Results in Rapid Catabolism of Δ6 and Wild-type ApoA-I in Transgenic Mice
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