Distinct Central Amphipathic α-Helices in Apolipoprotein A-I Contribute to the in Vivo Maturation of High Density Lipoprotein by Either Activating Lecithin-Cholesterol Acyltransferase or Binding Lipids*

(Received for publication, October 5, 1999, and in revised form, November 22, 1999)

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Recombinant adenoviruses with cDNAs for human apolipoprotein A-I (wild type (wt) apoA-I) and three mutants, referred to as Δ4-5A-I, Δ5-6A-I, and Δ6-7A-I, that have deletions removing regions coding for amino acids 100–143, 122–165, and 144–186, respectively, were created to study structure/function relationships of apoA-I in vivo. All mutants were expressed at lower concentrations than wt apoA-I in plasma of fasting apoA-I-deficient mice. The Δ6-5A-I mutant was found primarily in the lipid-poor high density lipoprotein (HDL) pool and at lower concentrations than Δ4-5A-I and Δ6-7A-I that formed more buoyant HDL particles. At an elevated adenovirus dose and earlier blood sampling from fed mice, both Δ5-6A-I and Δ6-7A-I increased HDL-free cholesterol and phospholipid but not cholesteryl ester. In contrast, wt apoA-I and Δ4-5A-I produced significant increases in HDL cholesteryl ester. Further analysis showed that Δ6-7A-I and native apoA-I could bind similar amounts of phospholipid and cholesterol that were reduced slightly for Δ5-6A-I and greatly for Δ4-5A-I. We conclude from these findings that amino acids (aa) 100–143, specifically helix 4 (aa 100–121), contributes to the maturation of HDL through a role in lipid binding and that the downstream sequence (aa 144–186) centered around helix 6 (aa 144–165) is responsible for the activation of lecithin-cholesterol acyltransferase.

Epidemiological studies have shown that the levels of high density lipoprotein cholesterol (HDL-C)† and the major protein of HDL, apolipoprotein A-I (apoA-I), are inversely correlated with the incidence of coronary artery disease (1, 2). ApoA-I circulates as a 243-amino acid protein (3) and plays an important role in determining the concentrations of plasma HDL-C. This is accomplished through the ability of apoA-I to promote the removal of cholesterol and phospholipids from cells and subsequently activate the enzyme lecithin-cholesterol acyltransferase (LCAT) (4, 5) that catalyzes the formation of cholesteryl esters (CE) on HDL. Additional interactions of apoA-I with the cell surface scavenger receptor class B, type I (6), and possibly with cholesteryl ester transfer protein may also influence circulating HDL-C levels.

Domains of apoA-I required for lipid association and the activation of LCAT have been the focus of many in vitro investigations. The initial binding of apoA-I to lipids is thought to occur via the extreme N-terminal (aa 44–64) and C-terminal (aa 220–243) amphipathic α-helices that may or may not require additional interactions with other regions of the protein (7–10). In the lipop-bound state the central helices (aa 100 to 186) have been proposed to contain a putative hinge domain (11, 12) thought to enable apoA-I to adopt distinct conformations on HDL, as well as the major LCAT activating domain (13–19).

We have previously generated a series of mutants in which a pair of adjacent helices were deleted sequentially within the central domain of apoA-I (20). These mutants denoted here as Δ4-5A-I, Δ5-6A-I, and Δ6-7A-I contain deletions in which amino acids 100–143, 122–165, and 144–186 have been removed, respectively. Our rationale in choosing these deletions was that removing a pair of helices would minimally modify the periodicity of the amphipathic α-helices and their putative interactions. Subsequent characterization of these mutants in vitro confirmed that the amphipathic α-helices within residues 100–186 were involved in interactions with phospholipids and may contribute to the overall lipid binding capacity of the apoA-I in the formation of HDL (21). Removal of helices 4 and 5 (aa 100–143) caused a slight decrease in LCAT activation in vitro, whereas deletion of either helices 5 and 6 (aa 122–165) or 6 and 7 (aa 144–186) almost abolished cholesterol esterification (18). This finding is in agreement with previous work suggesting that helix 6 (aa 144–165) is necessary for optimum LCAT activation (17) although other domains of apoA-I may also be involved (9, 22, 23). Despite the insights gained from these in vitro studies,
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**EXPERIMENTAL PROCEDURES**

**Production and Screening of First Generation Recombinant Adenoviruses**—The cDNAs for firefly luciferase (luc), wt apoA-I, ΔΔ-5A-I, ΔΔ-6A-I, and ΔΔ-7A-I were subcloned into the vector pCA13 under the control of the cytomegalovirus promoter (Microbix Biosystems Inc., Toronto, Ontario, Canada). Recombinant adenovirus (Ad5) constructs carrying these cDNAs were prepared following co-transfection of 293 cells (Microbix Biosystems Inc.) with the plasmid pJM17 and the appropriate pCA13 plasmid. LipofectAMINE (Life Technologies, Inc.) was used as the transfection reagent. After an 8-h incubation with the transfection mixture in Opti-MEM (Life Technologies, Inc.), the 293 cells were incubated overnight with Eagle’s minimum essential medium containing 10% fetal bovine serum and then overlaid with 0.65% SeaPlaque agar (FMC BioProducts, Rockland, ME) in Eagle’s minimum essential medium containing fetal bovine serum (5%), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). Plaques were picked (1–2 weeks later) from the agar and resuspended in sterile phosphate-buffered saline (PBS, 137 mM NaCl, 8.2 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl) containing CaCl2 (0.68 mM), MgCl2 (0.50 mM) (PBS2+), and glycerol (10%). These plaques were used to infect subsequent confluent monolayers of 293 cells in order to amplify the recombinant adenoviruses. Polymerase chain reaction of DNA prepared from SDS- (0.5%) and Pronase (0.05%)-lysed 293 cells infected with the appropriate Ad5 construct was performed to detect the apoA-I cDNAs or luciferase cDNA (luc).

All adenoviruses were subjected to several rounds of amplification prior to a final purification on two successive CsCl gradients. The purified virus stocks were dialyzed extensively against PBS containing 10% glycerol and aliquoted through sterile 0.22-μm filters (Millipore Corp.), placed in SDS sample buffer, and analyzed by SDS-PAGE. The presence of the apoA-I proteins in each of the FPLC Superdex 200 fractions was detected by chemiluminescence (West Pico SuperSignal substrate, Pierce) after incubation with horseradish peroxidase-conjugated anti-apoA-I antibody (Amersham Pharmacia Biotech). The relative intensities of the apoE signals were determined with software from Bio-Rad (Quantity One, version 4.1.0).

**Gel Filtration Chromatography and Sample Analysis**—Plasma (500 μl) isolated following the different Ad5 injections (96 h) was loaded on two Superdex 200 columns (analytical grade, Amersham Pharmacia Biotech) connected in series with a total bed volume of approximately 400 ml and void volume of 100 ml. The columns were standardized with a mixture of high and low molecular weight markers of known Stokes’ diameters (Amersham Pharmacia Biotech). Plasma was passed down the columns at a flow rate of 0.1 ml per min, and 5-ml fractions were collected. The VLDL and low density lipoprotein component of the plasma appeared in the void volume (fractions 9–12) on these columns. HDL2 particles were localized in fractions 15–19 (12.1 ± 1.02 nm), larger HDL3 particles in fractions 20–23 (9.7 to 8.2 nm), and smaller HDL fractions containing albumin were in fractions 24–28. Total cholesterol was determined for the various fractions. Briefly, samples were concentrated (5-fold by lyophilization) and resuspended in PBS, and cholesterol levels were quantified by standard enzymatic kits as described. Aliquots (100 μl) from each fraction were analyzed for apo-A-I by slot blot (Bio-Rad Bio-Dot SF unit) analysis. The nitrocellulose was probed with biotinylated monoclonal antibodies directed against apoA-I (a combination of 4H1 (against the extreme N terminus) and 5F6 (against the central region)). The antibodies were biotinylated with Sulfo-NHS-Biotin (Pierce). The presence of the apoA-I proteins in each of the FPLC Superdex 200 fractions was detected by chemiluminescence (Pierce SuperSignal substrate) following treatment with streptavidin-conjugated horseradish peroxidase (Amersham Pharmacia Biotech). No background signal could be detected as indicated by analysis of plasma fractions isolated following luc-Ad5 injections. Lipid levels (TC, FC, CE, and PL) in the HDL2/HDL3 FPLC fractions were measured as described earlier.

**Western Blot Analysis Probing for Either the Human apoA-I Proteins or Human apoE**—Plasma samples were subjected to discontinuous gradient density ultracentrifugation. Briefly, each sample (0.5 to 1.0 ml) was brought to a final volume of 3 ml (1 mM EDTA) with the addition of potassium bromide (1 g of KBr) and sucrose (50 mg). The discontinuous gradient consisted of plasma (3 ml on bottom) layered successively with ρ = 1.21 g/ml KBr (2 ml, ρ = 1.08 g/ml KBr (3 ml), and ρ = 1.00 g/ml KBr (3 ml top)). The tubes were spun for 18 h at 35,000 rpm on a Beckman L8–70M ultracentrifuge at 8 °C, and fractions (1 ml) were collected from top to bottom. The densities of the fractions were determined by comparing the readings measured with a refractometer (Fisher) to values obtained for solutions with known densities.

Aliquots of each of the fractions were dialyzed against PBS (0.025 μm filter disks, Millipore Corp.), placed in SDS sample buffer, and analyzed by 12% SDS-PAGE. The gels were either stained for protein (GELOCODE Blue Stain, Pierce) or transferred to nitrocellulose for Western blot analysis probing for either the human apoA-I proteins or mouse apoE. The concentrations of the apoA-I proteins (wild-type and mutant) in the different lipoprotein fractions from very low density lipoproteins (VLDL) to lipid-poor HDL species were measured by radiolabelling as described previously (12, 24).

**Non-denaturating Gradient Gel Electrophoresis**—The discontinuous gradient density fractions were subjected to non-denaturing gradient gel electrophoresis followed by Western blot analysis to size the HDL species formed by wt apoA-I or the central domain deletion mutants. The samples were dialyzed against PBS before application to the gels. The gradient gels were run for 2200 V-h and included high molecular weights.
Fasting Plasma Lipid Levels Accompanying Expression of ApoA-I Variants in ApoA-I-deficient Mice—We first established that wt apoA-I reached physiological levels (170 ± 40 mg/dl) in the plasma of fasted apoA-I-deficient mice 96 h post-Ad5 injection at a dose of 2 × 10^9 pfu. Under the same conditions, all apoA-I mutants were expressed at significantly lower levels. The highest expression was observed for Δ4-5A-I, followed by Δ6-7A-I, and lowest for Δ5-6A-I. No impairment in secretion rates of the apoA-I central domain deletion mutants were detected following injection of COS-7 cells in culture (>95% of all apoA-I forms were secreted in the media as measured by Western blot analysis 3 days postinfection, data not shown). This suggests that all apoA-I mutants are folded normally and secreted equally. When we increased the Ad5 doses and analyzed plasma in the fed state at earlier times postinjection (within 40 h), the levels of the apoA-I mutants obtained were similar to or greater than those found for wt apoA-I (Table I, 40-h fed column). The time course of expression showed that all mutants had early peak levels that decreased markedly by day 6 postinjection, whereas wt apoA-I reached a peak concentration 10 days postinjection and remained circulating for over 30 days (Fig. 1).

Fasting Plasma Lipid Levels Accompanying Expression of ApoA-I Variants—Expression of the apoA-I mutants in plasma taken from fasted apoA-I-deficient mice (96 h postinjection) was not accompanied by any increase in plasma lipid levels but rather by moderate decreases (Table II). In contrast, wt apoA-I produced significant increases in plasma cholesterol and phosphatidylcholines. This phenomenon in plasma cholesterol was due exclusively to an increase in the HDL-CE concentration. The control luc-Ad5-injected mice had slightly decreased lipid levels compared with non-injected mice, but this did not reach statistical significance. Therefore, the decrease in plasma lipid levels following expression of the apoA-I mutants, and in particular Δ5-6A-I (Table II), was significant (p < 0.05) and not explained by an effect of the adenovirus vector alone.

| Ad5 construct | 96-H fasted (n) | 40-H fed (n) |
|---------------|-----------------|--------------|
| Luciferase    | 0.32 ± 0.22 (2) | ND           |
| wt apoA-I     | 172 ± 43 (4)*   | 178 ± 130 (3)* |
| Δ4-5A-I       | 27 ± 6 (4)*     | 643 ± 206 (3)* |
| Δ5-6A-I       | 3.6 ± 2.3 (7)*  | 405 ± 230 (3)* |
| Δ6-7A-I       | 9.3 ± 4.2 (3)*  | 198 ± 90 (4)* |

* Adenovirus dose = 2 × 10^9 pfu. 
† Adenovirus dose = 1 × 10^10 pfu. 
ND, not determined.

Effect of Δ4-5A-I and Δ5-6A-I Expression on Mouse Plasma ApoE Levels—We tested the possibility that the reduced concentration of lipids in plasma of apoA-I-deficient mice following expression of the apoA-I mutants was correlated with decreases in the concentrations of other apolipoproteins normally found in these mice. It was previously reported that apoA-I-deficient mice have increased HDL apoE levels comprising 25% of the total HDL protein compared with control C57BL/6 mice in which apoE on HDL was barely detectable (28). We observed that plasma apoE levels (primarily in the HDL pool) were significantly reduced following expression of either Δ4-5A-I (Fig. 2A, lanes 6 and 7) or Δ5-6A-I (Fig. 2A, lanes 8–10) 96-h postinjection with 2 × 10^9 pfu of each recombinant adenovirus. However, injection with luc-Ad5 had no effect on the circulating apoE levels in apoA-I-deficient mice (Fig. 2A, lanes 1 and 2 compared with lanes 3–5 for non-injected mice). On average, the plasma apoE levels were reduced by 50% following expression of Δ4-5A-I and Δ5-6A-I as shown in Fig. 2B, and this occurred primarily in the HDL pool (data not shown). Similar results were obtained upon injection of either wt apoA-I or Δ6-7A-I which also promoted significant lowering of apoE plasma levels (not shown).

FPLC Analysis of ApoA-I-deficient Mouse Plasma following Expression of Human ApoA-I Wild-type and the Central Domain Deletion Mutants—Plasma samples isolated from fasted apoA-I-deficient mice 96 h following injection of the various

TABLE I
The protein concentrations of native human apoA-I and the three mutants in the plasma of apoA-I-deficient mice

Each Ad5 construct was administered via the tail vein of apoA-I-deficient mice at either one of the two doses as described under "Experimental Procedures." The number (n) of mice used for each mutant and either treatment is indicated. There are significant increases in the plasma protein concentrations of the apoA-I mutants when a higher Ad5 dose is combined with earlier sampling (40 h postinjection) and maintenance of the fed state.
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TABLE II

Fasted plasma lipid levels and the ratio of esterified to total cholesterol in apoA-I-deficient mice prior to or following injections of either luciferase or the various apoA-I recombinant adenovirus constructs

| Ad5 injection \( (* \) | Plasma lipid levels | CET/TC |
|---------------------|------------------|--------|
|                      | TC \( \text{mg/dl} \) | FC \( \text{mg/dl} \) | CE \( \text{mg/dl} \) | PL \( \text{mg/dl} \) |
| None (8)            | 53 ± 5           | 26 ± 5   | 27 ± 6 | 66 ± 12 | 0.51 ± 0.09 |
| Luciferase (3)      | 44 ± 3           | 23 ± 2   | 21 ± 5 | 46 ± 6  | 0.47 ± 0.08 |
| wt apoA-I (4)       | 115 ± 14         | 34 ± 11  | 81 ± 26b | 181 ± 25b | 0.70 ± 0.06b |
| Δ5-5A-I (3)         | 41 ± 14          | 24 ± 12  | 17 ± 4 | 44 ± 10 | 0.43 ± 0.10 |
| Δ5-6A-I (4)         | 27 ± 4           | 15 ± 3   | 12 ± 1b | 28 ± 5b | 0.44 ± 0.03 |
| Δ6-7A-I (3)         | 47 ± 4           | 33 ± 5   | 14 ± 2  | 46 ± 3  | 0.29 ± 0.05 |

* All adenovirus constructs were injected at a final titer of 2 \( \times 10^9 \) pfu. Plasma was sampled 96 h postinjection from a given number \( (n) \) of fasted (9–11-h) apoA-I-deficient mice. The lipid and CE/TC ratio values for wt apoA-I and mutants are shown. Low concentrations of Δ5–6A-I and Δ6–7A-I (see Table I) reduce the already low plasma lipid levels found in apoA-I-deficient mice, whereas wt apoA-I increases plasma CE and PL as well as the CE/TC ratio.

* Values that are statistically significant at \( p < 0.05 \) (Student’s \( t \) test) from noninjected apoA-I deficient mice are indicated.

Fig. 2. Δ4-5A-I and Δ5-6A-I expression decreases apoE levels in the plasma of apoA-I-deficient mice. A, Western blot of a 12% SDS-reducing polyacrylamide gel of whole fasted mouse plasma. Each lane contains 1.5 μl of plasma from either luc:Ad5-injected apoA-I-deficient mice, non-injected apoA-I-deficient mice, or mice injected with Δ4-5A-I:Ad5 or Δ5-6A-I:Ad5 as indicated. The gels were transferred to nitrocellulose (Bio-Rad mini trans-blot transfer cell) and probed with a polyclonal anti-mouse apoE antibody or a combination of biotinylated monoclonal anti-human apoA-I antibodies as described under “Experimental Procedures.” B, the relative mean intensities of Western blot densitometric scans of the combined luc:Ad5 and non-injected apoA-I signals were compared with the apoE signals obtained following expression of Δ4-5A-I and Δ5-6A-I. The average intensity (± S.E.) of the apoE signals following Δ4-5A-I and Δ5-6A-I expression (96-h postinjection with 2 \( \times 10^9 \) pfu) are given as a percentage of the average signal obtained for the combined luc:Ad5 injected and non-injected mice.

Ad5 constructs (2 \( \times 10^9 \) pfu) were subjected to FPLC analysis. The total cholesterol lipoprotein profiles observed following expression of Δ5-6A-I and Δ6-7A-I at levels between 3 and 10 mg/dl were similar (not shown) to the profile observed with luc:Ad5 (Fig. 3, A). In contrast, Δ4-5A-I expressed at much higher levels (Fig. 3, C; 43 mg/dl) resulted in a significant increase in HDL cholesterol. HDL cholesterol was further increased with expression of wt apoA-I (Fig. 3, C; 168 mg/dl). Native apoA-I was found predominantly in the HDL2 size range (fractions 14–19), whereas Δ4-5A-I was located in a smaller HDL pool comprising small HDL2 (fractions 17–19) and large HDL3 (fractions 20–22) as shown by slot blot analysis below and above Fig. 3, respectively.

To compare the HDL lipid profiles obtained following expression of wt apoA-I and Δ4-5A-I, the distributions of HDL TC, FC, CE, and PL were normalized per mg of apoA-I loaded on the columns and are expressed as μg of lipid per fraction (Fig. 4). From these data it is clear that Δ4-5A-I formed mature HDL but appeared to bind proportionally less lipid (cholesterol and phospholipid) than did wt apoA-I in the HDL pool.

Fractional Cholesterol Esterification Rate of ApoA-I-deficient Mouse Plasma following Expression of Wild-type ApoA-I and Δ4-5A-I—Despite the overall reduction in lipids found in the Δ4-5A-I HDL pool, a significant proportion of CE was found. This prompted us to compare the ability of apoA-I-deficient mouse plasma expressing either wt apoA-I or Δ4-5A-I to esterify cholesterol. The fractional cholesterol esterification rate (FER) of apoA-I-deficient mouse plasma without apoA-I (Ad5-injected mice) or following expression of either wt apoA-I or Δ4-5A-I was compared with control mouse plasma (wild-type C57BL/6 mice) (Fig. 5). The levels of wt apoA-I and Δ4-5A-I obtained following Ad5 injections were quantified by radioimmunoassay, and equal amounts of expressed protein (10 μg) in equal plasma volumes were used for the experiment. Compared with control mouse plasma, apoA-I-deficient plasma had a 65% reduction in FER of exogenously added \(^{3}H\)HFC (Fig. 5) in agreement with a previous report (29). The latter study also demonstrated that the marked decrease of CE concentration observed in apoA-I-deficient plasma resulted primarily from a decrease in apoA-I activator protein and not from a decrease in LCAT mass. We observed significant increases in the FER of apoA-I-deficient mice plasma expressing Δ4-5A-I and wt apoA-I (Fig. 5), although Δ4-5A-I was not as effective as wt apoA-I in activating LCAT in vivo.

Distribution of ApoA-I Central Domain Deletion Mutants in HDL Density Subclasses—The lipoprotein density distribution profiles of the three apoA-I mutants were compared with wt apoA-I following separation of the lipoproteins from plasma expressing the various apoA-I proteins. All injections were with 2 \( \times 10^9 \) pfu of Ad5, and plasma from fasted mice was collected 96 h postinjection. Native apoA-I formed very buoyant lipoproteins (down to 1.04 g/ml) with the majority of the protein found in the HDL2 subclasses (Fig. 6). Interestingly, both Δ4-5A-I and Δ6-7A-I exhibited similar density distribution profiles and were found associated with the small HDL2 and large HDL3 subclasses. However, only approximately 20% of Δ5-6A-I was

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found in the buoyant HDL_{2/3} pool, and the remaining protein was located in the very high density subclass (VHDL lipid-poor or lipid-free fractions).

Size of HDL Formed by the ApoA-I Central Domain Deletion Mutants—We observed that each of wt apoA-I, Δ4-5A-I, and Δ6-7A-I formed normal HDL particles (11–12 nm), either HDL2 (Fig. 7, lanes A, D, and F, respectively) or HDL3 (Fig. 7, lanes B, E, and G, respectively). However, proportionally more of the two central domain deletion mutants were found in HDL3 than wt apoA-I, confirming quantification by radioimmunoassay (Fig. 6). Very little Δ5-6A-I was detectable by Western blot analysis in the HDL2/3 subclasses, but it was found predominantly in lipid-poor (d > 1.16 g/ml) HDL fractions of much smaller size (Stokes’ diameter between 7 and 8 nm, Fig. 7, lane H).

Morphology of HDL Formed by the ApoA-I Central Domain Deletion Mutants—The isolated HDL fractions (from 1.06 to 1.12 g/ml) were also examined by negative staining electron microscopy (Fig. 8). The HDL isolated from apoA-I-deficient mice injected with the control luc.Adn vector (Fig. 8A) were spherical in shape as judged by the characteristic hexagonal packing array. This confirmed the result of an earlier study that detected only spherical HDL in the plasma of apoA-I-deficient mice (28). When HDL proteins were subjected to 12% SDS-PAGE, Coomassie staining and Western blot analysis demonstrated that apoE was a major HDL protein in the apoA-
I-deficient background (data not shown). The HDL (1.06 g/ml) isolated following injections of wt apoA-I Ad5 were also spherical (Fig. 8B) but larger than those normally found in apoA-I-deficient mice. Greater than 95% of the protein in this HDL fraction was native human apoA-I (colloidal blue staining and Western blot analysis of SDS-PAGE, data not shown).

Fig. 8, C and D, depicts HDL isolated following expression of Δ4-5A-I. In the more buoyant fraction (d = 1.07 g/ml, Fig. 8C), rolls of stacked HDL indicative of discoidal particles were present. However, the majority of Δ4-5A-I formed spherical HDL at a higher density (1.11 g/ml, Fig. 8D) in which very few discoidal HDL were observed. In contrast, both Δ5-6A-I and Δ6-7A-I formed predominantly discoidal HDL as shown in Fig. 8, E and F, respectively (d = 1.11 g/ml). The HDL did not exhibit the tight hexagonal packing that HDL containing wt apoA-I and Δ4-5A-I showed at this same density. Therefore, Δ5-6A-I and Δ6-7A-I induced the greatest population of discoidal HDL in vivo when compared with HDL formed with either wt apoA-I (no discoidal HDL) or Δ4-5A-I (small population of discoidal HDL).

Effect of the ApoA-I Central Domain Deletion Mutants on Plasma Lipid Levels in the Fed State—High concentrations of the apoA-I mutants were obtained when earlier sampling (40 h post-Ad5 injection) of plasma from mice maintained in the fed state (chow diet) was combined with an increase in the adeno-virus dose administered (1 × 10¹⁰ pfu; Table I, last column). These modifications enabled us to compare the ability of each mutant to transport plasma lipid in relation to wt apoA-I. The wt apoA-I adenovirus dose was kept at 2 × 10⁹ pfu since the protein concentration was already at or slightly above physiological levels. Sets of either 3 or 4 mice (all females between 3 and 6 months of age) were used to study each apoA-I variant under these conditions. The increased plasma lipid mass was confined to the HDL pool upon expression of all apoA-I forms (data not shown). Mice expressing wt apoA-I had the greatest increase in HDL-CE mass 40 h after Ad5 injection (Table III). There was also a significant increase in the HDL-CE concentration accompanying expression of Δ4-5A-I. In contrast, Δ5-6A-I and Δ6-7A-I did not increase the plasma CE concentrations although they both produced large increases in PL and FC. Interestingly, at these higher concentrations the plasma CE levels in apoA-I-deficient mice decreased from their normal values with Δ6-7A-I expression and were further lowered to values that approached zero when Δ5-6A-I was expressed (Table III).

To evaluate the ability of each mutant to associate with HDL lipids, we compared the mass ratio of lipids (TC, FC, CE, and PL) to apoA-I for each mutant to the values obtained for wt apoA-I. As shown in Fig. 9, Δ4-5A-I associated with significantly fewer of each lipid on a mass basis than did wt apoA-I.
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Fig. 8. Morphology of HDL species formed prior to or following in vivo expression of the apoA-I variants. Electron microscopy of negatively stained HDL fractions isolated by discontinuous gradient density ultracentrifugation. The HDL were prepared for EM as outlined under “Experimental Procedures.” The bar in each panel represents 50 nm. All images were taken at ≥30,000 magnification with the exception of E that was taken at ×70,000 magnification. A is HDL (p = 1.076 g/ml) isolated from apoA-I-deficient mice injected with the control lucAd5 construct. B–F represent HDL formed following injections of the indicated human apoA-I Ad5 constructs. B, wt apoA-I HDL (p = 1.06 g/ml); C, Δ4–5A-I HDL (p = 1.07 g/ml); D, Δ4–5A-I (p = 1.11 g/ml); E, Δ5–6A-I HDL (p = 1.11 g/ml); F, Δ6–7A-I HDL (p = 1.11 g/ml).

We have observed that all three apoA-I central domain deletion mutants are expressed at lower levels than native apoA-I in the plasma of apoA-I-deficient mice 96 h postinjection when the mice are fasted (Table I). Our results suggest that this is a consequence of their enhanced clearance from the plasma and not a result of impaired synthesis and secretion. Indeed, all mutants are secreted as efficiently as wt apoA-I from COS-7 cells following infection with recombinant adenoviruses in culture (not shown) and reach high concentrations, similar to and in some cases exceeding wt apoA-I levels, at early times postinjection in plasma sampled from fed mice (Table I). Furthermore, the time course of expression (Fig. 1) shows that the plasma concentrations of the apoA-I mutants decrease rapidly between 3 and 6 days, whereas wt apoA-I levels increase up to day 10 before starting a more gradual decline. Therefore, the low concentrations in plasma samples taken from fasted mice (96 h postinjection) must have resulted primarily from their enhanced clearance due to deficiencies in the abilities of these mutants to accumulate CE or bind lipids and not from impaired production by the liver.

The amphipathic α-helices are responsible for the lipid binding properties of apoA-I (30) and the class Y helix as compared with classes G* and A* appears to have the highest affinity for lipids (31). Two class Y helices are localized to the C terminus (aa 209–241) and are predicted to be important components for the interaction of apoA-I with lipids (8, 32). This is supported by the enhanced clearance of various apoA-I C-terminal truncation mutants when injected as lipid-free apoA-I into mice (33) or rabbits (34). Whereas it has been suggested by Mishra et al. (8) that all helical domains of apoA-I contribute to lipid binding, their data indicate that the central region of the protein exhibits a much weaker affinity for lipids than the C-terminal domain (aa 209–241).

The present in vivo study, however, demonstrates that helix 4 (aa 100–121) within the central domain also contributes significantly to the lipid binding abilities of apoA-I. According to the FPLC profile (Fig. 4), Δ4–5A-I associates with proportionally fewer lipids in the HDL pool compared with wt apoA-I, and the HDL are shifted to a smaller size (Fig. 3). The apparently reduced lipid binding of Δ4–5A-I becomes more evident at higher expression levels. The concomitant increases in plasma lipids in the HDL pool (Table III) observed at high mutant concentrations shows that, although Δ4–5A-I increases significantly the plasma CE concentrations (discussed later), it has a decreased ability to associate with lipids compared with wt apoA-I (Fig. 9). Since lipid binding is severely reduced for Δ4–5A-I and only slightly reduced for Δ5–6A-I and normal for Δ6–7A-I (Fig. 9), we conclude that helix 4 (aa 100 to 121) accounts for the majority of the lipid binding mediated by the central domain of apoA-I. This is the first direct in vivo evidence that helix 4 (aa 100–121), a class Y helix, is required for efficient lipid binding of apoA-I leading to the formation of circulating plasma lipoproteins.

There is evidence in the literature to support our conclusion that helix 4 is required for efficient lipid binding by apoA-I, whereas downstream sequences within the central domain are not as critical. One study demonstrated that a 44-mer synthetic peptide corresponding to helices 4–5 but not peptides containing helices 5–6 or 6–7 could lower the enthalpy of the gel to liquid crystalline phase transition of dimyristoylphosphatidylcholine multilamellar vesicles (8). As well, in vitro studies from our laboratory support a role for helix 4 in binding lipids. First, an epitope comprising aa 100–143 is sensitive to the phospholipid and cholesterol environment in well defined reconstituted lipoprotein particles containing two apoA-I molecules (Lp2A-I)...

DISCUSSION

Recombinant adenoviruses containing native and mutant cDNAs were generated to study structure/function relationships of apoA-I in vivo in apoA-I-deficient mice. This approach provides new insights into the roles of apoA-I central domain amphipathic α-helices in the maturation of HDL in vivo and shows that, between residues 100 and 186, two distinct functional domains can be distinguished that are primarily responsible for either lipid binding or the activation of LCAT. Our results also clarify discrepancies between previous in vitro studies addressing the LCAT activation domain of apoA-I and provide new information on amino acids within this region that are important to the overall lipid binding abilities of apoA-I.

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adenovirus constructs were injected at a dose of 1 pfu. The lipid to apoA-I mass ratio was determined in apoA-I-deficient deletion mutants with lipids in the HDL pool in the fed state. As shown in Table I, apoA-I or the other two mutants following tail vein injections of wt apoA-I and Δ4–5A-I increase the circulating CE concentrations in the plasma, whereas Δ6–7A-I and Δ6–7A-I have the opposite effects and cause further reductions in the CE concentrations.

Interestingly, in contrast to helix 4, the downstream sequence (aa 122–186) of the central domain contributes very little to the lipid binding as discussed above. This observation is consistent with the notion that this sequence contains the hinge domain that contains an important lipid-binding helix (aa 100–143) and that its loosely lipid-bound amphipathic α-helices are important for the efficient activation of LCAT (discussed below). The low lipid binding affinity of this region also explains why apoA-II is able to displace these central helices on lipoprotein particles (36). The sequential deletions of helix pairs have allowed us to distinguish the contribution of each helix to either or both lipid binding and LCAT activation. At high concentrations, Δ6-6A-I and Δ6-7A-I increase markedly the concentrations of plasma HDL-free cholesterol and phospholipid but not cholesteryl ester (Table III). The HDL formed by these two mutants are predominantly discoidal (Fig. 8, E and F) and migrate exclusively to the preβ position on agarose gels (not shown). On the other hand, despite a lower binding capacity for lipids, Δ4-5A-I significantly increases the plasma CE mass, forms spherical HDL particles (Fig. 8D), and migrates to both the preβ and α positions on agarose gels (similar to wt apoA-I). Together, these data demonstrate that the C-terminal half (aa 144–186) of the central domain is critical for the conversion of FC to CE in vivo, whereas the N-terminal half (aa 100–143) is not.

Many in vitro experiments have been performed to identify the minimal sequence requirements for apoA-I activation of LCAT. Some studies suggested a broad region of apoA-I between aa 96 and 186 or a smaller region between aa 95 and 125 (13). Other studies have shown that aa 144–186 (17, 18, 22, 37) are necessary, although additional reports have implicated contributions by N-terminal (38) and C-terminal sequences (10). In contrast to previous results (16, 19), our in vivo observations show that the N-terminal portion (aa 100–143) of the central domain that contains an important lipid-binding helix (aa 100–121) is not essential for the activation of LCAT. Our data also demonstrate that helix 6 (aa 144–165), a helix absent from Δ6-6A-I and Δ6-7A-I, is required for the conversion of preβ particles to mature CE-rich α-migrating HDL. This is the first in vivo evidence that helix 6 contributes little to lipid binding but appears to be essential for LCAT activation. Our findings are consistent with previous in vitro studies showing that the major LCAT activation domain is contained within aa 144–186 (17, 18, 22, 37). The reduced lipid association of this domain (aa 144–186) may enable apoA-I to interact directly with LCAT in vivo and/or allow LCAT to more easily access the underlying phospholipid fatty acid acyl chains. This concept is supported by the study of Sorci-Thomas et al. (39) where replacement of helix 6 (aa 145–165) with helix 10 (aa 220–241) produced an apoA-I variant that has a 5-fold decrease in its ability to activate LCAT despite a higher affinity for lipids.

Plasma lipoproteins from mice expressing Δ4-5A-I (Fig. 5) as well as reconstituted lipoproteins formed with this mutant (18) could activate LCAT with 50% efficiency compared with wt apoA-I. Based on the reduced lipid association of Δ4-5A-I compared with wt apoA-I, we propose that the lower FER for this mutant relative to the native protein is mostly a consequence of lower concentrations of lipid substrates (i.e. phospholipid fatty acyl chains) available for the LCAT-dependent reaction in the

### Table III

| Ads construct | TC | FC | CE | PL | CE/TC |
|---------------|----|----|----|----|-------|
| None (4)     | 53 ± 6.8 | 24 ± 7.5 | 30 ± 6.9 | 70 ± 14 | 0.56 ± 0.11 |
| wt apoA-I (3) | 151 ± 51<sup>b</sup> | 72 ± 28<sup>b</sup> | 80 ± 23<sup>b</sup> | 250 ± 100<sup>b</sup> | 0.53 ± 0.02 |
| Δ4-5A-I (3) | 167 ± 20<sup>b</sup> | 99 ± 27<sup>b</sup> | 67 ± 16<sup>b</sup> | 310 ± 62<sup>b</sup> | 0.40 ± 0.10 |
| Δ5-6A-I (3) | 190 ± 95<sup>b</sup> | 205 ± 99<sup>b</sup> | -15 ± 30<sup>b,c</sup> | 344 ± 180<sup>b</sup> | -0.07 ± 0.04<sup>b,c</sup> |
| Δ6-7A-I (4) | 165 ± 50<sup>b</sup> | 148 ± 59<sup>b</sup> | 17 ± 9<sup>c</sup> | 256 ± 90<sup>b</sup> | 0.13 ± 0.12<sup>b,c</sup> |

<sup>a</sup> Mean ± S.E. All adenovirus injections are at a dose of 1 × 10<sup>9</sup> pfu with the exception of wt apoA-I Ad5 that is injected at a dose of 2 × 10<sup>9</sup> pfu.

<sup>b</sup> All plasma lipid values and CE/TC ratios following high expression levels (see Table I) of wt apoA-I or each mutant are statistically different at p < 0.05 from non-injected mice.

<sup>c</sup> In addition, lipid values and CE/TC ratios for the mutants that are different from wt apoA-I at p < 0.05 are shown (Student’s t test). At these high concentrations, both wt apoA-I and Δ4–5A-I increase the circulating CE concentrations in the plasma, whereas Δ6–6A-I and Δ6–7A-I have the opposite effects and cause further reductions in the CE concentrations.

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**Fig. 9.** Association of wt apoA-I and the three central domain deletion mutants with lipids in the HDL pool in the fed state. The lipid to apoA-I mass ratio was determined in apoA-I-deficient mouse plasma following injections of the recombinant human apoA-I adenoviruses. Plasma was sampled 40 h postinjection from mice maintained in the fed state as outlined under "Experimental Procedures." All adenovirus constructs were injected at a dose of 1 × 10<sup>9</sup> pfu with the exception of wt apoA-I Ad5 that was injected at 2 × 10<sup>9</sup> pfu. A, wt apoA-I; B, Δ4-5A-I; C, Δ5-6A-I; D, Δ6-7A-I. The lipid values (TC, FC, CE, or PL) for each mutant were compared with the corresponding wt apoA-I value in A for statistical significance (Student’s t test) at either * p < 0.05 or ** p ≤ 0.1. Bars with no symbols are not statistically different from the corresponding wt apoA-I value (p > 0.1).

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HDL pool. However, secondary interactions of LCAT with residues 100–143 on apoA-I cannot be ruled out. Also, the deletion of helices 4 and 5 may produce an apoA-I variant that cannot accommodate the same size of CE core as wt apoA-I during HDL maturation. Nonetheless, this domain that contains aa 100–143 only contributes minimally to the LCAT activation by apoA-I.

Analysis of plasma samples isolated from fasted apoA-I-deficient mice 96 h postinjection with the recombinant adenovirus has also demonstrated the specific contributions of central helices to the formation and stability of HDL. Interestingly, despite its inability to incorporate CE into HDL, Δ6-7A-I forms buoyant lipoprotein particles similar to Δ4-5A-I in both density distribution (Fig. 6, ▼ and ■) and size (Fig. 7, lanes E, F, and C, D), although both mutants are shifted to a higher density relative to wt apoA-I (Fig. 6 ▽). In contrast, Δ5-6A-I is found predominantly in lipid-poor, less buoyant (Fig. 6, ▲), and much smaller HDL (Fig. 7 ▼). Therefore, removal of helices 5 and 6 has the biggest impact not only on the circulating apoA-I levels but also on the ability to maintain a stable conformation on HDL. We postulate that the slight decrease in lipid binding of this mutant compared with wt apoA-I (Fig. 9), combined with its inability to accumulate any CE (even less than Δ6-7A-I), generates an unstable apoA-I protein that is subsequently cleared more rapidly in fasted apoA-I-deficient mice.

Low plasma lipids are observed when the apoA-I mutants, in particular Δ5-6A-I, are expressed in low concentrations (Table II). As well, the CE/TC ratios, normally between 0.4 and 0.6 in the plasma of apoA-I-deficient mice, are reduced to values that approach zero (Table III) when Δ5-6A-I and Δ6-7A-I are expressed at high concentrations. These dominant negative effects are likely explained by the observed reduction in mouse apoE levels (Fig. 2) and possibly other apolipoproteins, in the HDL pool that normally contribute to plasma lipid levels in apoA-I-deficient mice. Therefore, these mutants provide useful tools to investigate the potential mechanisms that could underlie the dominant negative effects on HDL-C produced by some apoA-I variants. Future studies are planned to address this more thoroughly.

In summary, the in vivo results presented here demonstrate that the domain containing helices 4 and 5, and within it the class Y helix 4 (aa 100–121), is necessary for the efficient lipid binding of apoA-I and stabilization of lipoprotein particles. Therefore, this domain together with the C-terminal class Y amphipathic α-helices (aa 209–241) is important for the association of apoA-I with lipids. At the same time, helices 4 and 5 are not essential for LCAT activation while the downstream sequence (aa 144–186) is required for the activation of this enzyme. This is also the first in vivo demonstration that aa 144–186 contribute little to apoA-I lipid binding but are required for incorporation of CE into the HDL pool. Furthermore, deletion of this region prevents the conversion of pre-β migrating discoidal to α-migrating spherical particles in vivo. Based on these observations, we conclude that two distinct functional domains exist within the central region (aa 100–186) of apoA-I. The N-terminal sequence (aa 100–143) contains an important lipid binding domain while the more C-terminal sequence (aa 144–186) contains the major LCAT activation domain.

Acknowledgments—We are grateful to Peter Rippstein and Ann Fook Yang for assistance with the electron microscopy and preparation of the carbon-coated Formvar grids. We also thank the Animal Care staff at the University of Ottawa Heart Institute for their assistance in this study and Dan Sparks, Ross Milne, and Ruth McPherson for critical reading of the manuscript.

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Distinct Central Amphipathic α-Helices in Apolipoprotein A-I Contribute to the in Vivo Maturation of High Density Lipoprotein by Either Activating Lecithin-Cholesterol Acyltransferase or Binding Lipids
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J. Biol. Chem. 2000, 275:5043-5051.
doi: 10.1074/jbc.275.7.5043

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