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 Kₘ about 2-fold compared with wild type apoA-I, whereas deletion of the central domain (123–186) had no effect on either Kₘ or V₉max. 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.

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 interspersed 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 microsolvulization (17–19). The fact that peptides corresponding to the hydrophobic α-helical regions of the apoA-I molecule can also mediate membrane microsolvulization (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 formation 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) 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 risk 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, 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.

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1 The abbreviations used are: apo, apolipoprotein; ABCA1, ATP-binding cassette transporter A1; HDL, high density lipoprotein; FC, free cholesterol; PL, phospholipid; WT, wild type; Trx, thioredoxin; MEM, minimal essential medium; ACAT, acyl-CoA cholesterol acyltransferase.
Lipoprotein and Structure of Apo-A-I

Several laboratories have used engineered apoA-I variants to examine the contributions of different regions of the apoA-I molecule to membrane microsolvation (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 microsolvation 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]Cholesterol (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 BioWhitaker (Walkersville, MD). RPMI 1640, Eagle’s modified MEM, and phosphate-buffered saline were purchased from CellGro (Herdon, 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 cm)) of apoA-I was 1.44.

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: Δ1–43, Δ44–65, Δ44–126, Δ123–166, Δ190–243, and Δ223–243. 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 sonication 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 NaHCO3. 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 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% CO2). For efflux experiments, these cells were seeded in 12-well plates and grown to 80–90% confluence. They were then labeled with either 3 μCi/ml [3H]cholesterol or 5 μCi/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 μg/ml CP-113,818 ACAT inhibitor, 5 μCi/ml [3H]Cholesterol or 5 μCi/ml [3H]Choline chloride, and 25 μg/ml dialyzed lipoprotein lipase. For double-labeling, the cells were incubated with 20 μCi/ml [3H]Choline chloride and 3 μCi/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 μg/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 μCi/ml [3H]Cholesterol or 5 μCi/ml [3H]Choline chloride as described before (17). Cholesterol-enriched cells were prepared by incubating the radiolabeled fibroblasts with an FC loading medium containing cholesteryl ester lipopid 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 μg/ml CP-113,818 ACAT inhibitor and 0.3 mM 8-(4-chlorophenylthio)-cAMP to up-regulate the expression of ABCA1 (35).

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The equivalent 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 μg/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 at designated time intervals before (17). For FC-enriched J774 macrophages, the equivalent values were 30–40 and 90–110 μg/mg of protein, respectively.

Radioactivity 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 μg/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 μg/mg of protein, respectively.

Radioactivity was measured 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 PLC/PL efflux (without apoA-I) as follows: (counts/min in medium at 4 h/counts in cells at t = 0) × 100. Keff and Vmax 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 isopropl alcohol and its integrity was assessed by agarose gel electrophoresis. 20 μg of RNA was separated in a 1% agarose gel containing formaldehyde and transferred to nylon membranes and cross-linked by UV irradiation. 32P-hпробes extending from residues 711 to 1034 for human ABCA1 and 971 to 1294 for mouse ABCA1 were used to measure expression. The probes were 32P-radioabeled 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 rehybridized 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-hydroxysterol, 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 ± 0.2%/6 h) and fibroblasts (from 1.7 ± 0.1 to 12.4 ± 0.6%/6 h) with 20 μg/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 μg/ml (16, 17). Thus, initially WT apoA-I and the various deletion mutants were incubated with J774 macrophages at a saturating concentration of 20 μg/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 μg/ml of either WT or mutant apoA-I are depicted in Fig. 1A 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 μg/ml apoA-I, exposure of the cells to 2 μg/ml apoA-I (approximately the K₅₀ 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 (Δ190–243 and Δ223–243) resulted in an 80–90% reduction in both FC and PL efflux from these cells.

Furthermore, at this low concentration of apoA-I, significant reduction in FC and PL efflux (20–30%) occurred with N-terminal (Δ1–43) deletion or removal of residues 44–65. However, removal of the central domain of apoA-I (Δ44–126 and Δ123–166) 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 μg/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 μg/ml) of either WT or mutant apoA-I, FC efflux was essentially the same. The only exception was the C-terminal deletion (Δ223–243) 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 μg/ml of the apoA-I mutants (Fig. 4). Removal of either the N-terminal domain (Δ1–43) or the hydrophobic helix
Reduced both FC and PL efflux by 30–40%, whereas deletion of the central domains (44–126 and 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 (190–243 or 223–243) led to an 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

| ApoA-I peptide | Cholesterol efflux relative to apoA-I |
|---------------|--------------------------------------|
| (Ac-n1-n2-NH2) Fibroblasts* 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 |

* Data from Ref. 17.

(44–65) reduced both FC and PL efflux by 30–40%, whereas deletion of the central domains (44–126 and 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 (190–243 or 223–243) led to an 80–90% reduction in both FC and PL efflux from these cells (Fig. 4, A and B).

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peptides. Among the various peptides, only those representing the hydrophobic 22-mer N-terminal helix (4–65) 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 Δ223–243 mutant when compared with WT apoA-I (15).

Exposure of fibroblasts and macrophages to 2 μg/ml of these proline-insertion mutants reduced both FC and PL efflux to the proline insertion mutants relative to 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 μg/ml, in accord with previous reports (16, 19, 28). The K m values (≈3 μg of apoA-I/ml = 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 μg/ml (Table II). The efflux concentration curve for Δ123–166 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 μg/ml and 11.6 ± 1% FC efflux/4 h) derived for Δ123–166 apoA-I are quite similar to those for WT apoA-I. The N-terminal deletion mutant Δ1–43 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 Δ1–43 apoA-I. In contrast to the case with fibroblasts, the V max for FC efflux from J774 cells is lower for Δ1–43 than for WT apoA-I (Table II). The difference in FC efflux from J774 cells to WT and Δ1–43 apoA-I persisted over a range of con-
centrations (Fig. 6) and the higher $K_m$ for $\Delta^H_9004$1–43 apoA-I in both types of cells indicates that the affinity of this mutant for ABCA1 and/or lipid (see “Discussion”) is low compared with WT apoA-I. Strikingly, elimination of the C-terminal domain of apoA-I ($\Delta^H_9004$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 $\mu$g/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^H_9004$193–243, $\Delta^H_9004$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 $\alpha$-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. 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

![FIG. 6. Effect of apoA-I concentration on lipid efflux from J774 macrophages and human fibroblasts.](image-url)
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 net chemistry of lipid efflux is 1–3 μg of apoA-I/ml (Table II) and the Kd value for binding of WT apoA-I to ABCA1 is 2 μg/ml (24). This similarity of Kd for PL and FC indicates that apoA-I binds to ABCA1 independently of both the cell type (Figs. 2 and 4) and the type of lipid abundance in the cell. It is also apparent that the reduction in efflux caused by 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 type of lipid abundance in the cell. 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 type of lipid abundance in the cell.

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 microsolvulation via ABCA1. This is confirmed by the data in Figs. 2 and 4 showing 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 and the type of lipid abundance in the cell. 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 type of lipid abundance in the cell. 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 type of lipid abundance in the cell.

**Removal or disruption of the C-terminal region of apoA-I**
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 Δ223–243 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 Kd 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 microsolubilization 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 Km for efflux is observed for the Δ1–43 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, Km and Vmax for the Δ123–166 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 Δ123–166 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.

REFERENCES
1. Barter, P. J., and Rye, K. A. (1996) Atherosclerosis 121, 1–12
2. Schultz, J. R., and Rubin, E. M. (1994) Curr. Opin. Lipidol. 5, 126–137
3. Rader, D. J. (2003) Am. J. Cardiol. 92, 423–428
4. Oram, J. P. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 720–727
5. Yancrey, P. G., Bortnick, A. E., Kellner-Weibel, G., Llera-Moya, M., Phillips, M. C., and Rothblat, G. H. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 712–719
6. Marcel, Y. L., and Koss, R. S. (2003) Curr. Opin. Lipidol. 14, 151–157
7. Sviridov, D., and Nestel, P. (2002) Atherosclerosis 161, 245–254
8. Rye, K. A., and Barter, P. J. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 421–428
9. Brewer, H. B., Jr., and Santamarina-Fojo, S. (2003) Am. J. Cardiol. 91, 3E–11E
10. Singaraja, R. R., Brunham, L. R., Visscher, H., Kastelein, J. J., and Hayden, M. R. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 1322–1329
11. Wang, N., and Tall, A. R. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 1178–1184
12. Lund-Katz, S., Liu, L., Thauhnai, S. T., and Phillips, M. C. (2003) Front Biosci.
Biochim. Biophys. Acta 1531, 4–46
13. Brouillette, C. G., Anantharamai, G. M., Engler, J. A., and Borhani, D. W. (2001) Biochim. Biophys. Acta 1531, 4–46
14. Palgunachari, M. N., Mishra, V. K., Lund-Katz, S., Phillips, M. C., Adeeye, S. O., Alluri, S., Anantharamai, G. M., and Segrest, J. P. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 328–338
15. Saito, H., Dhanasekaran, P., Nguyen, D., Holvoet, P., Lund-Katz, S., and Phillips, M. C. (2003) J. Biol. Chem. 278, 23227–23232
16. Liu, L., Bortnick, A. E., Nickel, M., Dhanasekaran, P., Subbaiah, P. V., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (2003) J. Biol. Chem. 278, 42976–42984
17. Gillotte, K. L., Zaiou, M., Lund-Katz, S., Anantharamai, G. M., Holvoet, P., Dhoest, A., Palgunachari, M. N., Segrest, J. P., Weisgraber, K. H., Rothblat, G. H., and Phillips, M. C. (1999) J. Biol. Chem. 274, 2021–2028
18. Gillotte, K. L., Davidson, W. S., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (1998) J. Lipid Res. 39, 1918–1928
19. Gillotte-Taylor, K., Nickel, M., Johnson, W. J., Francone, O. L., Holvoet, P., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (2002) J. Biol. Chem. 277, 11811–11820
20. Mendez, A. J., Anantharamai, G. M., Segrest, J. P., and Oram, J. F. (1994) J. Clin. Investig. 94, 1698–1705
21. Remaley, A. T., Thomas, F., Stenik, J. A., Demosky, S. J., Bark, S. E., Neufeld, E. B., Bocharov, A. V., Vishnyakova, T. G., Patterson, A. P., Eggeman, T. L., Santamaria-Fujo, S., and Brewer, H. B. (2003) J. Lipid Res. 44, 828–836
22. Yaney, P. G., Bielecki, J. K., Johnson, W. J., Lund-Katz, S., Palgunachari, M. N., Anantharamai, G. M., Segrest, J. P., Phillips, M. C., and Rothblat, G. H. (1995) Biochemistry 34, 7955–7965
23. Oram, J. F., and Yokoyama, S. (1996) J. Lipid Res. 37, 2473–2491
24. Chroni, A., Liu, T., Fitzgerald, M. L., Freeman, M. W., and Zannis, V. I. (2004) Biochemistry 43, 2126–2139
25. Fitzgerald, M. L., Morris, A. L., Chroni, A., Mendez, A. J., Zannis, V. I., and Freeman, M. W. (2004) J. Lipid Res. 45, 287–294
26. Chroni, A., Liu, T., Gorkshkova, I., Kan, H. Y., Uehara, Y., Von Eckardstein, A., and Zannis, V. I. (2003) J. Biol. Chem. 278, 6719–6730
27. Burgess, J. W., Frank, P. G., Franklin, V., Liang, P., McManus, D. C., Desforges, M., Rassart, E., and Marcel, Y. L. (1999) Biochemistry 38, 14524–14533
28. McManus, D. C., Scott, B. R., Frank, P. G., Franklin, V., Schultz, J. R., and Marcel, Y. L. (2000) J. Biol. Chem. 275, 5043–5051
29. Panagotopulos, S. E., Witting, S. R., Horace, E. M., Hui, D. Y., Maiorano, J. N., and Davidson, W. S. (2002) J. Biol. Chem. 277, 39477–39484
30. Lund-Katz, S., and Phillips, M. C. (1986) Biochemistry 25, 1562–1568
31. Scanu, A. M., and Edelstein, C. (1971) Anal. Biochem. 44, 576–588
32. Weiszweiler, P. (1987) Clin. Chim. Acta 169, 249–254
33. Anantharamai, G. M. (1986) Methods Enzymol. 128, 627–647
34. Bortnick, A. E., Rothblat, G. H., Steudl, G., Hoppe, K. L., Royer, L. J., McNeilis, J., and Francone, O. L. (2000) J. Biol. Chem. 275, 28634–28649
35. Favari, E., Bernini, F., Tarugi, P., Franceschini, G., and Calabresi, L. (2002) Biochem. Biophys. Res. Commun. 299, 801–805
36. Markwell, M. A., Haas, S. M., Beier, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 87, 206–210
37. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Med. Sci. 37, 111–117
38. Scanu, A. M., and Edelstein, C. (1971) Anal. Biochem. 44, 576–588
39. Schulman, B. A., and Kim, P. S. (1996) Nat. Struct. Biol. 3, 682–687
40. Saito, H., Dhanasekaran, P., Nguyen, D., Deridder, E., Holvoet, P., Lund-Katz, S., and Phillips, M. C. (2004) J. Biol. Chem. 279, 20974–20981
41. Oda, M. N., Forte, T. M., Ryan, R. O., and Voss, J. C. (2003) Nat. Struct. Biol. 10, 455–460