

Influence of ApoA-I Structure on the ABCA1-mediated Efflux of Cellular Lipids*

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The influence of apolipoprotein (apo) A-I structure on ABCA1-mediated efflux of cellular unesterified (free) cholesterol (FC) and phospholipid (PL) is not well understood. To address this issue, we used a series of apoA-I mutants to examine the contributions of various domains in the molecule to ABCA1-mediated FC and PL efflux from mouse J774 macrophages and human skin fibroblasts. Irrespective of the cell type, deletion or disruption of the C-terminal lipid-binding domain of apoA-I drastically reduced the FC and PL efflux (~90%), indicating that the C-terminal amphipathic α -helix is required for high affinity microsolvubilization of FC and PL. Deletion in the N-terminal region of apoA-I also reduced the lipid efflux (~30%) and increased the K_m about 2-fold compared with wild type apoA-I, whereas deletion of the central domain (Δ 123–166) had no effect on either K_m or V_{max} . These results indicate that ABCA1-mediated lipid efflux is relatively insensitive to the organization of the apoA-I N-terminal helix-bundle domain. Alterations in apoA-I structure caused parallel changes in its ability to bind to a PL bilayer and to induce efflux of FC and PL. Overall, these results are consistent with a two-step model for ABCA1-mediated lipid efflux. In the first step, apoA-I binds to ABCA1 and hydrophobic α -helices in the C-terminal domain of apoA-I insert into the region of the perturbed PL bilayer created by the PL transport activity of ABCA1, thereby allowing the second step of lipidation of apoA-I and formation of nascent high density lipoprotein particles to occur.

3). The protective action of apoA-I is due primarily to its role in reverse cholesterol transport (4, 5). Reverse cholesterol transport comprises the following processes: 1) cholesterol efflux from cells in peripheral tissues including macrophages in the arterial wall, 2) lecithin:cholesterol acyltransferase-mediated esterification of HDL-associated cholesterol, and 3) receptor-mediated delivery of this cholesterol ester to the liver for excretion from the body (6, 7). The function of apoA-I in each of these steps is dependent upon its physical state at each stage. Regarding step 1, lipid-free (poor) apoA-I (8) appears to be the initial acceptor for cellular free cholesterol (FC) and phospholipids (PL) released by the activity of the ATP-binding cassette transporter A1 (ABCA1) (9, 10). Mutations in the ABCA1 gene lead to Tangier disease and Familial HDL deficiency and these patients are characterized by sterol deposition in macrophages and increased atherosclerosis (4, 5). A direct correlation between the expression of ABCA1 and apoA-I-mediated cellular lipid efflux has been revealed, emphasizing the importance of this transporter in the formation of HDL (9–11). However, the role of the structure of apoA-I in facilitating the export of cellular FC and PL via ABCA1 is not well established.

The human apoA-I molecule is a single polypeptide chain with 243 amino acid residues consisting of a series of homologous 11- and 22-mer amino acid repeats that are often interposed with proline-containing segments. The 22-mer repeats form amphipathic α -helices (6, 12, 13) and investigations of the secondary structure of apoA-I identified helical segments 44–65 and 220–241 in the N- and C-terminal domains, respectively, as being particularly hydrophobic and having the highest lipid binding affinities (14). The tertiary structure of apoA-I consists of two domains: a helix bundle domain comprising the N-terminal and central α -helices (residues 1–186) and a strongly lipid-binding C-terminal domain (residues 187–243) (15). An apoA-I molecule is thought to associate initially with the surface of a membrane via the latter domain, after which the helical bundle opens enhancing lipid-protein interaction (15). When apoA-I molecules interact with a cell expressing ABCA1, low levels of cellular FC and PL are removed and used to create various HDL particles (16), in a process we have termed membrane microsolvubilization (17–19). The fact that peptides corresponding to the hydrophobic α -helical regions of the apoA-I molecule can also mediate membrane microsolvubilization (20–23) implies that apoA-I/lipid interactions play a critical role. The ability of apoA-I to bind lipids is likely to be important in both the acquisition of PL and FC via ABCA1 and in the retention of these lipids to form stable HDL particles. Importantly, hydrophobic interactions between apoA-I and ABCA1 are also likely to be significant in the overall process of PL and FC efflux (24, 25).

Human apolipoprotein (apo)¹ A-I, the major protein of high density lipoprotein (HDL), is an anti-atherogenic molecule. Thus, high plasma levels of apoA-I are correlated with a low incidence of coronary heart disease (1) and studies in transgenic mice and rabbits have demonstrated that the expression of human apoA-I inhibits the development of atherosclerosis (2,

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¹ The abbreviations used are: apo, apolipoprotein; ABCA1, ATP-binding cassette transporter A-1; HDL, high density lipoprotein; FC, free cholesterol; PL, phospholipid; WT, wild type; Trx, thioredoxin; MEM, minimal essential medium; ACAT, acyl-CoA:cholesterol acyltransferase.

Several laboratories have used engineered apoA-I variants to examine the contributions of different regions of the apoA-I molecule to membrane microsolvubilization (17, 26, 27). Thus, the C-terminal region of apoA-I is essential for optimal ABCA1-mediated lipid efflux although there may be cell-specific effects because C-terminal truncation of apoA-I has been reported to significantly reduce lipid efflux from macrophages, but not from fibroblasts (27). In addition to this uncertainty about the quantitative contribution of the apoA-I C-terminal domain, there is disagreement about the role of the helix bundle domain. Thus, on the one hand, deletion of central α -helices has been reported to have no effect on lipid efflux from macrophages (28) but, on the other hand, it has been claimed that central α -helices are essential for ABCA1-mediated lipid efflux in the same cell type (26). Despite the presence of a strongly lipid-binding α -helix in the N-terminal region of the apoA-I molecule, deletion of the N-terminal domain has been asserted to have a minimal effect on ABCA1-mediated FC and PL efflux from macrophages (26, 28, 29). It is apparent that the use of apoA-I variants has not yet explained how the apoA-I domain structure influences ABCA1-mediated lipid efflux from cells. An important limitation of prior studies using engineered apoA-I molecules in assays of ABCA1-mediated lipid efflux is that they were conducted with saturating concentrations of apoA-I in the extracellular medium. Under this condition, the effects of alterations in apoA-I structure tend to be minimized so that the contributions of the various apoA-I domains to lipid efflux become difficult to detect and quantitate.

In the current study, we have addressed some of these limitations by exploring the roles of the apoA-I helix bundle and C-terminal domains in ABCA1-mediated FC and PL efflux using more than one cell type and engineered apoA-I molecules at a range of concentrations. Both the N- and C-terminal domains of apoA-I play a significant role in eliciting lipid efflux from either macrophages or fibroblasts. Analysis of the efflux kinetics provides insights into the likely contributions of apoA-I/ABCA1 and apoA-I/PL interactions to the overall microsolvubilization process.

EXPERIMENTAL PROCEDURES

Materials

Fetal bovine serum, gentamicin, 8-(4-chlorophenylthio)-cAMP, 9-*cis*-retinoic acid, and 22-hydroxycholesterol were purchased from Sigma. Bovine serum albumin was obtained from Intergen (Purchase, NY). [1,2-³H]Cholesterol (51 Ci/mmol), and [*methyl*-³H]choline chloride (86 Ci/mmol) were obtained from PerkinElmer Life Sciences. Minimum essential medium (MEM) buffered with 25 mM Hepes, pH 7.4 (MEM-Hepes), was obtained from BioWhittaker (Walkersville, MD). RPMI 1640, Eagle's modified MEM, and phosphate-buffered saline were purchased from CellGro (Herndon, VA). The acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Pfizer CP-113,818, was a gift from Pfizer Inc. (Groton, CT).

Methods

Preparation of ApoA-I—HDL was isolated from fresh plasma of normolipidemic donors by sequential ultracentrifugation as described earlier (30). Human HDL was delipidated in ethanol/diethyl ether (31) and apoA-I was isolated by anion exchange chromatography (32). Prior to use, the purified protein stored in lyophilized form at -20°C was resolubilized in guanidine hydrochloride (6 M) and dialyzed extensively against Tris buffer (10 mM Tris, 150 mM NaCl, 1.0 mM EDTA, pH 7.4). The protein concentration was determined by measuring its absorbance at 280 nm; the mass extinction coefficient (ml/(mg \cdot cm)) of apoA-I was 1.13.

Expression and Purification of ApoA-I Mutants—The construction of plasmids for expressing wild type (WT) apoA-I and its mutants, and the isolation and purification of these proteins have been described previously (15). The following deletion mutants were used in this study: Δ 1-43, Δ 44-65, Δ 44-126, Δ 123-166, Δ 190-243, and Δ 223-243. The point mutants, L230P and L230P,L233P,Y236P were constructed, expressed, and purified similarly. Briefly, the cDNA for either WT or

apoA-I mutant was ligated into a thioredoxin (Trx) fusion expression vector pET32a(+) and transformed into *Escherichia coli* strain BL21(DE3). These transformed cells were cultured in LB medium at 37°C and expression of the fusion protein Trx-apoA-I was induced with isopropyl- β -D-thiogalactopyranoside for 3 h. After sonicating the bacterial pellet, the lysate was centrifuged and the supernatant was loaded onto a nickel-chelating, histidine-binding resin column (Novagen). The Trx-apoA-I fusion protein bound to the column was eluted, pooled, and dialyzed against 20 mM NH_4HCO_3 . Subsequently, the fusion protein was complexed with dimyristoyl phosphatidylcholine (to prevent non-specific cleavage), and then cleaved with thrombin to release the Trx. The mixture was then lyophilized, delipidated, and dissolved in 6 M guanidine hydrochloride solution. Trx was separated from apoA-I by gel filtration chromatography on a Sephacryl S-300 column. Further purification (>95%) of the proteins was done by gel filtration with Superdex 75 and/or anion exchange chromatography with Q-Sepharose.

Preparation of ApoA-I Peptides—Peptides representing different segments of apoA-I were synthesized as described in detail previously (33) and their solutions were prepared by dissolving known amounts of peptides in Tris buffer.

Preparation of Cell Monolayers—J774 murine macrophages were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 0.5% gentamicin, and incubated at 37°C in a humidified chamber (95% air, 5% CO_2). For efflux experiments, these cells were then seeded in 12-well plates and grown to 80–90% confluence. They were then labeled with either 3 $\mu\text{Ci/ml}$ [³H]cholesterol or 5 $\mu\text{Ci/ml}$ [³H]choline chloride as described previously (16). Cholesterol-enriched monolayers were prepared by incubating the cells for 24 h in RPMI medium supplemented with 1% fetal bovine serum, 2 $\mu\text{g/ml}$ CP-113,818 ACAT inhibitor, 3 $\mu\text{Ci/ml}$ [³H]cholesterol or 5 $\mu\text{Ci/ml}$ [³H]choline chloride, and 25 $\mu\text{g/ml}$ acetyl-low density lipoprotein. For double labeling, the cells were incubated with 20 $\mu\text{Ci/ml}$ [³H]choline chloride and 3 $\mu\text{Ci/ml}$ [¹⁴C]cholesterol for 48 h. After labeling, the cells were washed with MEM-Hepes and incubated with RPMI medium containing 0.2% (w/v) bovine serum albumin, 2 $\mu\text{g/ml}$ CP-113,818 ACAT inhibitor, and 0.3 mM 8-(4-chlorophenylthio)-cAMP for 12 h, to up-regulate the expression of ABCA1 (34).

Human skin fibroblasts (GM 3468A) were maintained in Eagle's MEM supplemented with 10% fetal bovine serum and 0.5% gentamicin and incubated at 37°C . For the efflux experiments, the fibroblasts were seeded in 12-well plates and grown to confluence and then labeled with 3 $\mu\text{Ci/ml}$ [³H]cholesterol or 5 $\mu\text{Ci/ml}$ [³H]choline chloride as described before (17). Cholesterol-enriched cells were prepared by incubating the radiolabeled fibroblasts with an FC loading medium containing cholesterol/phospholipid liposomes for an additional 24 h (17). After labeling the cells for either 24 (cholesterol efflux) or 48 h (phospholipid efflux), the cells were washed with MEM-Hepes and incubated for 12 h in Eagle's MEM containing 0.2% (w/v) bovine serum albumin, 2 $\mu\text{g/ml}$ CP-113,818 ACAT inhibitor and 22-hydroxycholesterol (5 $\mu\text{g/ml}$), and 9-*cis*-retinoic acid (10 μM) to up-regulate ABCA1 (35).

The concentration of the protein in each well was determined by a modification of the Lowry method (36). To determine the degree of radiolabeling of the cells at time zero ($t = 0$), appropriate wells were washed with phosphate-buffered saline, dried, and the lipids were extracted with isopropyl alcohol. The radioactivity of the extracted lipids was measured by liquid scintillation counting (16). These extracted lipids were analyzed for FC by gas-liquid chromatography and for choline-containing PL by an enzymatic assay (Wako Bioproducts, Richmond, VA). The total FC and choline-PL contents of the fibroblasts at $t = 0$ were 20–30 and 50–70 $\mu\text{g/mg}$ cell protein, respectively. The equivalent values for FC-enriched fibroblasts were 40–60 μg of FC/mg of protein and 120–140 μg of choline-PL/mg of protein (19). Typical values for J774 cells were 5–15 μg of FC/mg of protein and 40–60 μg of choline-PL/mg of protein. For FC-enriched J774 macrophages, the equivalent values were 30–40 and 90–110 $\mu\text{g/mg}$ of protein, respectively.

Efflux of Cellular Cholesterol and Phospholipid—Cells prepared as described above were washed with MEM-Hepes and incubated with or without WT or mutant apoA-I under the indicated conditions of concentration and time. To determine the FC efflux, aliquots were removed from the incubation medium at specific time points, filtered, and radioactivity was determined by liquid scintillation counting. For PL efflux, radioactivity was measured after extraction of the lipids from aliquots of the filtered incubation medium by the procedure of Bligh and Dyer (37). The percent of FC and PL efflux were calculated after subtracting the background FC/PL efflux (without apoA-I) as follows: (counts/min in medium at 4 h/cpm in cells at $t = 0$) \times 100. K_m and V_{max} values were calculated by fitting the curves obtained by plotting the fractional lipid

efflux values obtained at 4 h and different concentrations of apoA-I to the Michaelis-Menten equation.

mRNA Isolation and Northern Blot Analysis—Total RNA was isolated from unlabeled fibroblasts and J774 macrophages treated as described above using the TRIzol reagent. The extracted RNA was precipitated by isopropyl alcohol and its integrity was assessed by agarose gel electrophoresis. 20 μ g of RNA was separated in a 1% agarose gel containing formaldehyde and transferred to nylon membranes and cross-linked by UV irradiation. 324-bp probes extending from residues 711 to 1034 for human ABCA1 and 971 to 1294 for mouse ABCA1 were used to measure expression. The probes were 32 P-radio-labeled using the random priming kit (Stratagene, La Jolla, CA), hybridized overnight, and the bands were quantified using a PhosphorImager (Storm, Amersham Biosciences). The blot was stripped and quantified with an actin probe so that the level of ABCA1 mRNA could be normalized to that of actin.

RESULTS

ABCA1-mediated Cholesterol and Phospholipid Efflux from J774 Cells—In the current study, J774 macrophages and human fibroblasts have been used as models to examine the role of apoA-I in ABCA1-mediated efflux studies. J774 cells and fibroblasts were treated with cAMP and 9-*cis*-retinoic acid plus 22-hydroxycholesterol, respectively, for ABCA1 induction. An ~6-fold increase in ABCA1 expression was obtained with this induction (data not shown). All efflux experiments were performed with cells where ABCA1 was up-regulated in this fashion: induction of ABCA1 increased cholesterol efflux ~7-fold in both J774 cells (from 1.9 ± 0.4 to $14.3 \pm 0.2\%$ /6 h) and fibroblasts (from 1.7 ± 0.1 to $12.4 \pm 0.6\%$ /6 h) with 20 μ g/ml apoA-I in the extracellular medium. To better understand the influence of the different domains in the apoA-I molecule on the efficacy of the lipid efflux process, deletion mutants lacking domains along the apoA-I molecule were incubated with the cells. We have shown previously that the rate of FC and PL release from J774 cells and fibroblasts is maximal at concentrations of apoA-I ≥ 10 μ g/ml (16, 17). Thus, initially WT apoA-I and the various deletion mutants were incubated with J774 macrophages at a saturating concentration of 20 μ g/ml and the efflux of PL and FC was monitored. Efflux to recombinant (WT) and human plasma apoA-I was similar (data not shown) and hence, WT apoA-I was used for all the comparisons in the study. The relative FC and PL efflux (normalized to the values for WT apoA-I) from ABCA1-stimulated J774 cells exposed to 20 μ g/ml of either WT or mutant apoA-I are depicted in Fig. 1, A and B. In the case of WT apoA-I, approximately equal masses of cellular PL and FC were released in a 4-h incubation; this corresponds to a 2:1 FC/PL molar ratio. It is apparent that deletion of the central domains (residues 44–126 and 123–166) did not influence FC or PL efflux. These results agree with an earlier investigation in which it was shown that the elimination of the central domain of apoA-I had a minimal effect on ABCA1-dependent lipid efflux from THP-1 macrophages (27). Deletion of the N-terminal hydrophobic α -helix (residues 44–65) and the N-terminal region (residues 1–43) led to a slight reduction in FC efflux relative to WT apoA-I (Fig. 1A). In contrast, removal of the C-terminal domain reduced FC efflux by ~50% under the same experimental conditions where excess apolipoprotein was present in the extracellular medium (Fig. 1A). Strikingly, the effects of removing either residues 190–243 or 223–243 had either no or an insignificant effect on PL efflux (Fig. 1B). In contrast to the results shown in Fig. 1 for 20 μ g/ml apoA-I, exposure of the cells to 2 μ g/ml apoA-I (approximately the K_m value) revealed dramatic differences between lipid efflux to WT apoA-I and the deletion mutants (Fig. 2, A and B). Under this condition, truncation of the C-terminal domain clearly affected the ability of apoA-I to stimulate FC efflux. Deletion of this region ($\Delta 190$ –243 and $\Delta 223$ –243) resulted in an 80–90% reduction in both FC and PL efflux from these cells.

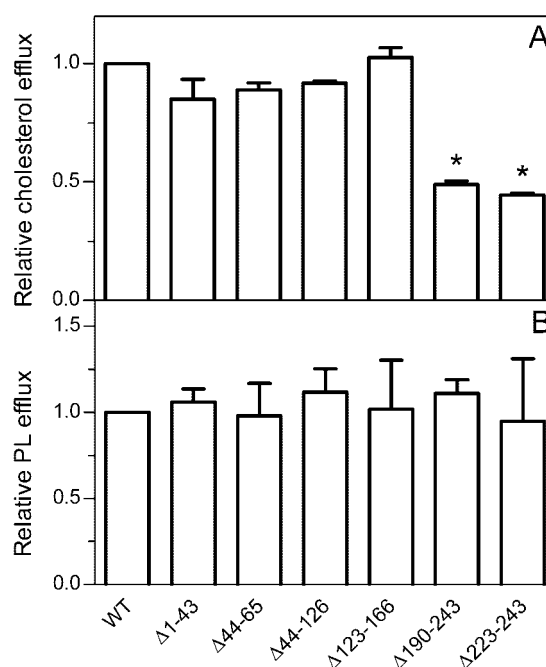


FIG. 1. Relative cholesterol and phospholipid efflux from J774 macrophages to 20 μ g/ml WT and deletion mutants of apoA-I. J774 macrophages were labeled with 3 μ Ci/ml of [3 H]cholesterol or 5 μ Ci/ml of [3 H]choline chloride as described under "Experimental Procedures" and then incubated with 0.3 mM 8-(4-chlorophenylthio)-cAMP overnight. Efflux was initiated by the addition of 20 μ g/ml WT or deletion mutants of apoA-I. After a 4-h incubation, aliquots of the media were collected, filtered, and extracted and the 3 H-radioactivity was determined. The values represent the cholesterol (A) and phospholipid (B) efflux from cells relative to WT apoA-I (set to 1.0). After 4 h of incubation, the cholesterol (FC) and phospholipid (PL) efflux was typically 9.2 ± 2.1 and $2.1 \pm 0.3\%$, respectively, for macrophages incubated with 20 μ g/ml of WT apoA-I. All data represent the mean \pm S.D. of two independent experiments each performed in triplicate. A one-way analysis of variance using the Dunnett's test was performed to determine the significant differences in efflux from WT apoA-I. *, significantly different at $p < 0.01$.

Furthermore, at this low concentration of apoA-I, significant reduction in FC and PL efflux (20–30%) occurred with N-terminal ($\Delta 1$ –43) deletion or removal of residues 44–65. However, removal of the central domain of apoA-I ($\Delta 44$ –126 and $\Delta 123$ –166) did not affect the lipid efflux at this low concentration of apoA-I. The ability of apoA-I variants lacking the central domain to stimulate FC and PL efflux similarly to WT at both concentrations (2 and 20 μ g/ml) indicates that this domain is not critical for lipid efflux.

ABCA1-mediated Cholesterol and Phospholipid Efflux from Human Fibroblasts—To determine whether the effects of the various domains of apoA-I in lipid efflux are sensitive to cell type, experiments similar to those described above with J774 macrophages were conducted with fibroblasts. It is apparent from Fig. 3A that when the fibroblasts were incubated with a saturating concentration (20 μ g/ml) of either WT or mutant apoA-I, FC efflux was essentially the same. The only exception was the C-terminal deletion ($\Delta 223$ –243) mutant where there was a slight reduction (~20%) in FC efflux. In contrast, none of the deletions in the apoA-I molecule significantly affected PL efflux under the same conditions (Fig. 3B). These results are generally consistent with those of an earlier study in which it was shown that neither C-terminal nor central domain mutations of apoA-I alter lipid efflux from fibroblasts (27). However, as was seen with J774 cells (Fig. 2), significant effects on FC and PL efflux from fibroblasts were seen when the cells were exposed to 2 μ g/ml of the apoA-I mutants (Fig. 4). Removal of either the N-terminal domain ($\Delta 1$ –43) or the hydrophobic helix

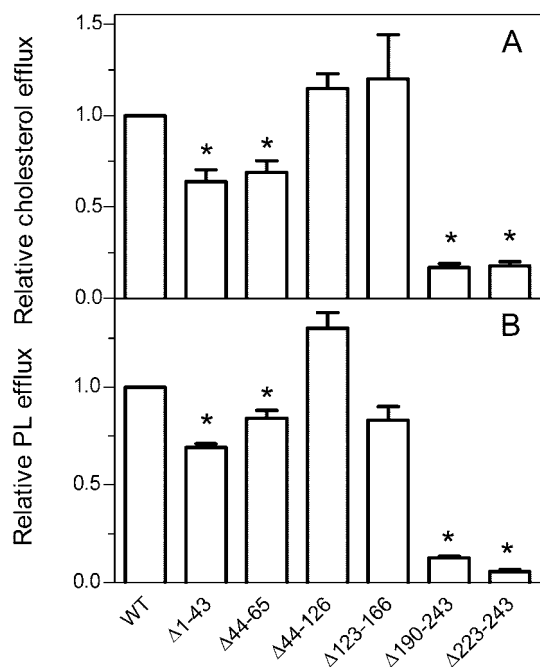


FIG. 2. Relative cholesterol and phospholipid efflux from J774 macrophages to 2 $\mu\text{g/ml}$ of WT and deletion mutants of apoA-I. J774 cells were labeled with 3 $\mu\text{Ci/ml}$ of [^3H]cholesterol or 5 $\mu\text{Ci/ml}$ of [^3H]choline chloride as described under "Experimental Procedures" and incubated with 2 $\mu\text{g/ml}$ WT or deletion mutants of apoA-I. Relative efflux and the statistically significant variations (*, $p < 0.01$) were determined as described in the legend to Fig. 1. After 4 h of incubation, the FC (A) and PL (B) efflux was typically 3.0 ± 0.7 and $0.9 \pm 0.2\%$, respectively, for J774 macrophages incubated with 2 $\mu\text{g/ml}$ of WT apoA-I.

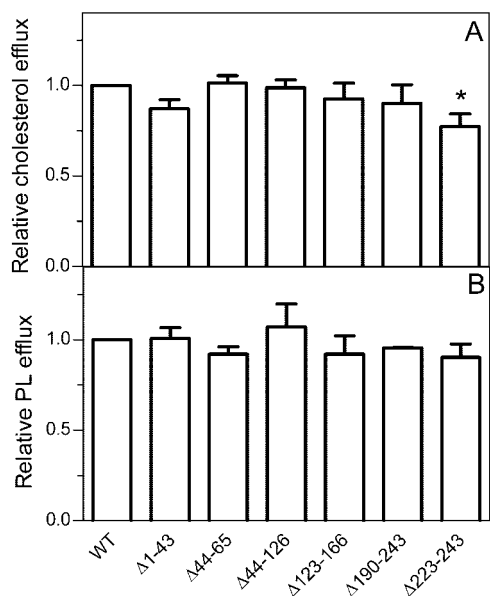


FIG. 3. Relative cholesterol and phospholipid efflux from fibroblasts to 20 $\mu\text{g/ml}$ of WT and deletion mutants of apoA-I. Human fibroblasts were labeled with 3 $\mu\text{Ci/ml}$ of [^3H]cholesterol or 5 $\mu\text{Ci/ml}$ of [^3H]choline chloride as described under "Experimental Procedures" and then incubated with 10 μM 22-hydroxycholesterol and 20 μM 9-*cis*-retinoic acid overnight to up-regulate the expression of ABCA1. Efflux was initiated by the addition of 20 $\mu\text{g/ml}$ of WT or deletion mutants of apoA-I. After 4 h incubation, aliquots of the media were collected, filtered, and extracted and the ^3H -radioactivity was determined. After 4 h of incubation, the cholesterol (A) and phospholipid (B) efflux was typically 9.1 ± 1.06 and $4.4 \pm 1.0\%$, respectively, for fibroblasts incubated with 20 $\mu\text{g/ml}$ of WT apoA-I. Statistically significant variations (*, $p < 0.01$) were determined as described in the legend to Fig. 1.

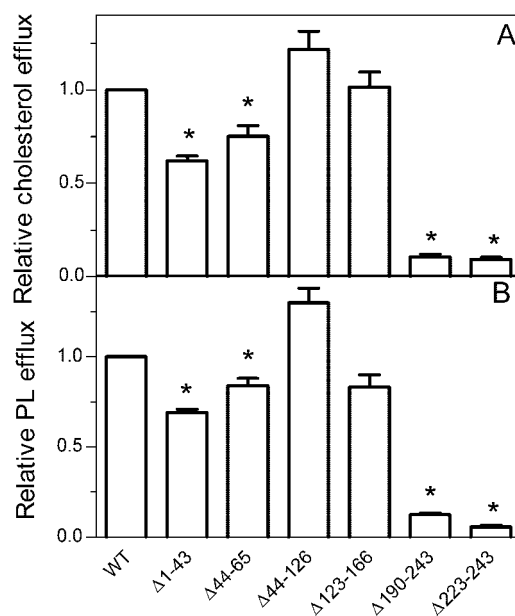


FIG. 4. Relative cholesterol and phospholipid efflux from fibroblasts to 2 $\mu\text{g/ml}$ of WT and deletion mutants of apoA-I. Human fibroblasts were labeled with 3 $\mu\text{Ci/ml}$ of [^3H]cholesterol or 5 $\mu\text{Ci/ml}$ of [^3H]choline chloride as described under "Experimental Procedures" and efflux was initiated by the addition of 2 $\mu\text{g/ml}$ of either WT or deletion mutants of apoA-I. Relative efflux and the statistically significant variations (*, $p < 0.01$) were determined as described in the legend to Fig. 3. After 4 h of incubation, the FC (A) and PL (B) efflux was typically 4.6 ± 0.6 and $2.7 \pm 0.1\%$, respectively, for fibroblasts incubated with 2 $\mu\text{g/ml}$ of WT apoA-I.

TABLE I
Cholesterol efflux to peptide segments of apoA-I

Peptides representing the indicated segments of human apoA-I were incubated at 50 $\mu\text{g/ml}$ for 4 h with either cholesterol-enriched fibroblasts or cAMP-induced J774 macrophages containing [^3H]cholesterol. Values are the mean from two independent experiments performed in triplicate.

ApoA-I peptide (Ac-n1-n2-NH ₂)	Cholesterol efflux relative to apoA-I	
	Fibroblasts ^a	J774 macrophages
	%	
1-33	0	3
44-65	46	27
44-87	13	9
66-87	5	0
99-120	0	0
121-142	0	0
143-164	0	0
165-186	0	0
187-208	0	0
209-241	66	61
220-241	0	2

^a Data from Ref. 17.

(44-65) reduced both FC and PL efflux by 30-40%, whereas deletion of the central domains ($\Delta 44-126$ and $\Delta 123-166$) had no effect (Fig. 4, A and B). The major role in the lipid efflux played by the C-terminal of apoA-I is demonstrated by the fact that deletion of the C-terminal domain residues ($\Delta 190-243$ or $\Delta 223-243$) led to a 80-90% reduction in both FC and PL efflux from these cells (Fig. 4, A and B).

Effect of Synthetic Peptides Representing Different Regions of ApoA-I on FC Efflux—To further delineate the structural features essential for ABCA1-mediated lipid efflux, peptides representing each of the 22-residue amphipathic-helical segments of apoA-I were examined for their ability to mediate FC efflux. It is apparent from Table I that the full-length apoA-I molecule is much more effective in removing cellular lipids than apoA-I

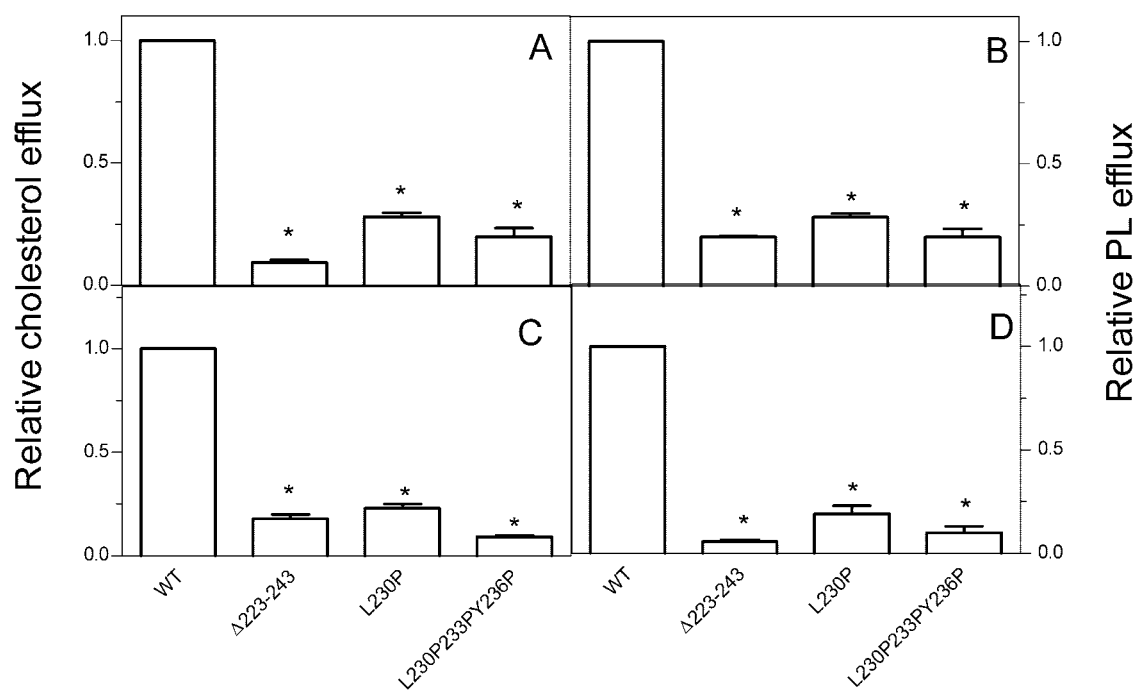


FIG. 5. Effect of apoA-I C-terminal helix mutations on cholesterol and phospholipid efflux from J774 macrophages and human fibroblasts. Human fibroblasts (A and B) and J774 macrophages (C and D) were labeled with either [^3H]cholesterol (3 $\mu\text{Ci}/\text{ml}$) or [^3H]choline chloride (5 $\mu\text{Ci}/\text{ml}$) as described under "Experimental Procedures." Relative cholesterol and PL efflux was measured to the apoA-I mutants added at 2 $\mu\text{g}/\text{ml}$. Statistically significant variations (*, $p < 0.01$) were determined as described in the legend to Fig. 1.

peptides. Among the various peptides, only those representing the hydrophobic 22-mer N-terminal helix (44–65) and the 33-mer C terminus (209–241) had a significant ability to remove FC from both fibroblasts and J774 macrophages. These are the apoA-I peptides with the greatest ability to interact with lipids (14). These results agree with a recent study that revealed that only those peptides corresponding to apoA-I helices 1, 9, and 10 were efficient at eliciting FC efflux (38). Peptides representing central domains in the apoA-I molecule were completely ineffective in eliciting FC efflux from both types of cells. There is excellent correlation between these results and those from the experiments with apoA-I mutants (Figs. 1–4) confirming that N- and C-terminal domains of apoA-I are key players in ABCA1-dependent lipid efflux.

Influence of C-terminal Helix Disruptions on Lipid Efflux—To further confirm the crucial role of the C-terminal α -helical domain in ABCA1-mediated lipid efflux, instead of simply deleting the segment (223–243), we introduced point mutations designed to disrupt this α -helix while minimally changing the primary structure of apoA-I. Because proline insertions into an α -helix in a molten globular protein only disrupts the particular helix involved (39), we prepared mutants L230P and L230P,L233P,Y236P by site-directed mutagenesis. CD measurements (data not shown) indicated that these mutants exhibited the same decrease in α -helix content as the $\Delta 223$ –243 mutant when compared with WT apoA-I (15). Exposure of fibroblasts and macrophages to 2 $\mu\text{g}/\text{ml}$ of these proline-insertion mutants reduced both FC and PL efflux to the low levels seen with the $\Delta 223$ –243 mutant in both types of cells (Fig. 5). These results further confirm that the strongly lipid binding C-terminal domain is vital for the promotion of lipid efflux and that it must fold into an amphipathic α -helix to be functional. Again, the effects of the mutations were less apparent if saturating concentrations (20 $\mu\text{g}/\text{ml}$) were employed (data not shown). In this case, lipid efflux from fibroblasts to these proline insertion mutants was similar to that obtained with WT apoA-I (data not shown). However, a 30–40% decrease in lipid efflux to the proline insertion mutants relative to

WT apoA-I was observed with J774 cells under these saturating conditions (data not shown).

Concentration Dependence of Lipid Efflux to ApoA-I Variants—To further investigate the contributions of the apoA-I domains and their lipid affinities in stimulating efflux, the concentration dependence of FC and PL efflux to apoA-I and its variants was determined. The FC and PL efflux was monitored as a function of time with fibroblasts and macrophages to ascertain the initial velocity conditions. After an initial 5-min lag there was simultaneous release of FC and PL from J774 cells at a constant rate for up to 8 h (data not shown); similar results have been reported for fibroblasts (18). Fig. 6, A–D, depicts representative plots of FC and PL efflux at 4 h from fibroblasts and J774 cells as a function of apoA-I concentration. V_{max} and K_m values (Table II) were derived by fitting these hyperbolic velocity-substrate curves to the Michaelis-Menten equation. Optimal FC or PL efflux is observed from cells treated with WT apoA-I (Fig. 6, A and B) and saturation is reached at a protein concentration of ≥ 5 $\mu\text{g}/\text{ml}$, in accord with previous reports (16, 19, 28). The K_m values (~ 3 μg of apoA-I/ml $\cong 10^{-7}$ M) for FC and PL efflux from J774 cells are not significantly different, pointing to the coordinated release of FC and PL from these cells. A similar effect is observed for fibroblasts where the K_m values are ~ 1.5 $\mu\text{g}/\text{ml}$ (Table II). The efflux concentration curve for $\Delta 123$ –166 apoA-I (Fig. 6A) is indistinguishable from that of WT apoA-I, consistent with a minor role for this domain in ABCA1-mediated FC efflux. Also, the K_m and V_{max} values (1.1 ± 0.2 $\mu\text{g}/\text{ml}$ and $11.6 \pm 1\%$ FC efflux/4 h) derived for $\Delta 123$ –166 apoA-I are quite similar to those for WT apoA-I. The N-terminal deletion mutant $\Delta 1$ –43 apoA-I is also effective in promoting FC efflux from fibroblasts although the K_m value is 2-fold higher than that for WT apoA-I (Table II). However, there are no significant differences in FC efflux attained at saturating concentrations (V_{max}) of both WT and $\Delta 1$ –43 apoA-I. In contrast to the case with fibroblasts, the V_{max} for FC efflux from J774 cells is lower for $\Delta 1$ –43 than for WT apoA-I (Table II). The difference in FC efflux from J774 cells to WT and $\Delta 1$ –43 apoA-I persisted over a range of con-

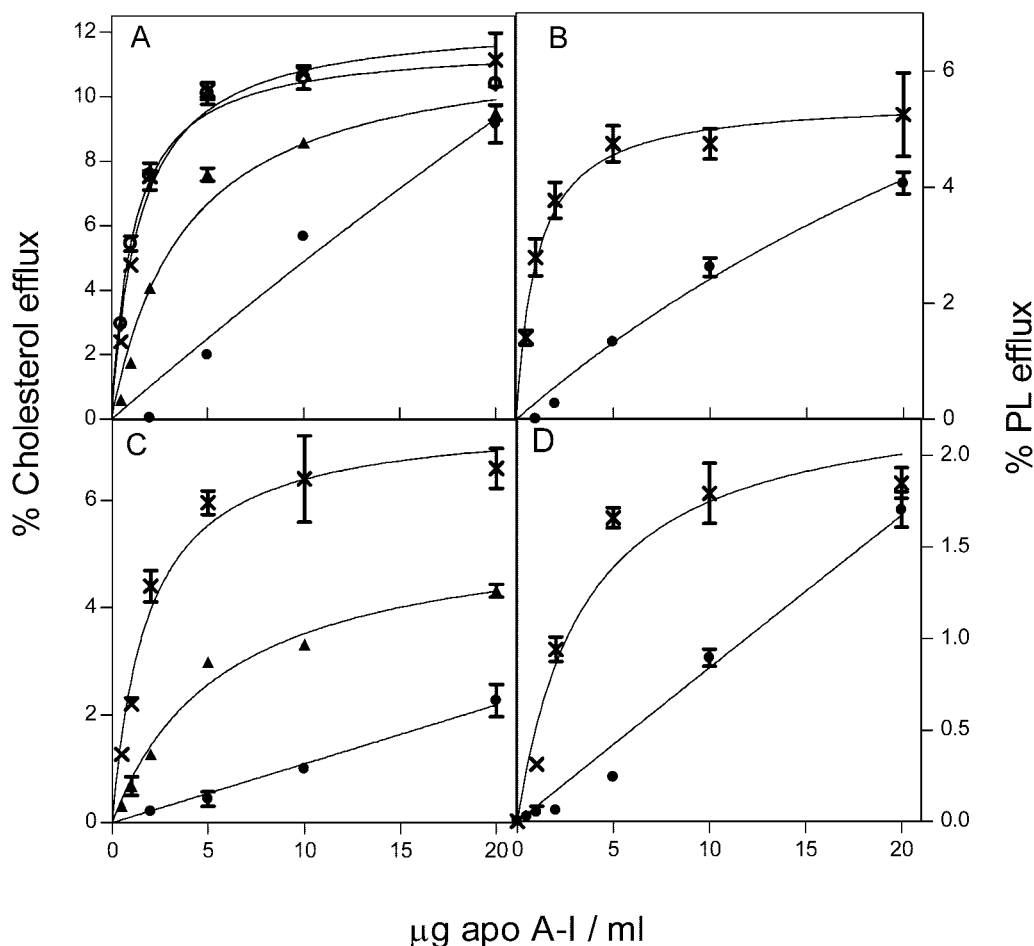


FIG. 6. Effect of apoA-I concentration on lipid efflux from J774 macrophages and human fibroblasts. Fibroblasts and J774 macrophages were labeled with either [^3H]cholesterol (3 $\mu\text{Ci}/\text{ml}$) or [^3H]choline chloride (5 $\mu\text{Ci}/\text{ml}$) as described under "Experimental Procedures." These labeled cells were then treated with either 8-(4-chlorophenylthio)-cAMP (J774 macrophages, panels C and D) or 22-hydroxycholesterol and 9'-cis-retinoic acid (fibroblasts, panels A and B) to up-regulate ABCA1 as described in the legends to Figs. 1 and 3. The lipid efflux was then initiated by the addition of WT or deletion mutants of apoA-I (0–20 $\mu\text{g}/\text{ml}$). After 4 h incubation, the medium was removed, filtered, and extracted for lipids and ^3H -radioactivity was determined. Curves were generated by fitting the fractional efflux at 4 h, measured at various concentrations of WT apoA-I or deletion mutant (except $\Delta 223$ –243 apoA-I), to the Michaelis-Menten equation. The curve for $\Delta 223$ –243 apoA-I was obtained by fitting the fractional efflux to a one-phase exponential equation. Data represent FC efflux (panels A and C) and PL efflux (panels B and D) to WT apoA-I (\times), $\Delta 1$ –43 apoA-I (\blacktriangle), $\Delta 123$ –166 apoA-I (\circ), and $\Delta 223$ –243 apoA-I (\bullet).

centrations (Fig. 6C) and the higher K_m for $\Delta 1$ –43 apoA-I in both types of cells indicates that the affinity of this mutant for ABCA1 and/or lipid (see "Discussion") is low compared with WT apoA-I. Strikingly, elimination of the C-terminal domain of apoA-I ($\Delta 223$ –243) induces a linear dependence of FC and PL efflux on protein concentration in both types of cells (Fig. 6). These results indicate that ABCA1-mediated efflux to this mutant is a low affinity process further confirming the importance of the C-terminal domain.

Influence of FC Enrichment of Cells on ABCA1-mediated Lipid Efflux—Because variation in lipid efflux from different types of cells may be affected by differences in cell FC content, we examined the effects of FC enrichment of fibroblasts and J774 cells on ABCA1-mediated efflux. In response to FC loading, a 2–2.5-fold enhancement in fractional PL and FC efflux to WT apoA-I was observed for fibroblasts and macrophages (16, 17, 27). It is clear that removal of segments in either the N-terminal or the central domains of apoA-I did not significantly affect the lipid efflux from FC-loaded fibroblasts and macrophages when they were exposed to 20 $\mu\text{g}/\text{ml}$ apoA-I (Fig. 7). In contrast, C-terminal deletion reduced FC and PL efflux by 30–40% with these cells. It is interesting to note that cellular cholesterol content enhanced the sensitivity of lipid efflux to C-terminal truncations in the apoA-I molecule. Thus,

at saturating concentrations of apoA-I, PL efflux to C-terminal deletion mutants ($\Delta 193$ –243, $\Delta 223$ –243) was hardly altered in unloaded cells (Figs. 1B and 3B), whereas a 30–50% reduction compared with WT apoA-I was observed in FC-loaded cells (Fig. 7, B and D).

DISCUSSION

The focus of this study was to identify the structural domains of apoA-I that determine its ability to elicit FC and PL efflux from ABCA1-stimulated cells and examine the contributions of apoA-I/PL and apoA-I/ABCA1 interactions. ApoA-I is known to be a primary ligand for ABCA1 (6, 26, 29). The predominant secondary structural element present in apoA-I is the amphipathic α -helix and prior work has established the importance of such hydrophobic helices in the lipid efflux process (4, 6, 11). The fact that only those peptides (Table I) that represent the strongly hydrophobic N- and C-terminal regions are efficient in the lipid efflux process points to the importance of hydrophobic interactions. Such interactions could be between apoA-I and ABCA1 and/or between apoA-I and PL. The latter interaction is required for apoA-I to acquire and/or retain PL and FC to create nascent HDL particles. Recent evidence (24, 25) indicates that apoA-I can form a high affinity complex with ABCA1 and that this is the first step in a two-step process of FC

TABLE II
Influence of deletion mutations on the apoA-I-mediated efflux of cholesterol and PL

K_m (μg of apo/ml) and V_{max} (% FC efflux/4 h) values were generated by fitting the fractional efflux at 4 h measured at various concentrations of either WT apoA-I or the deletion mutants to the Michaelis-Menten equation. The values are the mean \pm S.D. from at least two complete velocity concentration curves.

ApoA-I	J774 cells				Fibroblasts			
	FC efflux		PL efflux		FC efflux		PL efflux	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
WT	9.4 \pm 1.8	2.8 \pm 0.9	2.3 \pm 0.2	3.4 \pm 0.3	11.9 \pm 0.7	1.6 \pm 0.3	5.5 \pm 0.2	1.1 \pm 0.2
Δ 1-43	6.3 \pm 1.0	7.4 \pm 2.03	ND ^a	ND	11.8 \pm 1.1	3.8 \pm 1.0	ND	ND

^a ND, not determined.

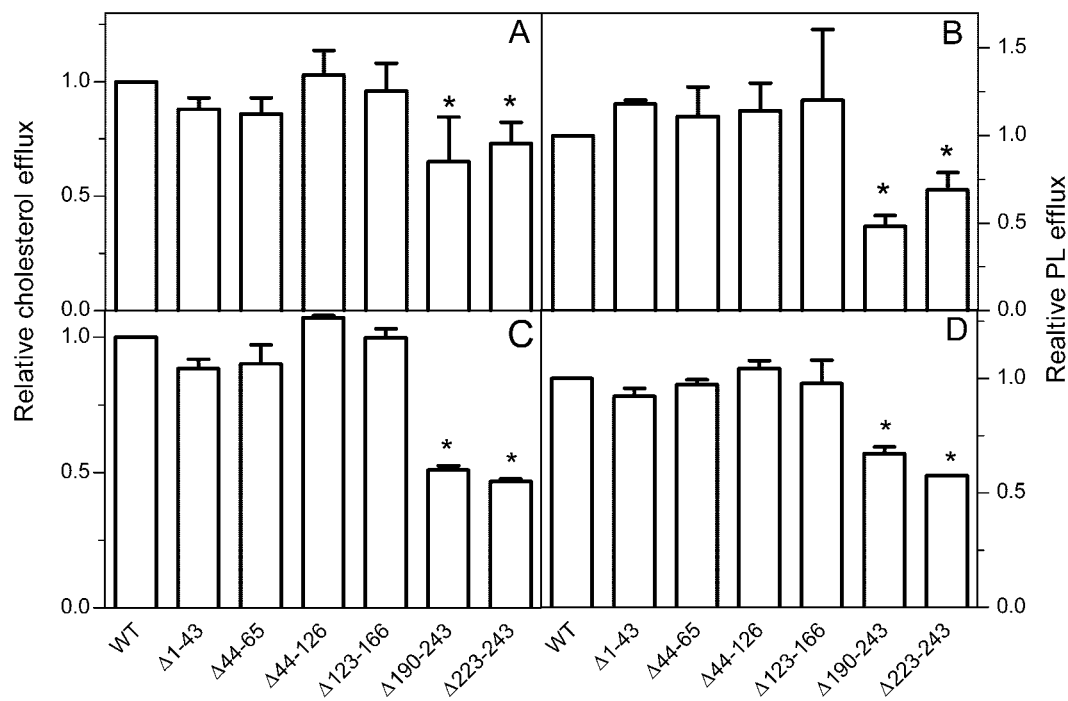


FIG. 7. Relative cholesterol and phospholipid efflux from cholesterol-loaded J774 macrophages and human fibroblasts to WT and deletion mutants of apoA-I. Human fibroblasts (A and B) were radiolabeled with either [³H]cholesterol or [³H]choline chloride as described under "Experimental Procedures." FC-enriched cells were prepared by a 24-h incubation in the presence of low density lipoprotein and FC/PL dispersions and an ACAT inhibitor. For cholesterol loading of J774 macrophages (C and D), the cells were labeled with either [³H]cholesterol (3 $\mu\text{Ci/ml}$) or [³H]choline chloride (5 $\mu\text{Ci/ml}$) in the presence of acetyl-low density lipoprotein as described under "Experimental Procedures." Relative cholesterol and PL efflux and the statistically significant variations (*, $p < 0.01$) were determined as described in the legend to Fig. 1. After 4 h of incubation, the cholesterol and PL efflux was typically 20.6 \pm 2.4 and 4.5 \pm 0.6% for J774 macrophages and 5.0 \pm 0.4 and 1.3 \pm 0.4% for fibroblasts incubated with 20 $\mu\text{g/ml}$ of WT apoA-I. The initial FC contents of J774 macrophages and human fibroblasts were typically 42 \pm 10 and 55 \pm 7 $\mu\text{g/mg}$ of cell protein, respectively.

and PL efflux via ABCA1. The binding to ABCA1 is not very specific and apparently involves interactions of amphipathic helices with a hydrophobic site on the transporter (25). The second step involves lipidation of apoA-I and release of the nascent HDL particles into the extracellular medium. The kinetics of lipid efflux are affected by both steps and it is important to separate the two contributions. The K_m for PL and FC efflux is 1–3 μg of WT apoA-I/ml (Table II) and the K_d value for binding of WT apoA-I to ABCA1 is 2 $\mu\text{g/ml}$ (24). This similarity of K_m and K_d implies that the affinity of apoA-I for ABCA1 controls the overall efflux process. However, the K_d for binding of WT apoA-I to egg phosphatidylcholine small unilamellar vesicles, which can be regarded as a surrogate for the PL bilayer of a cell membrane, is also about 2 $\mu\text{g/ml}$ (40). Thus, the affinity of WT apoA-I for ABCA1 and a PL bilayer is similar and the kinetic parameters for WT apoA-I are not informative about the relative contributions of the two interactions to the overall efflux. However, by studying efflux to apoA-I molecules with altered structures it is possible to distinguish the contributions of different domains in the apoA-I molecule, and of apoA-I interactions with the transporter and the PL bilayer.

Here we discuss these issues in the context of the two-domain model we have proposed recently for the tertiary structure of apoA-I (15).

ApoA-I C-terminal Lipid-binding Domain—As summarized in the Introduction, it is well established that C-terminal α -helices are important for effective FC and PL microsolvubilization via ABCA1. This is confirmed by the data in Figs. 2 and 4 showing that deletion of residues 220–243 or 190–243 greatly reduces efflux of both FC and PL. Parallel effects on efflux of both lipids are expected because they are released simultaneously. It is also apparent that the reduction in efflux caused by the removal of the C-terminal domain of apoA-I occurs independently of both the cell type (Figs. 2 and 4) and the cholesterol content of the cells (Fig. 7). The data in Fig. 5 indicate that residues 220–241 at the C terminus of the apoA-I molecule have to be present as an amphipathic α -helix for efficient lipid efflux to occur. This requirement for the C-terminal domain to be in the appropriate conformation is consistent with α -helix formation in this region of the molecule being necessary for high affinity binding of apoA-I to lipids (40, 41).

Removal or disruption of the C-terminal region of apoA-I

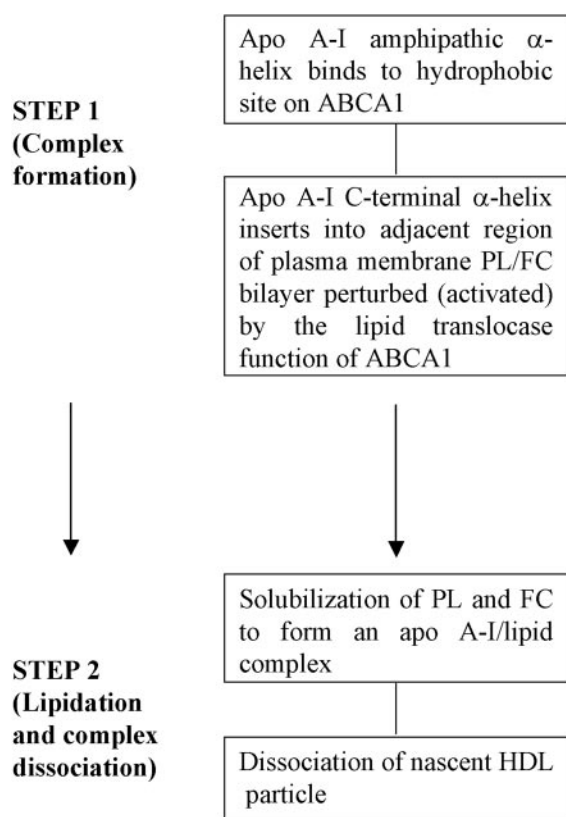


FIG. 8. Schematic of steps involved in ABCA1-mediated efflux of cellular lipids to apoA-I. This scheme is a refinement of the two-step model proposed by Freeman, Zannis, and colleagues (24, 25). The critical role played by the strongly lipid-binding C-terminal α -helix of apoA-I is included. The lipid-binding/solubilization process is apparently rate-limiting for the overall efflux. The molecular stoichiometry for ABCA1, apoA-I, PL, and FC is not known at this time.

results in low-affinity, non-saturable binding to PL small unilamellar vesicles as compared with WT apoA-I, which exhibits high-affinity, saturable binding: this effect occurs because formation of α -helix by the C-terminal region is required for high affinity binding (40). Removal of residues 223–243 in the C-terminal of apoA-I also causes lipid efflux to apoA-I to become low affinity. Thus, efflux to the Δ 223–243 apoA-I mutant exhibits a linear rather than hyperbolic dependence on protein concentration (Fig. 6). In striking contrast, removal of the C-terminal of apoA-I reduces the K_d of binding of apoA-I to ABCA1 only slightly and the binding is similar to that of WT apoA-I in being high affinity and saturable (see Fig. 2, D and E, in Ref. 24). Thus, the effects of C-terminal removal on the concentration dependence of apoA-I binding to ABCA1 are distinct from the effects on both the concentration dependence of efflux to apoA-I and the binding of apoA-I to a PL bilayer. This strongly suggests that the lipidation occurring as step 2 determines the dependence of efflux on apoA-I concentration. The reason that step 2 is defective in C-terminal mutants is because of the inability of such mutants to bind well to a PL bilayer and insert their hydrophobic α -helices among the PL molecules. Such penetration of the α -helices seems to be required for microsolvubilization to proceed and this helix insertion can occur where the membrane lateral compressibility is relatively high (17). This process is likely to occur where the PL packing is disturbed by ABCA1-mediated transport of PL molecules across the membrane.

ApoA-I N-terminal Helix Bundle Domain—The results in Figs. 2 and 4 establish that deletions in the N-terminal region (residues 1–43 and 44–65) of apoA-I reduce PL and FC efflux

slightly. These deletions disrupt the helix bundle organization as reflected by an increase in ANS binding (15), perhaps because of disruption of stabilizing interactions between N- and C-terminal α -helices. These structural changes reduce the affinity of apoA-I for PL vesicles by a factor of 2–4 (40) and a similar increase in the K_m for efflux is observed for the Δ 1–43 mutant (Table II). Thus, as was observed for the C-terminal mutants, mutations in the N-terminal domain have parallel effects on lipid efflux and the affinity for PL. In contrast, deletion of residues 1–41 appears to enhance the affinity of apoA-I for the ABCA1 binding site (24). Taken together, these observations again support the idea that step 2 in the reaction scheme proposed by Freeman, Zannis, and colleagues (24) controls the overall dependence of lipid efflux on apoA-I concentration.

Removal of the central helices (residues 44–126 and 123–166) in the apoA-I molecule does not have any deleterious effects on FC and PL efflux (Figs. 2 and 4). In agreement with this finding, K_m and V_{max} for the Δ 123–166 mutant are identical to those for WT apoA-I (Fig. 6A). This lack of effect on lipid efflux occurs despite the fact that the removal of central α -helices disrupts the apoA-I helix bundle organization (15). It follows that the precise tertiary structure of the apoA-I molecule does not determine the interaction with ABCA1. This finding is consistent with the fact that other apolipoproteins such as apoE and peptides with amphipathic α -helices can mediate lipid efflux via the ABCA1 transporter (4, 9). The affinity of the Δ 123–166 mutant for PL vesicles is essentially the same as that of WT apoA-I, confirming that this part of the molecule does not play a critical role in lipid binding. Helices spanning this region cannot mediate FC efflux (Table I), so the fact that their omission does not reduce the ability of apoA-I to mediate lipid efflux is unsurprising.

Summary—Our results with apoA-I mutants support the two-step model for ABCA1-mediated lipid efflux proposed by Freeman, Zannis, and colleagues (24, 25) while providing more insight into the lipidation process (see Fig. 8). The initial binding of apoA-I to ABCA1 can be mediated by helices in either the N-terminal helix bundle or the C-terminal domain of apoA-I. This binding to ABCA1 targets apoA-I to regions of the plasma membrane where transmembrane PL flux is occurring. Subsequently, there is binding of apoA-I helices to the perturbed lipid domain (with high lateral compressibility) created by ABCA1 activity and insertion of sufficiently hydrophobic helices among the PL and FC molecules in the membrane. The interaction of apoA-I with ABCA1 may promote the insertion of apoA-I C-terminal helices into the adjacent PL/FC milieu. Nascent HDL particles are formed by binding of PL and FC molecules to the apoA-I but, at this time, nothing is known about the stoichiometry of this solubilization and assembly process. More insight into this model will be provided by detailed characterization of the HDL particles released into the extracellular medium.

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