Structure-function studies of apoA-I variants: site-directed mutagenesis and natural mutations

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Abstract Five mutants of apolipoprotein A-I (apoA-I), apoA-I(Δ63–73), apoA-I(Δ140–150), apoA-I(63–73@140–150), apoA-I(R149V), and apoA-I(P143A) were compared with human plasma apoA-I for their ability to promote cholesterol and phospholipid efflux from HepG2 cells. A significantly lower capacity to promote cholesterol and phospholipid efflux was observed with lipid-free apoA-I(Δ63–73), while mutations apoA-I(Δ140–150) and apoA-I(P143A) affected phospholipid efflux only. When added as apoA-I/palmitoyloleoyl phosphatidylcholine (POPC) complex, mutations apoA-I(63–73@140–150) and apoA-I(Δ140–150) affected cholesterol efflux. None of the mutations affected α-helicity of the lipid-free mutants or their self-association. Five natural mutations of apoA-I, apoA-I(A95D), apoA-I (Y100H), apoA-I(E110K), apoA-I(V156E), and apoA-I (H162Q) were studied for their ability to bind lipids and promote cholesterol efflux. None of the mutations affected lipid-binding properties, cholesterol efflux, or α-helicity of lipid-free mutants. Two mutations affected self-association of apoA-I: apoA-I(A95D) was more prone to self-association, while apoA-I(E100H) did not self-associate. The following conclusions could be made from the combined data: *i*) regions 210–243 and 63–100 are the lipid-binding sites of apoA-I and are also required for the efflux of lipids to lipid-free apoA-I, suggesting that initial lipidation of apoA-I is rate limiting in efflux; *ii*) in addition to the lipid-binding regions, the central region is important for cholesterol efflux to lipidated apoA-I, suggesting its possible involvement in interaction with cells.—Sviridov, D., A. Hoang, W. Huang, and J. Sasaki. **Structure-function studies of apoA-I variants: site-directed mutagenesis and natural mutations. *J. Lipid Res.* 2002.** 43: **1283–1292.**

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Apolipoprotein A-I (apoA-I) is the principal apolipoprotein of HDL and a key element in the reverse cholesterol transport pathway. ApoA-I is a single polypeptide of 243 amino acids and its characteristic feature is that when bound to lipids it is organized into a series of 22-mer or 11-mer amphipathic α-helices (1). These helices are positioned either perpendicularly to the surface of the lipid disk according to the picket fence model (2) or parallel to the surface according to the belt (3) and hairpin (4) models. It follows from all three models that the correct secondary structure, i.e., correct length, amphipathicity, and orientation of the helices is essential for enabling apoA-I to maintain correct structure of HDL and to carry out its functions. That brings about the question of whether a specific amino acid sequence for apoA-I is required for carrying out its functions and how important this requirement might be in relation to that of secondary structure.

Mutagenized apoA-I, synthetic peptides, and monoclonal antibodies have been used to probe the structure-function relationship of apoA-I [for review see (5)]. These studies suggest that the central α-helical region (residues 137–186) is involved in LCAT activation, cellular cholesterol efflux, and interaction with a cell surface binding site (6–11). The C-terminus (residues 193–243) of apoA-I appears to play a role in protein–lipid interaction, cholesterol efflux from the plasma membrane, and in vivo HDL catabolism (11–15). The importance of individual amino acid residues for apoA-I function has also been examined by investigating naturally occurring apoA-I mutants. ApoA-I(L141R)$_{Pisa}$, apoA-I(P143R)$_{Giessen}$, apoA-I(V156E)$_{Oita}$, apoA-I(L159R)$_{Fin}$, apoA-I(R160L)$_{Oslo}$, apoA-I(P165R), and apoA-I(ΔE235)$_{Nichinan}$ have been shown to affect either LCAT activation or cholesterol efflux (16–

Abbreviations: apoA-I, apolipoprotein A-I; DMPC, dimyristoyl phosphatidylcholine; POPC, palmitoyloleoyl phosphatidylcholine; rHDL, reconstituted HDL.

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22). Most of these studies, however, did not address the issue of whether the effect of the mutation was due to its effect on the secondary structure of apoA-I or whether there is a requirement for a specific amino acid sequence for a particular function.

In this paper, we analyze the effect of a number of apoA-I mutations, either naturally occurring or made by site-directed mutagenesis, on the ability of apoA-I to promote cholesterol and phospholipid efflux. We have previously demonstrated that monoclonal antibody directed against the central part of apoA-I (residues 140–150) inhibits the efflux of intracellular cholesterol to human plasma (10). This finding was not confirmed, however, when we used truncated forms of apoA-I (11), presumably because the effects of truncation on the carboxyl-terminal half of apoA-I overshadowed the effect of removal of the targeted sequence. Therefore, we created apoA-I mutants more precisely targeting the region 140–150 of apoA-I; the mutations included those predictably affecting or not affecting the secondary structure of the region. Two mutations, Δ140–150 and P143A, were predicted to affect the secondary structure of the target region of apoA-I, while two others, R149V and 63–73@140–150, were not. The fifth mutation, Δ63–73, was designed as incurring changes to the secondary structure similar to the deletion of the target region, but located in a segment of apoA-I not thought to be involved in cholesterol efflux. It was recently demonstrated, however, that the region 63–73 might represent a second lipid-binding region of apoA-I (9). In addition to mutations created by site-directed mutagenesis, we also investigated five natural mutations of apoA-I identified during screening of blood samples from a population survey. These natural mutations were also located in the central part of apoA-I between residues 95 and 162; the effect of these mutations on apoA-I structure and functions has not been previously described.

MATERIALS AND METHODS

Site-directed mutagenesis and expression of recombinant apoA-I

Construction, expression, purification, and verification of the recombinant apoA-I mutants apoA-I(P143A), apoA-I(R149V), apopA-I (Δ63-73), apoA-I(Δ140–150), and apoA-I(63–73@140–150) are described in detail elsewhere (9, 23, 24). All apoA-I mutants were expressed in a baculovirus/insect cell expression system as described previously (24). Recombinant human apoA-Is containing natural mutations were expressed as a glutathione-*S*-transferase fusion protein in an *Escherichia coli* expression system as described previously (22, 25). Concentration of the proteins was measured according to Bradford (26). Human plasma apoA-I was isolated and purified as described previously (27).

Preparation of reconstituted HDL

The reconstituted HDL (rHDL) was prepared by the sodium cholate dialysis method according to Jonas et al. (28, 29) using palmitoyloleoyl phosphatidylcholine (POPC) (Sigma, Castle Hill, NSW, Australia), apoA-I, and sodium cholate (Sigma) in a molar ratio of 80:1:80. The characteristics of the particles were reported previously (9, 25).

Lipid efflux

Efflux of plasma membrane and intracellular cholesterol as well as cellular phospholipid to apoA-I modified by site directed mutagenesis was tested in a previously described model utilizing HepG2 cells (10, 11). Briefly, to label plasma membrane cholesterol and phospholipid, cells were incubated in serum-containing medium with [1α,2α(n)-^{3}H]cholesterol (Amersham Pharmacia Biotech; specific radioactivity 1.81 TBq/mmol, final radioactivity 75 KBq/ml) and [methyl-^{14}C]choline (Amersham Pharmacia Biotech; specific activity 2.1 GBq/mmol, final activity 0.2 MBq/ml) for 48 h in a CO_2 incubator. The cells were then washed and cooled on ice. Leibovitz L-15 serum-free medium containing [1-^{14}C]acetic acid sodium salt [(ICN; specific radioactivity 2.2 GBq/mmol, final radioactivity 18 MBq/ml) was added and cells were incubated for 3 h at 15°C to label newly synthesized (intracellular) cholesterol. Under these conditions, intracellular cholesterol trafficking is blocked while cholesterol biosynthesis proceeds (30, 31). After labeling, cells were incubated for 3 h or indicated periods of time at 37°C with serum-free medium containing 1,000-fold excess of unlabeled sodium acetate, and lipid-free apoA-I at a final concentration of 1 μM, or rHDL at a final POPC concentration of 80 μM. Lipids were extracted from aliquots of media and cells, and cholesterol and phospholipid were isolated by TLC as described previously (32).

Efflux of cellular cholesterol to apoA-I containing natural mutations was tested in a previously described model utilizing human skin fibroblasts (33). It has been previously shown that properties of cholesterol efflux from fibroblasts and HepG2 cells are similar (34). Fibroblasts, like HepG2 cells, contain ATP binding cassette transporter A1 (ABCA1) required for the efflux to lipid-free apoA-I (35). Loading with cholesterol can increase cholesterol efflux to fibroblasts, but both loaded and non-loaded fibroblasts exert features of ABCA1-dependent specific efflux to lipid-free apoA-I (36). Non-cholesterol-loaded fibroblasts have been used in this study; the efflux in the presence of lipid-free apoA-I was twice of that in its absence. Fibroblasts were labeled by incubating with [^{3}H]cholesterol (Amersham Pharmacia Biotech; specific radioactivity 1.81 TBq/mmol, final radioactivity 75 KBq/ml) for 48 h at 37°C in the presence of 10% fetal calf serum and then incubated with DMEM containing different cholesterol acceptors for 3 h at 37°C. The final concentration of apoA-I was 1 μM. Lipids from cells and medium were extracted by incubation with hexane-isopropanol (3:2 v/v) and counted.

Interaction of apolipoproteins with phospholipid liposomes

Solubilization of dimyristoyl phosphatidylcholine (DMPC) by apoA-I was studied as described previously (9, 33). Briefly, dry DMPC (Sigma) was sonicated in Tris buffer (pH 8.0) to form multilamellar liposomes. Apolipoproteins (final concentration 0.1 mg/ml) were pre-incubated for 10 min at 24°C and the reaction initiated by adding DMPC liposomes (final DMPC concentration 0.5 mg/ml). The reduction of absorption at 325 nm (which reflects the reduction in light scattering) was monitored for 1 h at 2 min intervals at 24°C to assess formation of apoA-I/DMPC complexes.

Circular dichroism studies

The α-helical content of apoA-I was determined by measuring circular dichroism spectra at protein concentration 0.1 mg/ml in 20 mM phosphate buffer (pH 7.4) at 25°C. Circular dichroism spectra were measured on a JASCO 810 spectropolarimeter. Data were collected from 185 nm to 250 nm at 0.5 nm intervals. The percentage of α-helical content of apoA-I was calculated by the equation of Chen et al. (37).

Cross-linking of apoA-I

Self-association of apoA-I was studied as described previously (38). In brief, proteins in PBS were mixed in solution with a 5.6 molar excess of dithiobis(succinimidyl propionate) (DSP) (Pierce, Rockford, IL) dissolved in DMSO. Samples were incubated for 30 min at room temperature and then quenched with Tris/HCl, (pH 7.3) (final concentration 50 mM), for 15 min and analyzed on 10% SDS-PAGE.

Statistics

All experiments were performed in quadruplicate (i.e., determination from four dishes) and reproduced two or three times. Background values for the cholesterol and phospholipid efflux (i.e., the amount of radioactivity released to the medium in the absence of an acceptor) were subtracted. Means ± SE are presented. Statistical significance of differences was determined by Student's two-tail *t*-test.

Prediction of the structural properties of the mutants

Predicted hydrophobicity (Kyte-Doolittle), average charge, and amphipathicity (Eisenberg) of the regions of apoA-I were calculated using Protean software (DNASTAR Inc). Wheel diagrams and predicted orientations of α-helices were generated using Antheprot v. 4.0 (Microsoft).

RESULTS

Cholesterol and phospholipid efflux to apoA-I mutants made by site-directed mutagenesis

Labeled cells were incubated for 3 h with equimolar (1 μM) concentrations of human plasma apoA-I or each of the five apoA-I mutants, and transfers of plasma membrane and newly synthesized intracellular cholesterol, and of cellular phospholipid, from cells to acceptor were assessed. When apoA-I was added in lipid-free form, efflux of both plasma membrane and intracellular cholesterol to four out of five mutants [apoA-I(Δ140-150), apoA-I(P143A), apoA-I(R149V), and apoA-I(63–73@140–150)] was similar to the efflux observed with wild-type apoA-I (**Figs. 1A**, **B**). Efflux of plasma membrane cholesterol to the mutant apoA-I(Δ63-73) was 15 times less than to the wild-type apoA-I, and efflux of intracellular cholesterol to this mutant was no more than efflux to the medium alone (Figs. 1A, 1B). When phospholipid efflux to lipid-free apoA-I was assessed, three mutations, apoA-I(Δ140–150), apoA-I(P143A), and apoA-I(Δ63–73), showed ∼50% inhibition of phospholipid efflux, whereas the two other mutations, apoA-I(R149V) and apoA-I(63–73@140–150), did not affect efflux of phospholipid (Fig. 1C).

The ability of lipid-free apoA-I to promote cholesterol efflux could be affected by mutations through a number of mechanisms. First, a mutation may affect α-helicity of the lipid-free form, which in turn may affect the initial microsolubilization of the microdomains of plasma membrane (39). No effect of the mutations on the secondary structure of lipid-free mutants was, however, found (**Table 1**). It should be mentioned that the effect of mutations on the secondary structure of lipid-free apoA-I might be different from their effect on that of lipid-bound apoA-I, where α-helicity is much higher. Second, a mutation may affect the ability of apoA-I to self-associate. Enhanced self-association may decrease the apparent concentration of a monomer apoA-I. No effect of mutations on self-association of lipid-free apoA-I was, however, found (**Fig. 2A**). The amount of monomer protein was 55–65% for the wild-type and all mutated apoA-I. This is consistent with our previous finding indicating that the carboxyl-terminal end of apoA-I is essential for its self-association (38). Third, the ability of apoA-I to promote cholesterol efflux could be affected by its lipid-binding properties (11, 39).

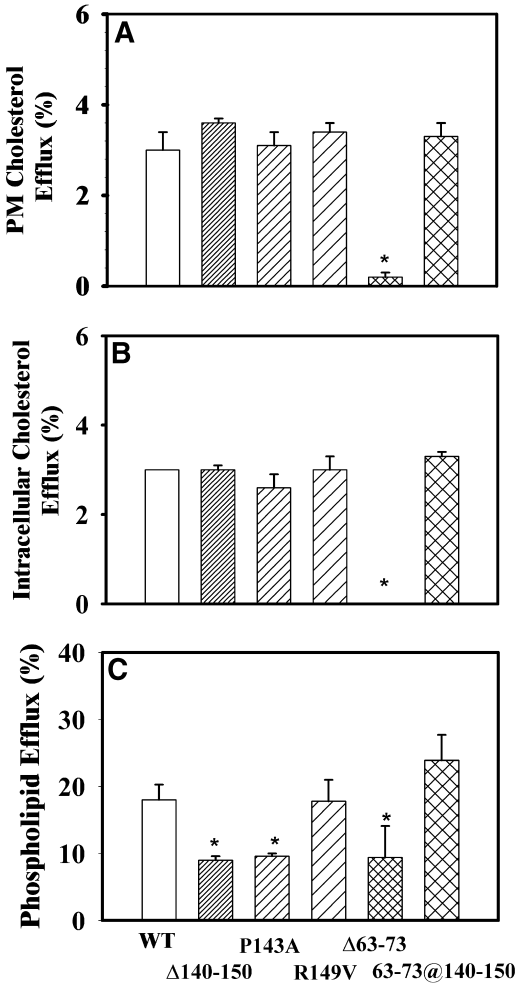

Fig. 1. Efflux of plasma membrane (A) or intracellular (B) cholesterol or phospholipid (C) to lipid-free apolipoprotein A-I (apoA-I). Plasma membrane or intracellular cholesterol or cellular phospholipid in HepG2 cells were labeled as described in Materials and Methods. Cells were incubated for 3 h at 37°C with serum-free medium containing lipid-free human plasma apoA-I or apoA-I mutants at a final concentration of 1 μM. The medium was then collected, and cells were washed and harvested. Lipids were extracted from aliquots of media and cells, and cholesterol and phospholipid were isolated by TLC. Cholesterol efflux is expressed as a percentage of labeled cholesterol moved from cells to media. All points are mean ± SEM of quadruplicate determinations. $*P < 0.01$. WT, wild type.

TABLE 1. α-Helical content of lipid-free apoA-I mutants

ApoA-I Mutant	α-Helical Content	Number of Amino Acids in α-Helices
	%	
ApoA-I(WT)	42	102
ApoA-I(Δ140–150)	48	111
ApoA-I(P143A)	42	102
ApoA-I(R149V)	42	102
ApoA-I(Δ63–73)	42	97
ApoA-I(63–73@140–150)	48	117
ApoA-I(A95D)	45	109
ApoA-I(Y100H)	41	100
ApoA-I(E110K)	44	107
ApoA-I(V156E)	43	104
ApoA-I(H162Q)	42	99

ApoA-1, apolipoprotein A-1.

We have reported previously that mutants apoA-I(P143A) and apoA-I(Δ63–73) are defective in their ability to bind phospholipid (9), which may explain their lesser activity in promoting phospholipid efflux. Mutant apoA-I(Δ140–150) was more efficient in binding phospholipid than was wild-type apoA-I (9); thus, its deficiency in promoting phospholipid efflux cannot be explained by lipid-binding properties alone.

To examine cholesterol efflux to apoA-I/PC complexes, apoA-I mutants were assembled into apoA-I/POPC (1:80 mol/mol) rHDL particles. We were unable to obtain stable rHDL particles with mutant apoA-I(P143A). Other mutants formed rHDL particles of similar size and composition, and their properties have been described previously (9). There was no cellular phospholipid efflux to rHDL particles already containing phospholipid. Efflux of plasma membrane cholesterol to three mutants, apoA-I(Δ140–150), apoA-I(R149V), and apoA-I(63–73@140–150), was 30–40% lower compared with that to wild-type apoA-I, whereas mutation apoA-I(Δ63–73) had no effect (**Fig. 3A**). When efflux of intracellular cholesterol was assessed, two mutations, apoA-I(Δ140–150) and apoA-I(63–73@140–150), inhibited efflux by ~50%, whereas two others, apoA-I(R149V) and apoA-I(Δ63–73), did not have a statistically significant effect (Fig. 3B).

To determine if rates of cholesterol efflux are affected by the mutations, time-course experiments were con-

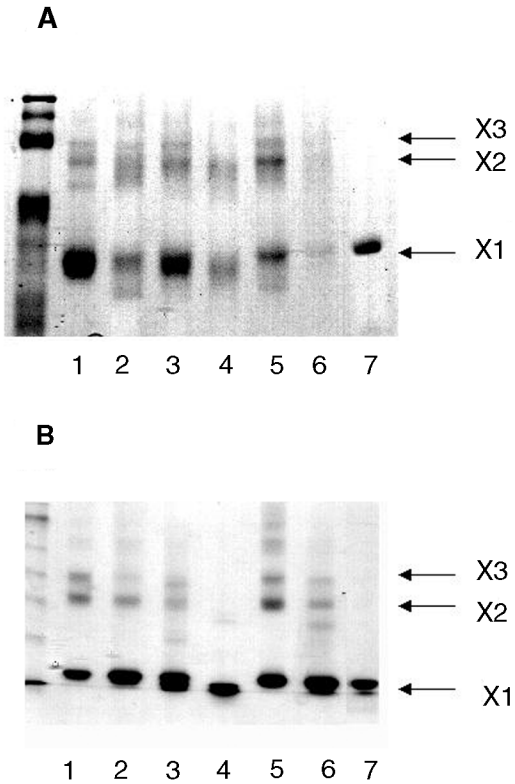

Fig. 2. Self-association of apoA-I mutants obtained with site-directed mutagenesis (A) or natural mutants of apoA-I (B). All proteins (35.3 μM) were cross-linked with 200 μM dithiobis (succinimidyl propionate) as described in Materials and Methods and separated using 10% SDS-PAGE. A: Lane 1, human apoA-I; Lane 2, apoA-I(P143A); Lane 3, apoA-I(R149V); Lane 4, apoA-I(Δ140-150); Lane 5, apoA-I(63-73@140-150); Lane 6, apoA-I(Δ63-73); Lane 7, human apoA-I not cross-linked. B: Lane 1, apoA-I(H162Q); Lane 2, apoA-I(V156E); Lane 3, apoA-I(E110K); Lane 4, apoA-I(Y100H); Lane 5, apoA-I(A95D); Lane 6, human apoA-I; Lane 7, human apoA-I not cross-linked.

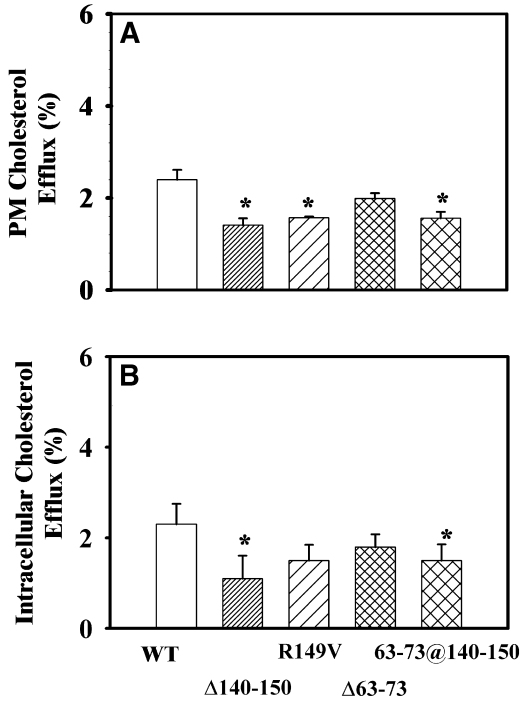

Fig. 3. Efflux of plasma membrane (A) or intracellular (B) cholesterol to reconstituted HDL (rHDL) containing apoA-I mutants. Plasma membrane or intracellular cholesterol in HepG2 cells was labeled as described in Materials and Methods. Cells were incubated for 3 h at 37°C with serum-free medium containing rHDL [apoA-I-palmitoyloleoyl phosphatidylcholine (POPC), 1:80, mol/mol] with human plasma apoA-I or apoA-I mutants at a final POPC concentration of 80 μM. The medium was then collected, and cells were washed and harvested. Lipids were extracted from aliquots of media and cells, and cholesterol and phospholipid were isolated by TLC. Cholesterol efflux is expressed as a percentage of labeled cholesterol moved from cells to media. All points are mean ± SEM of quadruplicate determinations. *$P < 0.05$.

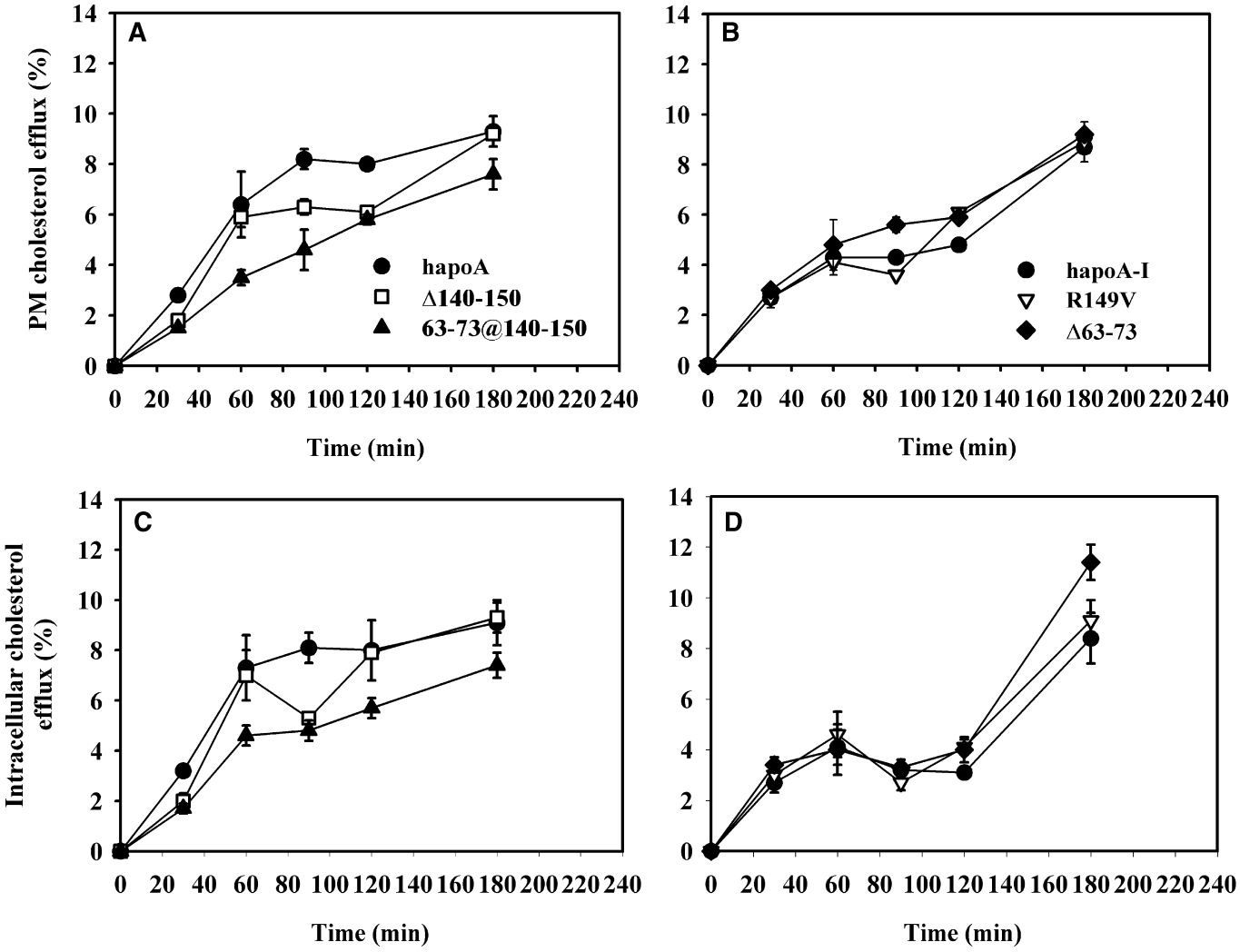

Fig. 4. Time course of the efflux of plasma membrane (A, B) or intracellular (C, D) cholesterol to rHDL containing apoA-I mutants. Plasma membrane or intracellular cholesterol in HepG2 cells was labeled as described in Materials and Methods. Cells were incubated for indicated periods of time at 37°C with serum-free medium containing rHDL (apoA-I-POPC, 1:80, mol/mol) with human plasma apoA-I or apoA-I mutants at a final POPC concentration of 80 μM. The medium was then collected, and cells were washed and harvested. Lipids were extracted from aliquots of media and cells, and cholesterol and phospholipid were isolated by TLC. Cholesterol efflux is expressed as a percentage of labeled cholesterol moved from cells to media. All points are mean ± SEM of triplicate determinations.

ducted to assess efflux of plasma membrane and intracellular cholesterol to rHDL containing wild-type or mutated apoA-I (**Fig. 4**). Only mutation apoA-I(63–73@140–150) significantly affected the rate of efflux of plasma membrane cholesterol ($P < 0.65$; Fig. 4A): initial velocities of cholesterol efflux (V_0) were 0.17 ± 0.04%/min and 0.06 ± 0.01%/min ($P < 0.001$) for wild-type apoA-I and for apoA-I(63–73@140–150), respectively. The same mutation caused inhibition of efflux of intracellular cholesterol (Fig. 4C): V_0 were 0.22 ± 0.06%/min and 0.09 ± 0.03%/min ($P < 0.001$) for wild-type apoA-I and for apoA-I(63–73@140–150), respectively.

Cholesterol and phospholipid efflux to apoA-I carrying natural mutations

We studied the properties of five natural apoA-I mutants: apoA-I(A95D)$_{Hita}$, apoA-I(Y100H)$_{Karatsu}$, apoA-I(E110K)$_{Fukuoka}$, apoA-I(V156E)$_{Oita}$, and apoA-I(H162Q)$_{Kurume}$. To evaluate the effect of the mutations on the ability of apoA-I to promote cholesterol efflux, wild-type or mutated apoA-I, lipid-free or incorporated into rHDL, was incubated with labeled human skin fibroblasts, and release of the labeled cholesterol from cells to extracellular acceptors was measured. None of the mutations affected the ability of apoA-I to promote cholesterol efflux when presented either as lipid-free proteins (**Fig. 5A**) or assembled into rHDL (Fig. 5B).

The effect of the mutations on the secondary structure of lipid-free apoA-I was also studied (Table 1). None of the mutations affected the secondary structure of lipid-free apoA-I (Table 1).

When self-association properties of the mutants were studied, three mutations, apoA-I(E110K), apoA-I(V156E), and apoA-I(H162Q), self-associated similarly to the wild-

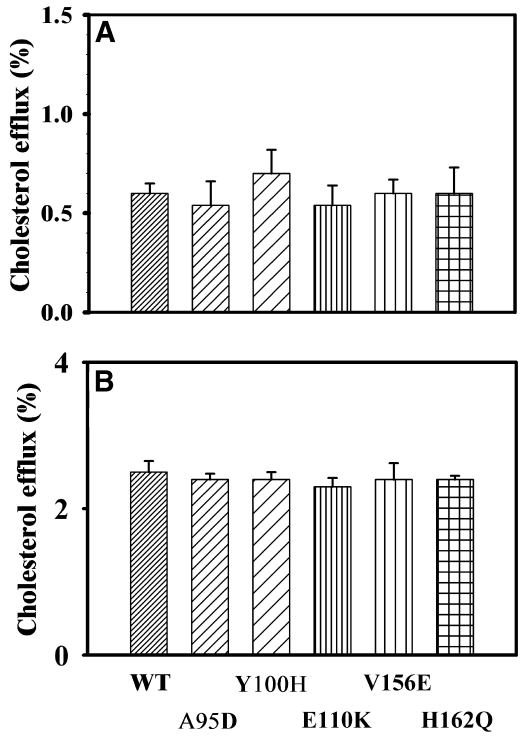

Fig. 5. Efflux of cholesterol to the natural mutants of apoA-I, lipid free (A) or incorporated into rHDL (B). Cholesterol in human skin fibroblasts was labeled as described in Materials and Methods. Cells were incubated for 3 h at 37°C with serum-free medium containing lipid-free human plasma apoA-I or apoA-I mutants (A) or rHDL (B) at a final protein concentration of 1 μM. The medium was then collected, and cells were washed and harvested. Lipids were extracted from aliquots of media and cells and counted. Cholesterol efflux is expressed as a percentage of labeled cholesterol moved from cells to media. All points are mean ± SEM of quadruplicate determinations.

type apoA-I. Sixty to seventy percent of the protein was present as a monomer, the rest forming dimers and trimers (Fig. 2B). It was demonstrated previously that self-association of proform of apoA-I does not progress past trimeric stage (38). Mutant apoA-I(A95D), however, under the same conditions, formed higher levels of aggregates, with almost 50% of protein self-associated (Fig. 2B, Lane 6). The mutant apoA-I(Y100H) had severely impaired ability to self-associate, with 97% of protein present as a monomer (Fig. 2B, Lane 4). These differences in self-association properties did not affect cholesterol efflux.

Lipid-binding properties of the mutants were assessed by measuring their ability to bind to DMPC suspension. None of the mutations had a statistically significant effect on the lipid-binding properties of apoA-I (**Table 2**).

TABLE 2. Interaction of DMPC with apoA-I mutants

ApoA-I Mutant	$K_{1/2}$ (Min^{-1})
ApoA-I(WT)	0.24
ApoA-I(A95D)	0.25
ApoA-I(Y100H)	0.22
ApoA-I(E110K)	0.27
ApoA-I(V156E)	0.28
ApoA-I(H162Q)	0.22

Experiments were performed as described in Materials and Methods. Half-life time ($t_{1/2}$) was determined from plots of fractional absorption at 325 nm (which reflects light scattering) against time (min) and data fitted to second-order kinetics of $[A] = 1/(1+kt)$. $K_{1/2} = 1/t_{1/2}$. DMPC, dimyristoyl phosphatidylcholine.

Delineation of the structure-function relationship of apoA-I

To analyze the structure–function relationship of apoA-I, we combined data on apoA-I mutations studied by our laboratories (10 mutations from this study and four mutations from our previous studies) (9, 11, 15, 22, 25, 40–42). The complete list of mutations we studied is given in **Table 3**. The mutations of apoA-I were analyzed for their ability to bind lipids and to promote cholesterol efflux to the lipid-free or lipid-associated forms. Eight mutants were naturally occurring mutations of apoA-I. Four of

TABLE 3. ApoA-I mutants: natural occurrence and predicted effect on secondary structure

Mutation	Natural Occurrence	HDL Levels	Predicted Effect on Secondary Structure	α-Helicity[a]	Reference
ApoA-I(Δ63–73)	No		Fuses two α-helices creating one 33-mer helix instead of two 22-mers	93	This paper (9)
ApoA-I(A95D)	$ApoA\text{-}I_{Hita}$	Normal	Turns α-helix	n.d.	This paper (40)
ApoA-I(Y100H)	$ApoA\text{-}I_{Karatsu}$	Normal	Not affected	96	This paper (41)
ApoA-I(ΔK107)	Yes	Low	Shortens and turns α-helix	90	(25)
ApoA-I(E110K)	$ApoA\text{-}I_{Fukuoka}$	Normal	Not affected	n.d.	This paper (42)
ApoA-I(P143A)	Similar to $apoA\text{-}I_{Giessen}$	Low	Fuses two α-helices creating one 44-mer helix instead of two 22-mers	n.a.	This paper (9)
ApoA-I(Δ140–150)	No		Fuses two α-helices creating one 33-mer helix instead of two 22-mers	86	This paper (9)
ApoA-I(63–73@140–150)	No		Not affected	100	This paper (9)
ApoA-I(R149V)	No		Not affected	86	This paper (9)
ApoA-I(V156E)	$ApoA\text{-}I_{Oita}$	Low	Not affected	91	This paper (18)
ApoA-I(H162Q)	$ApoA\text{-}I_{Kurume}$	Normal	Not affected	100	This paper (41)
ApoA-I(Δ210–243)	No		Shortens α-helix	84	(11, 38)
ApoA-I(Δ222–243)	No		Shortens α-helix	94	(11, 38)
ApoA-I(ΔE235)	$ApoA\text{-}I_{Nichinan}$	Low	Shortens and turns α-helix	85	(15, 22)

[a] In lipid-bound form (rHDL), percentage of α-helicity of wild type apoA-I.
rHDL, reconstituted HDL.

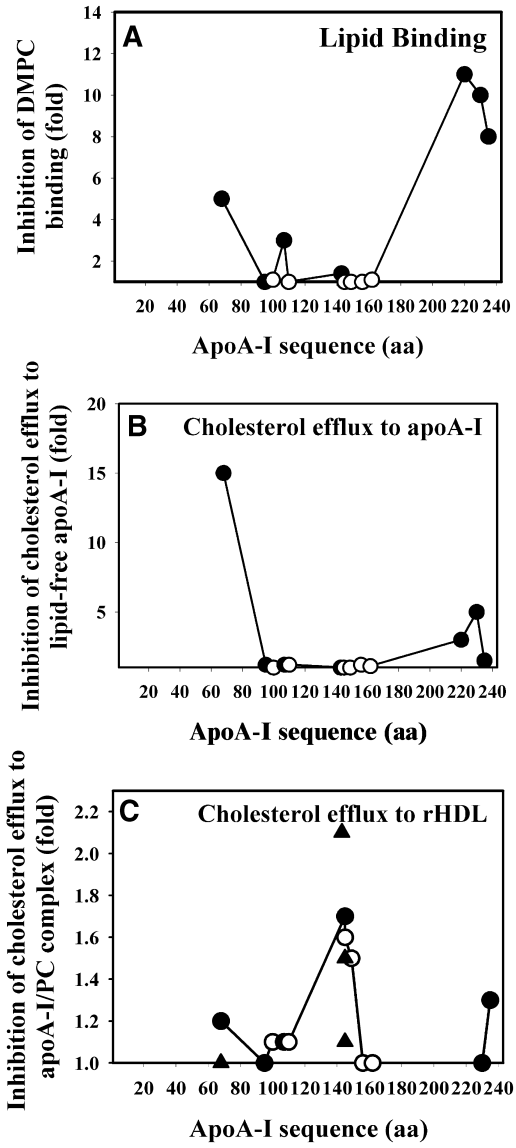

Fig. 6. Delineation of the structure-function relationship of apoA-I. Effects of mutation on the ability of apoA-I to bind lipids (A) or promote cholesterol efflux as lipid-free (B) or lipid-bound (rHDL) (C) acceptor are presented. Sequence of apoA-I is given on the *x* axis and the effects of the mutations are given on the *y* axis in folds (i.e., numerical value of a function determined with wild-type apoA-I divided to the same value determined with mutated apoA-I). Closed circles represent mutations affecting secondary structure; open circles represent mutations not affecting secondary structure; triangles (C only) represent effect of mutations on efflux of intracellular cholesterol.

them were associated with hypoalphalipoproteinaemia and four with normoalphalipoproteinaemia. The natural mutations described in this paper were identified during screening of blood samples from one of the population surveys. Because there was no follow-up, the effect of the mutations on the subsequent development of atherosclerosis and coronary heart disease could not be investigated.

To analyze the requirement for a specific amino acid sequence for apoA-I functions, mutations were subdivided into those predicted to either affect or not affect the secondary structure of 22- or 11-mer α-helical repeats of their lipid-bound form (Table 3). We considered the mutation to have an effect on secondary structure when it was predicted to change the size or orientation of the helix or introduce a kink into its amphipathic structure. The predicted effects of the mutations on the secondary structure are listed in Table 3. Eight mutations were predicted to affect the secondary structure of apoA-I, whereas the remaining six had no such effect. The effect of the mutations on the α-helical content of lipid-bound apoA-I (rHDL) has been investigated previously for most mutations and is also given in Table 3. With two exceptions, apoA-I(V156E) and apoA-I(R149V), α-helical content of the lipid-bound mutants that were predicted to affect secondary structure was lower than that of those predicted not to (Table 3). Neither the prediction based on molecular models nor that based on analysis of α-helicity is a reliable method of evaluating the true effect of the mutation on the structure of a lipid-bound protein in the absence of a high-resolution structure of the complex. However, considering the relatively high number of mutations analyzed, these methods may show at least a trend. Nevertheless, division of the mutants into those affecting and those not affecting secondary structure should be treated with caution.

The effect of the mutations on the ability of apoA-I to bind phospholipid is shown in **Fig. 6A**. Two regions of apoA-I appear to be critical in this regard: carboxyl-terminal end (210–243) and the region around residues 63 to 100.

The effect of the mutations on the ability of apoA-I to promote cholesterol efflux when added as lipid-free proteins is shown in Fig. 6B. The pattern of the effect of mutations on cholesterol efflux was very similar to that for lipid binding: the carboxyl-terminal end and residues around 63–73. Only those mutations affecting secondary structure inhibited both cholesterol efflux and lipid binding.

The effect of the mutations on the ability of apoA-I to promote cholesterol efflux when added as apoA-I/PC complex (rHDL) is shown in Fig. 6C. Some mutations within the sites involved in lipid binding and efflux to lipid-free apoA-I also affected cholesterol efflux to rHDL. However, the biggest effect was observed with mutations in the central region of apoA-I. Mutations of apoA-I were effective in inhibiting cholesterol efflux irrespective of their effect on secondary structure. Several mutations have been analyzed for their ability to promote efflux of cholesterol from intracellular compartments; mutations in the region 140–150 showed the biggest effect (Fig. 6D, triangles).

DISCUSSION

The present study continues our investigation into the putative features of apoA-I responsible for three of its functions: binding of lipids and cholesterol efflux from

plasma membrane and intracellular compartments. Five apoA-I mutants were created using site-directed mutagenesis. Lipid-binding properties of these mutants and their ability to activate LCAT were described previously (9). Here we studied these mutants in a cholesterol efflux assay. The first finding was that the region between amino acids 63 and 73 is essential to the ability of lipid-free apoA-I to promote cholesterol efflux. The deletion of this region affected neither the α-helical content of lipid-free apoA-I nor its self-associating properties, but it weakened the ability of apoA-I to bind lipid and reduced the stability of its complex with lipid (9), which may explain its effect on the efflux. Interestingly, however, the effect of the mutation on cholesterol efflux was greater than expected. We speculate that other mechanisms may be involved, such as interaction with ABCA1, the site of initial lipidation of apoA-I (43). The second finding was that the region 140–150 is important for the ability of apoA-I to promote cholesterol efflux to rHDL particles. This finding is consistent with our previous finding using a monoclonal antibody (10).

Five new natural mutations of apoA-I were analyzed in this study. These mutations were identified in a Japanese population, and recombinant proteins were synthesized based on their sequences. The mutations had no effect on the α-helical content of lipid-free forms of the mutants, their lipid-binding properties, or their ability to promote cholesterol efflux in lipid-free or lipid-bound forms.

To obtain a comprehensive picture of the effects of mutations on apoA-I functions, properties of the mutants described in this paper were combined with data derived from the same and other mutants previously reported by our laboratories. Analysis of lipid binding showed that two regions, the carboxyl terminus and region 63–73, may be involved. The carboxyl-terminal end of apoA-I is the most hydrophobic part of the protein (12) and its involvement in lipid binding has been repeatedly demonstrated by different methods (14, 44–46). It has also been predicted that a second lipid-binding region exists in the amino terminus of apoA-I (39, 47), which was confirmed in the present study. All available mutations on the carboxyl terminal affected secondary structure, making requirement for the specific amino acid sequence unclear. However, it is more likely that correct secondary structure of the amphipathic α-helices is the key factor determining the ability of apoA-I to bind lipids.

The two regions of apoA-I involved in lipid binding appear to be also involved in cholesterol efflux when lipid-free apoA-I was used as an acceptor. It follows that the initial lipidation of apoA-I is a necessary step in cholesterol efflux. When apoA-I is lipidated before interacting with cells, in addition to regions responsible for lipid binding, a third region in the middle of apoA-I appears to be involved. Mutations in this region also affected efflux of intracellular cholesterol. We hypothesize that this region might be involved in cholesterol efflux by means other than binding lipids, e.g., interaction with ABCA1 or caveolin, or stimulation of cholesterol trafficking or cell signaling.

On the basis of our data, we propose the following model of cholesterol efflux (**Fig. 7**). Two "sides" participate in efflux, an acceptor and a cell, and although our data support the acceptor "side," the cellular "side" of the model is based on the results of others (43, 48). The first step in efflux is lipidation of lipid-free apoA-I, mainly with phospholipid and possibly with small amounts of cholesterol. This step depends at least partially on the ability of apoA-I to accept lipids, which is determined by correct secondary structure of regions 210–243 and 63–73. A second factor is the release of cellular lipids and the lipidation of apoA-I, which is most likely determined by ABCA1. The product of the first step is discoid lipid-poor particles. These particles may be analogous to preβ_1-HDL, also a discoid lipid-poor particle (49) identified in human plasma by non-denaturing two-dimensional electrophoresis and claimed to be the initial acceptor of cel-

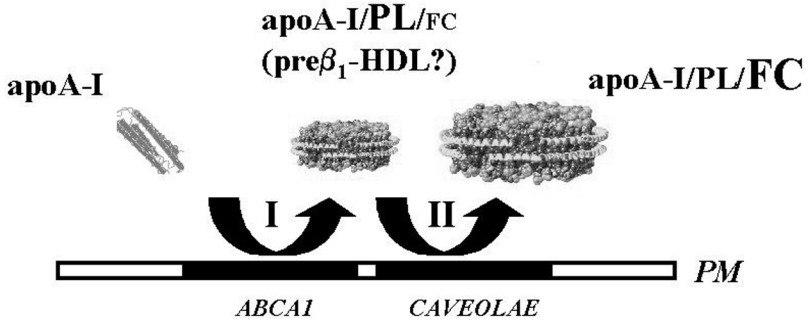

Fig. 7. Proposed model of cholesterol efflux. The first step in cholesterol efflux is the interaction of lipid-free apoA-I with the plasma membrane, which depends on the ability of apoA-I to bind lipids and on the ability of cells to release lipids and lipidate apoA-I. The former involves the lipid-binding regions of apoA-I (210–243 and 63–73); the latter most likely involves ATP binding cassette transporter A1 (ABCA1). The product of the first stage is minimally lipidated discoid particles resembling preβ_1-HDL. The second stage involves the interaction of these particles with regions of the plasma membrane, most likely caveolae, with efflux of more cholesterol and formation of fully lipidated discoid particles ready to interact with lecithin-cholesterol acyltransferase (LCAT). The second step also involves lipid-binding regions of apoA-I, but in addition it requires a specific sequence in the central part of apoA-I.

lular cholesterol (50). The second step is interaction of these particles with specific sites on the plasma membrane, leading to efflux of larger amounts of cellular cholesterol. The site on the plasma membrane where this interaction is most likely to occur is caveolae, the primary site of release of cellular cholesterol (51). This step also depends on the ability of apoA-I to bind lipids, but in addition may involve an unknown interaction that requires the central region of apoA-I. The product from the second step is fully lipidated discoid HDL, which is ready to interact with LCAT, take up more cellular cholesterol, and proceed along the reverse cholesterol transport pathway (Fig. 7). It is not clear what the contribution of the suggested mechanism into overall transfer of cholesterol from cells to HDL could be, but it may play a critical role in the formation of an HDL pool, which in turn may determine the efficiency of reverse cholesterol transport. Interestingly, the natural mutant apoA-I(L141R)$_{Pisa}$ showed an impaired ability to recruit cellular cholesterol; this was not caused by defective initial lipidation, but rather by a defect in the second stage of efflux, formation of fully lipidated HDL (16). Expression of apoA-I(Δ100–121) in vivo led to the formation of lipid-poor HDL by a mechanism not related to activation of LCAT (52). The central region of apoA-I appears to show the most plasticity, capable of dissociating from lipids and extending outside the particle (53), a property consistent with it being an "active site" interacting with enzymes and receptors. Although our observations deal with the "acceptor side" of the proposed model of cholesterol efflux, they are consistent with models suggested by Fielding et al. (48) and Wang et al. (43), who have investigated the "cellular side" of the same process.

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