Deletion of the C-Terminal Domain of Apolipoprotein A-I Impairs Cell Surface Binding and Lipid Efflux in Macrophage†

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ABSTRACT: The contribution of the amphipathic α-helices of apoA-I toward lipid efflux from human skin fibroblasts and macrophage was examined. Four apoA-I mutants were designed, each by deletion of a pair of predicted adjacent helices. Three mutants lacked two consecutive central α-helices [Δ(100−143), Δ(122−165), and Δ(144−186)], whereas the final mutant lacked the C-terminal domain [Δ(187−243)]. When compared to recombinant wild-type apoA-I and mutants with central domain deletions, Δ(187−243) exhibited a marked reduction in its ability to promote either cholesterol or phospholipid efflux from THP-1 macrophages. This mutant also demonstrated a decreased ability to bind lipids and to form lipoprotein complexes. In contrast, the four mutants and apoA-I equally supported cholesterol efflux from fibroblasts, albeit with a reduced capacity when compared to macrophages. Δ(187−243) bound poorly to the macrophage cell surface when compared to apoA-I, and competitive binding studies with the central domain and C-terminal deletions mutants showed that only Δ(187−243) did not compete effectively with [^{125}I]apoA-I. Omission of PMA during cholesterol loading enhanced cholesterol efflux to both apoA-I (1.5-fold) and the C-terminal deletion mutant (2.5-fold). Inclusion of the Sandoz ACAT inhibitor (58-035) during loading and, in the absence of PMA, increased and equalized cholesterol efflux to apoA-I and Δ(187−243). Surprisingly, omission of PMA during cholesterol loading had minimal effects on the binding of apoA-I or Δ(187−243) to the THP-1 cell surface. Overall, these results show that cholesterol efflux from cells such as fibroblasts does not require any specific sequence between residues 100 and 243 of apoA-I. In contrast, optimal cholesterol efflux in macrophages requires binding of the C-terminal domain of apoA-I to a cell surface-binding site and the subsequent translocation of intracellular cholesterol to an efflux-competent pool.

The mechanisms of HDL[1]-mediated cholesterol efflux are poorly characterized and likely vary among different cell types. Nevertheless, two mechanisms of cholesterol efflux from cells have been described. The first is diffusional efflux (*1*), in which cholesterol from the cell membrane transfers by passive diffusion through the aqueous phase to acceptors, principally lipoproteins. Although viewed as a passive process, diffusional efflux may be enhanced by the activity of LCAT (*2*, *3*) that functions to maintain the gradient of cholesterol between the cell surface and acceptor particle. Diffusional efflux is also enhanced by the acceptor phospholipid composition (*4*) and receptor-mediated tethering of the acceptor particle in the high cholesterol microenvironment of the cell surface (*5*, *6*). The second pathway is specific and involves binding of apolipoprotein A-I (apoA-I) or HDL to poorly characterized cell surface binding sites, mobilization of intracellular stores of cholesterol to efflux accessible pools at the cell surface (*7*, *8*), and consequent increases in diffusional efflux or even direct lipid transfer to the bound acceptor. In some cell types, for example, fibroblasts (*9*, *10*) and mouse peritoneal macrophage (*11*), this mobilization of cellular cholesterol is initiated by activation of protein kinase C. Activation of cAMP-dependent protein kinase has also been reported to stimulate the efflux of intracellular cholesterol in fibroblasts and bovine aortic endothelial cells (*12*).

To determine if the central and C-terminal domains of apoA-I participate in either cellular diffusional efflux or cholesterol mobilization, we have prepared four apoA-I mutants. The removal of a pair of adjacent helices was adopted for preparing the central deletions. This strategy was selected to minimize disruption of the predicted secondary

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[1] Abbreviations: ACAT, acyl coenzyme A:cholesterol acyl transferase; AcLDL, acetylated LDL; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CE, cholesterol esters; DMPC, dimyristoylphosphatidylcholine; FBS, fetal bovine serum; FC, free cholesterol; HDL, high-density lipoprotein; H-7, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine; PMA, phorbol 12-myristate 13-acetate; SR-B1, scavenger receptor class B, type I.

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structure, based on the sequence periodicity of the protein (*13*, *14*). The first three mutants lacked two consecutive central α-helices [Δ(100−143), Δ(122−165), and Δ(144−186)], whereas the final mutant lacked the C-terminal domain [Δ(187−243)]. We have previously shown that the central deletion mutants exhibit a reduced capacity to bind phospholipid but have retained the ability to form lipoproteins (*15*−*18*) and promote the diffusional efflux of cholesterol from normal quiescent fibroblasts (*18*).

Here, we have tested the following hypotheses. First, deletions in the central domain of apoA-I remove a putative hinge-domain that has been postulated to be important for the association of apoA-I with lipids (*15*, *19*). We have also tried to corroborate indirect evidence for the participation of the central domain in specific cellular cholesterol efflux (*20*−*23*). Second, we have tested the importance of the C-terminal domain in mediating binding to the cell surface and in controlling specific lipid efflux. This region includes an amphipathic α-helix with the highest lipid-binding affinity of the apoA-I domains (*24*), and mutants lacking this region elicit a decreased efflux from HepG2 cells, albeit without alteration in cell binding (*25*). The present results demonstrate that, in cholesterol-loaded THP-1 cells, lipid efflux is significantly decreased only to the C-terminal deletion mutant. The mechanism for the impaired efflux appears related principally to decreased binding to the cell surface and a decreased ability to promote the intracellular translocation of cholesterol to the cell surface. In contrast to THP-1 cells, when wild-type and mutants of apoA-I were added to fibroblasts, much reduced levels of cell surface binding and cholesterol efflux were found suggesting an important cellular specificity, possibly related to surface binding.

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl 2-oleyl phosphatidylcholine and cholesterol were obtained from Avanti Polar Lipids (Birmingham, AL). [1α, 2α-^{3}H]Cholesterol and [methyl-^{3}H]-choline chloride were purchased from DuPont NEN (Boston, MA). PMA, staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), and calphostin C were obtained from Sigma. All other reagents were of analytical grade. Human skin fibroblasts (GM00038B) and human THP-1 monocytes were obtained from the Coriell Institute for Medical Research (Camden, NJ) and from the American Type Culture Collection (no. ATCC TIB-202), respectively. The ACAT inhibitor, Sandoz 58-035, was a gift from Novartis.

Construction of the Carboxyl-Domain Deletion Mutant cDNA. Construction of the central deletion mutants was previously described (*15*). Deletion of nucleotides encoding residues 187−243 of apoA-I was performed in a similar manner using a 36-base oligonucleotide (5′-GCGGCG-GCGGGCGCCTCA/GCCGCCGTTCTCCTTGAG-3′). This oligonucleotide was complementary to 2 portions of 18 nucleotides each located upstream and downstream, respectively, of the DNA sequence to be deleted. The oligonucleotide was annealed to the denatured plasmid and served as a primer in the repair reaction that was performed according to the method of Kunkel et al. (*26*). The DNA of the corresponding mutant was verified by sequencing prior to its transfer into the expression vector.

Production of the Mutant Proteins. Wild-type and mutant apoA-I with an N-terminal extension Met-Arg-Gly-Ser-$(His)_6$-Met (apoA-I) were expressed in a bacterial system. Four mutants, Δ(100−143), Δ(122−165), Δ(144−186), and Δ(187−243), were produced as previously described (*15*). After purification on nitriloacetic acid agarose (NTA, Qiagen), the purified proteins were dialyzed against 5 mM NH_4HCO_3, 1 mM EDTA, and 0.02% NaN_3 and lyophilized. Proteins were then stored at −20 °C. Previous studies have documented the lack of effect of the N-terminal extension on wild-type apoA-I properties, including its ability to bind lipids, to activate LCAT, and to promote diffusional cholesterol efflux (*15*, *18*).

Cell Culture and Lipid Efflux. Human skin fibroblasts were cultured in a CO_2 incubator at 37 °C as previously described (*27*). They were maintained between passage 15−25 in DMEM low-glucose, 10% FBS, 4 mM glutamine, and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). For experiments performed with human skin fibroblasts, the cells in complete medium were seeded in 6-well plates at a density of 6×10^4 cells/well. After 48 h, the medium was replaced with DMEM supplemented with 5% FBS, 200 μg/mL cholesterol from a cholesterol-rich dispersion, and 50 μg/mL LDL. The lipid dispersion was prepared as described by Arbogast et al. (*28*) with a cholesterol/phospholipid molar ratio of 3. After 3 days of loading, the media was changed to DMEM containing 5% FBS and 15 μCi/mL [^{3}H]cholesterol dispersed in 0.1% ethanol (% final volume of media) for 24 h. Before each efflux experiment, the cells were washed three times with DMEM and then incubated with DMEM containing 2 mg/mL fatty-acid-free BSA (Sigma) for 4 h. For the efflux experiments, the cells were washed three times with DMEM and then incubated with DMEM containing the lipid-free apoprotein (1.70 μM) and 0.2% fatty-acid-free BSA. Media aliquots were taken at different times of incubation and treated as previously described (*24*). At the end of the experiment, the cells were solubilized in 2-propanol and the cholesterol content was determined by gas chromatography (*29*) after extraction of cellular lipids by the method of Bligh and Dyer (*30*). Results are expressed as the percentage of labeled cholesterol remaining in the cells as a function of time.

Human THP-1 monocytes were grown in complete RPMI 1640 medium containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μM β-mercaptoethanol, and 2 mM glutamine. To differentiate these cells into macrophages, the monocytes were seeded in 6-well plates at a density of 2×10^6 cells/well in the same medium containing 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) and incubated for 72 h. The cells were cholesterol-loaded and labeled for 48 h in 1 mL of RPMI 1640 supplemented with 2 mg/mL fatty-acid-free BSA (referred to as RPMI-BSA) and with either [^{3}H]cholesterol or [^{3}H]choline (10 μCi/mL final concentration) which had been preincubated for 30 min at 37 °C with 75 μg/mL (final concentration) acetylated LDL (AcLDL). After loading, the cells were incubated in RPMI-BSA for 24 h to allow equilibration of the label in free-cholesterol and esterified-cholesterol pools. In some experiments, PMA (100 nM) and/or the Sandoz ACAT inhibitor 58-035 (5 μg/mL) were included during the cholesterol loading and equilibration steps. On the day of the experiment, the cells were washed with RPMI 1640, and the lipid-free apoproteins (1.70 μM final) in RPMI-BSA were added to

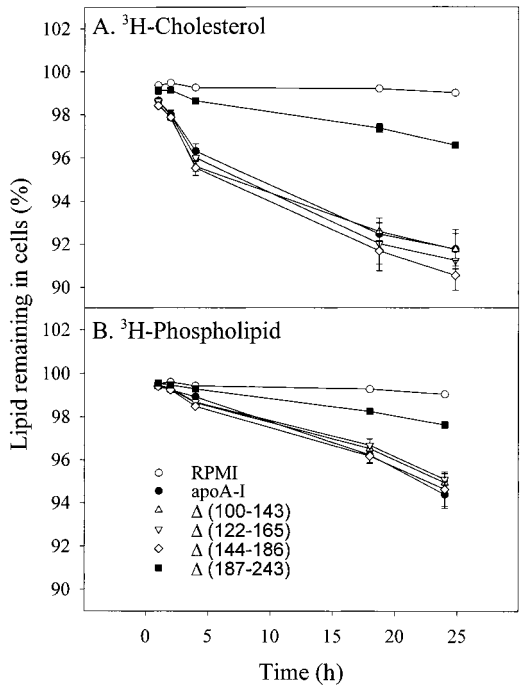

FIGURE 1: Cholesterol (panel A) and phospholipid (panel B) efflux from macrophage in the presence of lipid-free apolipoproteins. For the determination of cholesterol efflux, the cells were cholesterol loaded in the presence of [^{3}H]cholesterol (see Experimental Procedures) and then incubated with lipid-free apolipoproteins (1.70 μM). Aliquots of media were removed at the indicated time and counted. Efflux is expressed as the percentage of [^{3}H]cholesterol remaining in cells as a function of time. Experiments were performed in triplicate and are representative of three independent experiments. To examine phospholipid efflux the cells were cholesterol loaded in the presence of [^{3}H]choline labeling (see Experimental Procedures) and then incubated with the lipid-free apolipoprotein (1.70 μM). Aliquots of media were removed at the indicated times and the labeled lipids, extracted by the method of Bligh and Dyer (*30*), were counted. The efflux was expressed as the percentage of [^{3}H]phospholipid remaining in cells at the different times of incubation. Experiments were performed in triplicate and are representative of three independent experiments. After 24 h, both cholesterol and phospholipid efflux were significantly reduced for Δ(187−243) when compared to apoA-I ($p < 0.001$).

initiate lipid efflux. This apoA-I concentration was selected as saturating for efflux based on estimates of lipid-free apoA-I levels in interstitial fluids and plasma (*8*) and as reported by others as optimum for efflux assays (*7*, *8*). When included, the protein kinase C inhibitors Calphostin C (0.5 and 1.5 μM, final concentrations), staurosporine (50 and 150 nM, final concentrations), and H7 (50 and 150 μM, final concentrations) were added to the efflux assay at the same time as the lipid-free apolipoproteins. At the times indicated (Figures 1 and 4), samples of medium were removed and counted. For determinations of phospholipid efflux, the medium samples were further extracted to separate [^{3}H]-phospholipids from free [^{3}H]choline (*30*) prior to counting. Efflux is calculated as (medium ^{3}H-label/cellular ^{3}H-label at 0 time radioactivity) × 100.

Characterization of the Complexes Formed during Efflux. After efflux, the media was recovered and concentrated using Centricon concentrators (Amicon, Beverly, MA). Samples of media were loaded onto native polyacrylamide 8 to 25% gradient gels or on Beckman Spe Agarose gel. The electrophoresed proteins were transferred to nitrocellulose and were incubated with a combination of mAbs against apoA-I [2F1, 5F6, and A44 (*31*)], followed by an anti-mouse IgG HRP-conjugated polyAb (Jackson ImmunoResearch, Mississauga, Ontario). The presence of apoA-I was revealed by BM Chemiluminescence Blotting Substrate (Boehringer Mannheim). In some experiments, the concentrated media containing the proteins after efflux assay were electrophoresed on agarose gels, and the dried gels were then exposed to a tritium screen of a phosphorimager to demonstrate the association of labeled lipids with different apolipoprotein mutants.

DMPC Kinetics Analysis. The ability of the different proteins to clear a DMPC solution was determined as previously described (*15*).

Binding of apoA-I and D(187−243) to Cells. Macrophage and fibroblasts were cholesterol loaded as described above. After the loading period, the cells were incubated for 24 h with RPMI-BSA (macrophage) or DMEM-BSA (fibroblasts). For cell surface binding analysis, the cells were washed with the appropriate medium containing 1% fatty-acid-free BSA and then incubated for 3 h at 37 °C with radiolabeled apoA-I or Δ(187−243) (500 000 cpm/well, specific activity ≈ 8000 cpm/ng). Cells were then washed three times with medium (no added BSA) and solubilized overnight in 0.5 N NaOH. Radioactivity and protein content of the cell extracts were determined and the percentage of [^{125}I]apoA-I or Δ(187−243) bound to the cells was determined and expressed as a function of cell protein. The competition binding studies were performed in a similar manner with varying concentrations of the cold competing protein.

Miscellaneous Procedures. AcLDL was prepared from human LDL as described by Basu et al. (*32*). Phospholipid and free and total cholesterol levels in cells were determined with Boehringer Mannheim enzymatic kits. Protein determinations were performed as described by Markwell et al. (*33*). Lipid-free apolipoproteins were iodinated by the IODO-BEAD method (Pierce).

RESULTS

Lipid Efflux from Cholesterol-Loaded THP-1 Macrophage. To analyze the domain specificity of macrophage interaction with apoA-I, we examined lipid efflux mediated by apoA-I and deletion mutants [Δ(100−143), Δ(122−165), Δ(144−186), and Δ(187−243)]. To this end, human THP-1 monocytes were differentiated in the presence of phorbol ester and loaded with cholesterol using acetylated-LDL. This procedure resulted in a significant increase in the cellular cholesterol content compared to the nonloaded cells (97.0 ± 9.7 vs 48.1 ± 4.1 μg of total cholesterol/mg of cell protein, respectively). For cholesterol-loaded macrophages, the cholesterol pool comprised, on average, 40% CE and 60% FC.

When cholesterol-loaded THP-1 cells were incubated with the different lipid-free apolipoproteins, only the C-terminal deletion mutant exhibited a significantly reduced ability to promote cholesterol efflux. Compared to apoA-I and the other mutants, Δ(187−243) demonstrated a 70% decrease in its ability to remove cholesterol from macrophage after a 24 h incubation (Figure 1, panel A). After 24 h, about 8−9% of the cellular cholesterol was removed with apoA-I and the central deletion mutants, whereas only 3.4% was removed with Δ(187−243) [$p < 0.001$; comparison of apoA-I versus Δ(187−243)].

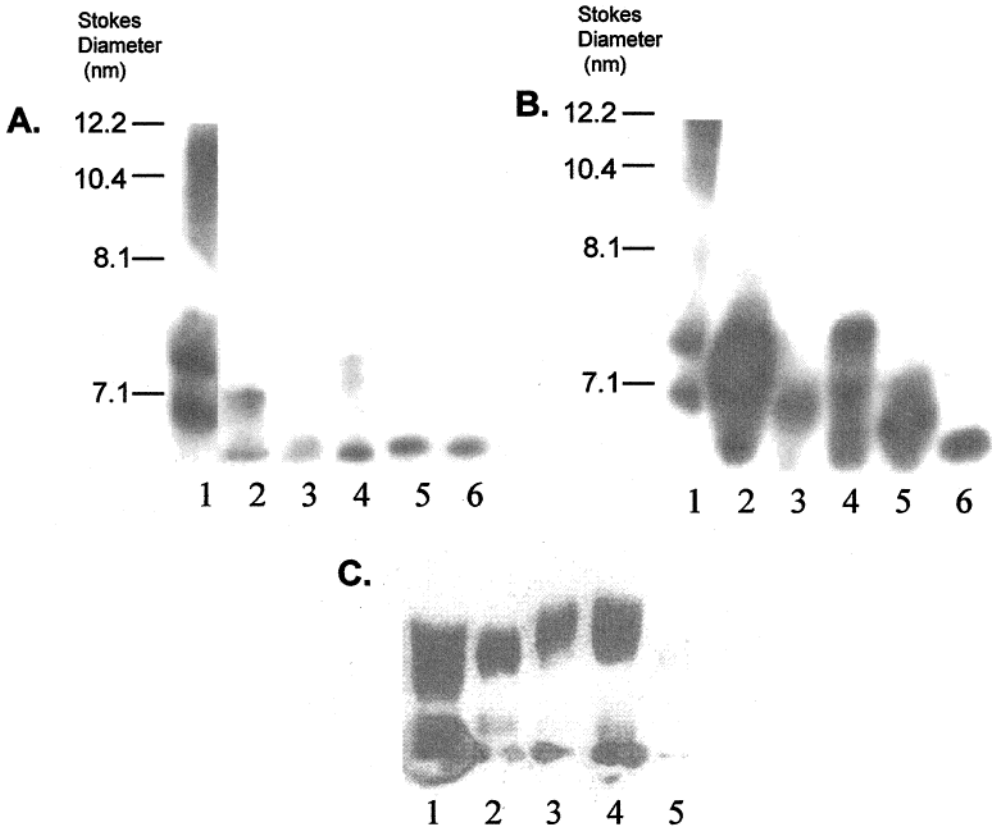

FIGURE 2: Characterization of the apolipoprotein−lipid complexes formed by incubation with macrophage. Samples of media obtained before (panel A) and after a 24 h incubation with cholesterol-loaded macrophages (panel B) were applied onto native polyacrylamide 8 to 25% gradient gels. The gels were transferred to nitrocellulose membranes, probed with mAbs against apoA-I (2F1, 5F6, and A44), and then detected using the ECL system (Amersham). Lanes 1−6 of both gels represent total HDL, apoA-I, Δ(100−143), Δ(122−165), Δ(144−186), and Δ(187−243), respectively. The enhanced signal observed with samples after efflux may be due to an increased immunoreactivity of the apoproteins after lipidation. In panel C, samples of media collected after a 24 h incubation with [^{3}H]cholesterol loaded THP-1 macrophages, were applied to a Beckman SPE agarose gel. The distribution of the tritium label in the gels was evaluated with a phosphorimage analyzer. Lanes 1−5 represent apoA-I, Δ(100−143), Δ(122−165), Δ(144−186), and Δ(187−243), respectively.

We also determined the efflux of phospholipid from cholesterol-loaded macrophages (Figure 1B). When compared to apoA-I, the C-terminal deletion mutant demonstrated a 70% reduction in its ability to promote phospholipid efflux after a 24 h incubation. Deletion of the central domains of apoA-I did not influence the efflux of phospholipids. Incubations in the presence of apoA-I or the central deletion mutants decreased cellular lipids by 5−6%. In contrast, Δ-(187−243) removed 2.4% [$p < 0.001$; comparison of apoA-I versus Δ(187−243)].

Complex Formation and Phospholipid Binding Properties of the Deletion Mutants. The decreased lipid efflux to Δ-(187−243) could result from defects in forming oligomeric lipoprotein structures or in the binding of phospholipids. To examine the abilities of Δ(187−243) and the central deletion mutants to form lipoprotein-like structures, media was obtained from macrophages after incubation with the apolipoproteins, concentrated, and then electrophoresed on an 8 to 25% native gradient gel to determine changes in particle size (Figure 2). The separated proteins were transferred to nitrocellulose and Western blotted with monoclonal antibodies against apoA-I [2F1, 5F6, and A44 (*34*, *35*)]. Figure 2A illustrates the native gradient gel for medium that has not been incubated with cells. At the concentration used in this experiment, apoA-I and Δ(122−165) (lane 2 and 4, respectively) appeared to form oligomers as demonstrated by the presence of a secondary band whereas the other apolipoproteins did not. Incubation with cells increased the apparent size of the wild-type apoprotein (panel B, lane 2) and the three central deletion mutants (lanes 3, 4, and 5) consistent with the notion that they bound lipids. In contrast, no increase in Δ(187−243) particle size was observed after incubation with cells (lane 6), indicating a requirement for the C-terminal region in forming lipoprotein-like structures. Medium samples were also collected following a 24 h incubation of apoA-I and the deletion mutants with [^{3}H]cholesterol-loaded macrophages and applied to agarose gels. The gels were exposed to an [^{3}H]phosphorimager screen. The signal derived indicated co-localization of the cholesterol label with apoA-I, Δ(100−143), Δ(122−165), and Δ(144−186) (lanes 1−4, respectively). This was, however, not the case for Δ-(187−243) for which a very weak signal was detected (lane 5). A very similar pattern was observed when the phospholipid distribution was examined in medium samples from [^{3}H]cholate-labeled cells (data not shown), indicating that full-length apoA-I and the central deletion mutants bind cellular lipids efficiently whereas the C-terminal deletion mutant does not.

We have previously demonstrated that Δ(122−165) and Δ(144−186) exhibit a reduced capacity to bind phospholipid when compared to apoA-I. In contrast, the phospholipid binding capacity of apoA-I was unaffected by deletion of residues 100−143 (*15*). To determine if the ability of Δ-(187−243) to bind phospholipids was impaired, DMPC

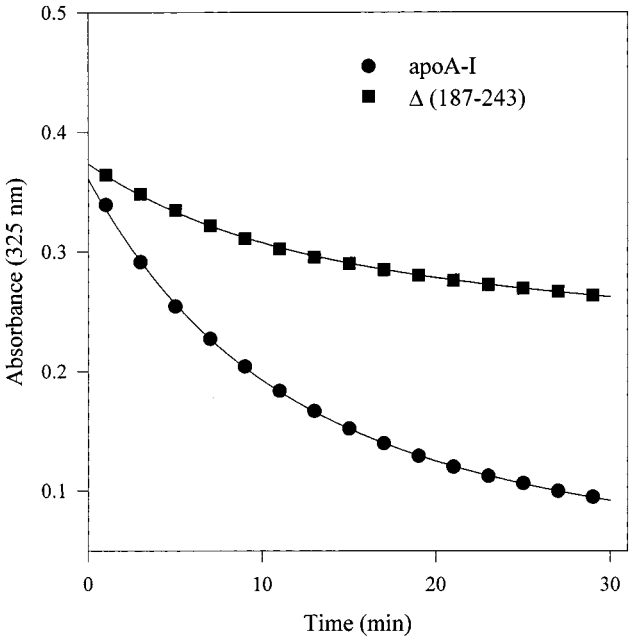

FIGURE 3: Kinetics of DMPC association with recombinant apolipoproteins. DMPC was solubilized in TBS pH 8 at 37 °C and added to the apoprotein (DMPC:apoA-I, 50:1, mol:mol) and preincubated for 10 min at 24 °C. The reaction was followed for 30 min at 325 nm in a temperature-controlled cell (24 °C).

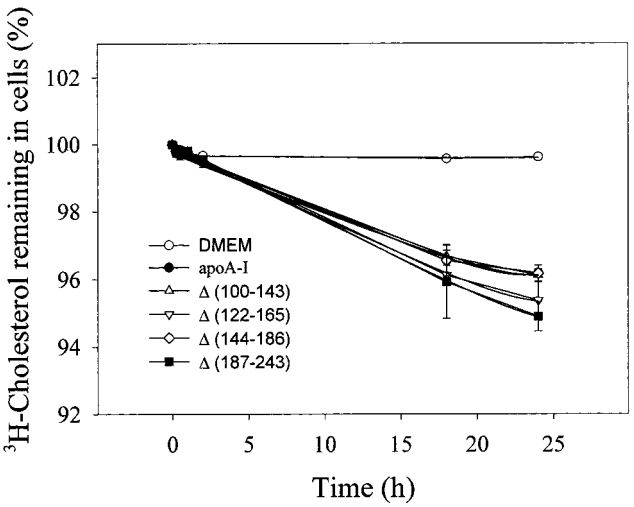

FIGURE 4: Cholesterol efflux from fibroblasts to lipid-free proteins. After cholesterol loading and labeling (see Experimental Procedures) the cells were incubated with lipid-free apolipoproteins (1.70 μM). Aliquots of media were removed at the indicated times and counted. Efflux is expressed as the percentage of [^{3}H]cholesterol remaining in cells at the different times of incubation. Experiments were performed in triplicate and are representative of three independent experiments.

clearance experiments were similarly performed. The kinetics of association with DMPC at 24 °C was followed by the decrease in the turbidity at 325 nm, which reflects the formation of discoidal complexes (Figure 3). We observed that Δ(187−243) displayed a 5.3-fold reduction in the kinetics of association with DMPC [$t_{1/2}$ of 60.3 min for Δ(187−243) versus 11.3 min for apoA-I]. In contrast, the Δ(122−165) and Δ(144−186) mutants exhibited about a 1.6-fold reduction in the ability to bind phospholipids (*15*). Therefore, when compared to apoA-I and the central deletion mutants, the Δ(187−243) mutant lacks the ability to bind

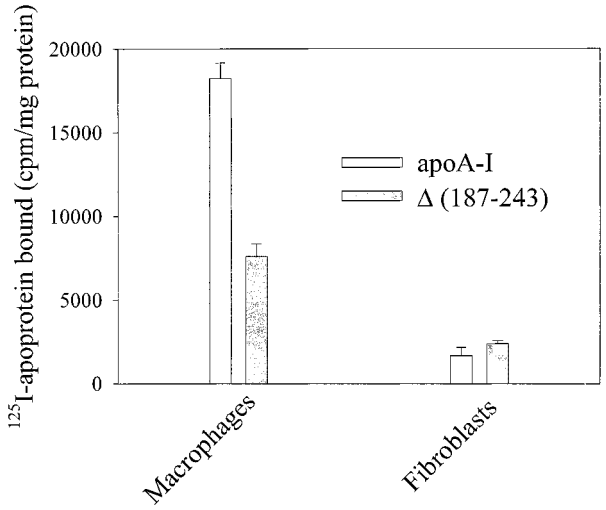

FIGURE 5: Association of apolipoproteins with THP-1 macrophage and fibroblasts. Macrophage or fibroblast monolayers were incubated with PBS containing 1% fatty acid BSA for 1 h at 4 °C. The cells were washed with RPMI containing 0.2% BSA and then incubated with ^{125}I-radiolabeled apoA-I or Δ(187−243) (500 000 cpm/well; specific activity ≈ 8000 cpm/ng) for 3 h at 37 °C. The wells were washed three times with PBS, solubilized with 0.5 N NaOH, and then counted in a γ-counter. Results represent the mean of three separate determinations.

cellular lipids and to form higher order complexes and also exhibits a markedly impaired phospholipid binding capacity.

Cholesterol Efflux from Cholesterol-Loaded Fibroblasts. We also examined cholesterol efflux to the lipid-free apolipoproteins in human skin fibroblasts. These cells have been used extensively in efflux studies but have a limited capacity to accumulate large amounts of cholesterol when compared to macrophage cell lines. Fibroblasts were loaded with cholesterol using LDL and cholesterol-enriched phospholipid dispersions (*28*). With this treatment, the cholesterol content of cells nearly doubled (from 15.3 ± 4.0 μg of FC/mg of cell protein in control cells to 26.9 ± 2.2 μg/mL in cholesterol-loaded cells) in agreement with the results of others (*36*). There was no significant CE accumulation in fibroblasts (data not shown). Cholesterol loading of fibroblasts significantly enhanced cholesterol efflux to lipid-free apoA-I (i.e., from undetectable levels in unloaded cells to a 5% decrease in the total cholesterol of loaded cells in 24 h). This result is in accord with that reported by Bielicki et al. (*37*). However, the levels of efflux from fibroblasts were much lower compared to those obtained with macrophage (4−5% for fibroblasts versus 8−9% for macrophage after 24 h). When the cholesterol-loaded cells were incubated with the same molar concentrations of apoA-I or the central or C-terminal deletion mutants, no significant difference in the rates of cholesterol efflux (Figure 4) or phospholipid efflux (data not shown) was observed. These results indicate that there are substantial differences in the mechanisms of lipid efflux in macrophages, which require the C-terminal of apoA-I, and fibroblasts, which do not.

Association of apoA-I and the Deletion Mutants with THP-1 Macrophages and Fibroblasts. We investigated the possibility that binding of the lipid-free apolipoproteins to the cell surface might correlate with the varied abilities of Δ(187−243) mutant to promote cholesterol efflux from macrophages and fibroblasts. As indicated in Figure 5, the association of Δ(187−243) mutant to the macrophage surface

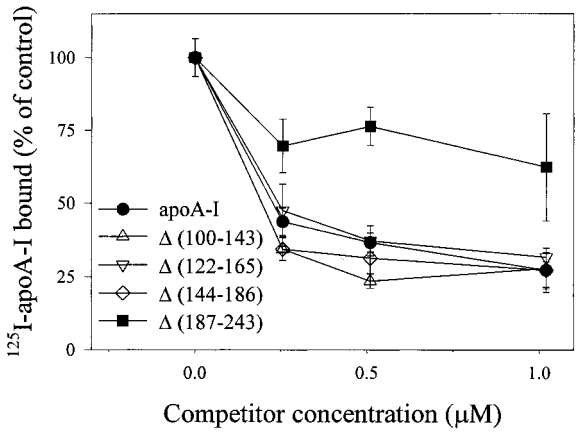

FIGURE 6: Competition between [^{125}I]apoA-I and the deletion mutants for binding sites on the THP-1 cell surface. Cholesterol-loaded THP-1 macrophages were incubated with ^{125}I-labeled apoA-I (500 000 cpm/well; specific activity ≈ 8000 cpm/ng) and varying concentrations of apoA-I and deletion mutants. Results are expressed as the percent of ^{125}I-labeled apoA-I bound as a function of the amount of competing protein concentration (100%, no competitor). The results are the average of quadruplicate wells and representative of three assays.

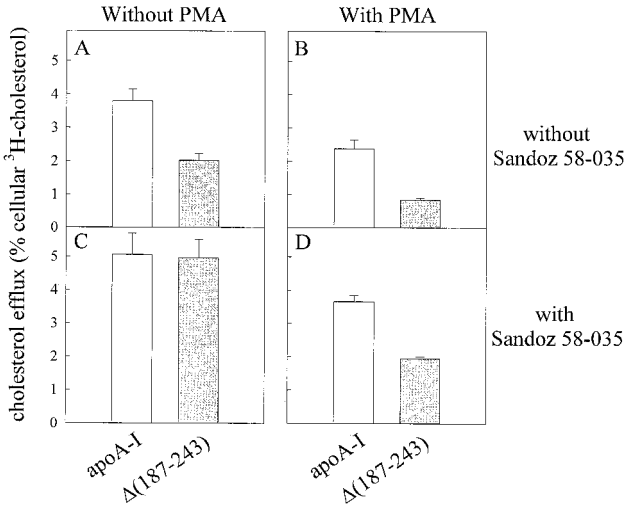

FIGURE 7: The effects of PMA and an ACAT inhibitor on cholesterol efflux from THP-1 macrophages. THP-1 cells were differentiated for 72 h in the presence of PMA as described in "Experimental Procedures". The cells were washed with RPMI 1640 and then incubated with RPMI-1640-BSA, acetylated LDL (75 μg/mL), and [^{3}H]cholesterol (10 μCi/well) in the absence (panels A and C) or presence of 100 nM PMA (panels B and D) for 48 h. In panels C and D, the Sandoz compound 58-035, an ACAT inhibitor (5 μg/mL) was also included during the loading step. After loading the cells were washed twice with RPMI−BSA and then lipid-free apoA-I or Δ(187−243) in RPMI−BSA was added to a final concentration of 1.7 μM and incubated at 37 °C for 3 h. Medium samples were collected and counted. Apolipoprotein-specific efflux was determined by subtracting efflux to RPMI−BSA alone and is expressed as a percentage of the total cellular label. All results represent the mean and SE of three separate determinations.

at 37 °C was reduced by about 58% when compared to the association of apoA-I. In contrast, the association of [^{125}I]-apoA-I with fibroblasts under identical conditions represented only 9.3% of that observed in THP-1 macrophages and there was no significant difference in the binding of apoA-I and Δ(187−243).

To determine if apoA-I and Δ(187−243), as well as the central deletion mutants, compete for the same binding site on the THP-1 cell surface, competition studies were performed. The cells were incubated in the presence of [^{125}I]-apoA-I and increasing concentrations of cold competing protein. As indicated in Figure 6, the central deletion mutants competed with the cell surface binding of [^{125}I]apoA-I as efficiently as cold apoA-I. In contrast, the Δ(187−243) mutant was a less efficient competitor, reducing the binding of apoA-I by about 40% when compared with excess cold apoA-I or the other mutants. These results suggest the possibility that at least two binding sites for apoA-I exist on the THP-1 cell surface. The major site binds apoA-I via the C-terminal tail; a second site may bind apoA-I by the N-terminal domain (residues 1−100) or any remaining amphipathic helix.

Effect of PMA and the ACAT Inhibitor (Sandoz 58-035) on Apolipoprotein-Mediated Efflux in THP-1 Macrophage. Differentiation of THP-1 monocytes to macrophages is usually performed by the addition of PMA to the growth medium for 72 h. Prolonged incubations (4 days) in the absence of PMA, following the differentiation step, have been reported to result in a decrease in cell viability (*38*). Long incubations with PMA (greater than 4 h), however, downregulates protein kinase C in many cell types (*39*−*41*), and this could adversely affect apolipoprotein-mediated cholesterol translocation to an efflux-competent pool in THP-1 macrophages. Consequently, following PMA-induced differentiation, we loaded the cells for 2 days with AcLDL in the absence or continued presence of PMA. Removal of PMA during this step resulted in marginal decreases in [^{3}H]-cholesterol labeling (8%) and cellular protein content (5%). The omission of PMA from the loading step increased cholesterol efflux by about 1.5-fold to apoA-I and by about 2.5-fold to Δ(187−243) when compared to cells loaded in the presence of PMA (Figure 7; compare panel A and panel B). Efflux to Δ(187−243), however, remained significantly lower than that to apoA-I in the absence of PMA (Figure 7A). Inclusion of inhibitors of protein kinase C (calphostin C, staurosporine and H-7) and cAMP-dependent protein kinase (H-7) to the efflux assay did not influence cholesterol efflux to either protein independently of the absence or presence of PMA during cholesterol loading (data not shown). Addition of PMA directly to the efflux assay with or without prior addition during cholesterol loading was also without effect on the rates of efflux (data not shown).

The impaired efflux to Δ(187−243) could be explained by a decreased ability to bind to the THP-1 cell surface (Figure 5) or by a decreased ability to mobilize cholesterol from an ACAT accessible pool. Table 1 demonstrates that the omission of PMA after the cholesterol loading step did not affect the binding at 4 °C of either apoA-I or Δ(187−243), although the cell association at 37 °C of Δ(187−243) was marginally reduced.

The ability of apoA-I and Δ(187−243) to mobilize cholesterol from an ACAT accessible pool was examined by including the Sandoz compound 58-035, an ACAT inhibitor during the 2 day cholesterol-loading step. As expected, inclusion of the ACAT inhibitor during loading significantly decreased the levels of esterified cholesterol with or without PMA addition (Table 1). In the presence of PMA during loading, the levels of free cholesterol increased but did not result in increased efflux. When the ACAT inhibitor was included in the absence of PMA, cholesterol

Table 1: Effects of PMA and the Sandoz Compound 58-035 on Apolipoprotein Binding and Cholesterol Levels in THP-1 Macrophages

		without PMA		with PMA	
		−	58-035	−	58-035
binding (4 °C)	apoA-I	3851 ± 824	3523 ± 311	3049 ± 382	3895 ± 551
[^{125}I]apoprotein/mg of protein	Δ187−243	839 ± 96	1013 ± 86	866 ± 229	1319 ± 89
cell association (37 °C)	apoA-I	19 119 ± 542	26 389 ± 1224	19 636 ± 1466	28 170 ± 470
[^{125}I]apoprotein/mg of protein	Δ187−243	7947 ± 764	12 128 ± 863	9615 ± 1206	13 397 ± 897
cellular cholesterol	FC	42.5 ± 7.7	44.0 ± 2.2	58.5 ± 4.2	70.7 ± 3.2
μg/mg of cell protein	CE	17.6 ± 4.0	4.2 ± 1.7	18.8 ± 2.1	ND[b]

[a] Not detected. [b] After differentiation, THP-1 cells were cholesterol-loaded in the absence or presence of PMA. In some wells the Sandoz ACAT inhibitor, 58-035 (5 μg/mL), was also included. The cells were washed and the cell cholesterol levels were determined. Duplicate plates were incubated at 4 or 37 °C with [^{125}I]apoA-I or [^{125}I]Δ (187−243) (1×10^6 cpm representing about 30 ng of protein) for 3 h. The cells were washed, solubilized in 0.5 N NaOH and counted in a γ-counter. Nonspecific binding was determined with a 100-fold excess of unlabeled apolipoprotein. Results represent the mean and SE of three separate determinations.

efflux to both apoA-I and Δ(187−243) increased and the difference between the two disappeared (Figure 7C), indicating that apoA-I, but not the C-terminal deletion mutant, is capable of promoting efflux of cholesterol from an ACAT-accessible pool. When PMA was present, however, the effect of the ACAT inhibitor was attenuated and efflux remained lower to Δ(187−243) than to apoA-I (Figure 7D). Loading in the presence of the ACAT inhibitor did not significantly change binding of either proteins at 4 °C, but significantly increased the cell association at 37 °C of both apoA-I and Δ(187−243) by 1.4−1.5-fold ($p \leq 0.01$). This increased cell surface association may partially explain the equal efflux to the two proteins noted under these conditions.

DISCUSSION

Our primary aim was to analyze the role of apoA-I amphipathic α-helices in (lipid-free) apolipoprotein-mediated lipid efflux and to examine the relationships between efflux and cell surface binding. Toward this goal, we have compared apoA-I binding and efflux in THP-1 macrophages, a cell type in which lipid efflux is active and well characterized (*38*, *42*), and human fibroblasts, which demonstrate cholesterol efflux when studied in growth arrest or after cholesterol loading (*43*).

In both cell types, efflux to the lipid-free apolipoproteins is unaffected by the deletion of any pair of adjacent helices between residues 100 and 186. We conclude, therefore, that the deletion of any pair of adjacent helices in the central domain does not affect the capacity of apoA-I to elicit efflux of phospholipids and cholesterol (Figure 1A and B) and to form lipoproteins (Figure 2). These results also suggest that the putative central hinge domain, located between residues 99 and 186 (*44*), does not serve any function in this pathway. Therefore, the observed inhibition of efflux to plasma apoA-I by an antibody reacting with the central domain (residues 137−144) (*20*) may not reflect inhibition of a ligand-cell interaction but rather a direct effect on apoA-I conformation.

Deletion of the C-terminal domain of apoA-I decreases both lipid efflux to the lipid-free apoprotein (Figures 1 and 7) and binding to the cell surface (Figure 5) in macrophage. In contrast the rate of cholesterol efflux in fibroblasts is lower (Figure 4) and supported equally by apoA-I and the C-terminal deletion mutant. In agreement, Gillotte et al. (*45*) reported similar levels of cholesterol efflux to apoA-I and to a deletion mutant lacking residues 190−243 of the C-terminal domain in human fibroblasts. The fact that apoA-I and the C-terminal deletion mutant bind equally to the fibroblast cell surface but at dramatically reduced levels compared to THP-1 macrophages (Figure 5) indicates the importance of cell surface events and cell type specificity in relation to apolipoprotein-mediated cholesterol efflux. In agreement with the literature, the rates of cholesterol efflux are significantly lower to lipid-free apoA-I proteins than to reconstituted lipoproteins containing apoA-I (*18*). The lipoproteins are more efficient due to the important contribution of diffusional efflux to increased cholesterol-binding capabilities of complexes containing phospholipids. Our observations also indicate that the distinction between specific (translocation-dependent) efflux to lipid-free apolipoproteins and diffusional efflux to lipoproteins is somewhat attenuated in cell such as macrophage, where apoA-I becomes significantly lipidated upon incubation (Figure 2). Several mechanisms of cell surface-apoA-I interaction have been proposed. Several groups have suggested a direct interaction between apoA-I and a cell surface receptor (*5*, *46*, *47*). In support of this concept, overexpression of the murine scavenger receptor class B type I (SR-BI) has been shown to markedly enhance the rate of cholesterol efflux in CHO cells (*5*). While differentiation of THP-1 into macrophages appears to down-regulate CLA-I (human homologue of SR-BI) expression in this cell line (*48*), cholesterol loading of these macrophages might lead to an upregulation of SR-BI transcription similar to that observed in rat liver Kupffer cells (*49*). SR-BI ligand binding specificity is not well-defined yet. It has been shown to bind anionic phospholipids and to a variety of lipoproteins (*50*) with varied apolipoprotein composition (*51*). Experiments with synthetic peptides have shown that the C-terminal domain of apoA-I has the highest affinity for phospholipids (*24*, *45*). Removal of this region could decrease the interaction of apoA-I (in HDL) with SR-BI, especially since SR-BI-mediated cholesterol efflux is a function of phospholipid content of the cholesterol acceptor (*6*). The presence of HB2, another HDL-binding protein, in THP-1 cells may also explain the observed differences between apoA-I and Δ-(187−243) binding. HB2 binds both HDL and lipid-free apoA-I and is upregulated with PMA-induced differentiation to macrophages. Most importantly, HB2 has been shown to bind a region within the carboxy-terminus of apoA-I (*52*). Alternatives other than cell surface receptors should also be considered, such as reduced cell association of the C-terminal deletion mutant to cholesterol enriched domains of the plasma membrane (*45*, *53*). The decreased initial binding of mutant Δ(187−243) to phospholipids is demonstrated in

Figure 3 and by other studies (*16*, *45*, *54*). The C-terminal domain of apoA-I may be essential for acquisition of lipids (i.e., initial binding of phospholipid and cholesterol) or for interaction with a cell surface site that eventually enables apoA-I to interact with cell lipids, thereby promoting lipid efflux. This mechanism is reminiscent of the apoA-I-induced microsolubilization of plasma membrane, which has been suggested to generate preβ-HDL (*55*), and agrees with our DMPC kinetic analysis (Figure 3). The similar and low level of apoA-I and Δ(187−243) binding to fibroblasts, when compared to binding to macrophage, could reflect differences in the number or type of receptors or specific membrane microdomains capable of binding apoA-I. In this regard, apoA-I-mediated efflux from fibroblasts is insensitive to trypsin treatment (*45*), which has led to the suggestion that apoA-I-mediated efflux occurs predominantly by membrane microsolubilization in this cell type. Lipid efflux from mouse peritoneal macrophages, in contrast, is fully inhibited by pretreatment with trypsin (*56*), arguing the importance of apoA-I-protein interactions in these cells.

The observed differences in the rates of cholesterol efflux in macrophages and fibroblasts could also reflect the abilities of the two cell types to accumulate or store excess cholesterol in different pools (*57*−*59*). THP-1 macrophages were shown recently to accumulate and metabolize triglycerides more actively than cholesterol esters compared to other macrophage cell lines (*38*). However, whereas cholesterol ester does not accumulate in fibroblasts, we observed more than a doubling of cholesterol ester upon loading of THP-1 cells. The increased cholesterol content of macrophages following loading may lead to the formation of cholesterol-enriched plasma membrane domains that have a higher affinity for apoA-I (*60*), particularly its C-terminal domain. Caveolae could be such a site where active cholesterol transfer occurs (*61*), and it has been reported that caveolin-1 expression increases in cholesterol-loaded fibroblasts (*62*). Whether macrophages contain caveolae is less certain at present with some groups reporting the absence of caveolin-1 and caveolae (*61*, *63*) and others their presence (*64*, *65*). Other explanations for the higher efflux in macrophage compared to that of fibroblasts (Figures 1 and 4) may involve the presence of the C-terminal binding site on macrophages and the active lipidation of apoA-I that occurs upon incubation with the macrophage (Figure 2).

In some cell types, including human skin fibroblasts (*9*, *10*) and mouse peritoneal macrophages (*11*), translocation of cholesterol to the cell surface is either increased by phorbol esters which activate protein kinase C or inhibited by protein kinase C inhibitors. Differentiation of THP-1 monocytes to macrophage is commonly performed over several days by incubations with PMA or other phorbol esters. In addition, phorbol esters are maintained during the cholesterol-loading step. Long-term incubations with phorbol esters, however, are well-known to downregulate protein kinase C (*38*−*40*), which could adversely affect cholesterol efflux in these cells. Consequently, we explored protocols in which PMA was omitted or maintained during the cholesterol-loading step. Omission of PMA during cholesterol loading increased efflux to both apoA-I and Δ(187−243) relative to loading protocols in the presence of PMA (Figure 7, panels A and B). However, the increased efflux was not inhibited by the inclusion of staurosporine, calphostin C, or H-7, nor was it increased by the addition of PMA to the efflux assay. Therefore, in contrast to the results in fibroblasts and other cell types, protein kinase C may not be directly involved in efflux but could, over longer periods of time, alter the synthesis of proteins involved in cholesterol translocation and/or efflux. In this regard, removal of PMA during cholesterol loading did not affect the binding (4 °C) or cell association (37 °C) of apoA-I or the Δ(187−243) mutant to the THP-1 cell surface (Table 1). It is perhaps not surprising that the mechanisms of efflux vary substantially within cell types. Kritharides and colleagues (*38*) have noted substantial differences in cholesterol metabolism when comparing mouse peritoneal macrophages and THP-1 cells. In support of our findings, these investigators also noted that PMA did not increase cholesterol efflux to apoA-I in THP-1 cells.

The ACAT inhibitor profoundly modifies the metabolism and subcellular distribution of cholesterol and, more importantly, increases the efflux competent pool of cholesterol at the cell surface. Inclusion of the ACAT inhibitor increased cholesterol efflux to both apoA-I and the Δ(187−243) mutant (Figure 7) and increased the proportion of free cholesterol in cells (Table 1). The increased efflux was most evident when PMA was not included during the cholesterol loading. Under these conditions, the levels of efflux to the Δ(187−243) mutant increased to those determined for apoA-I (Figure 7, panels C and D). Inclusion of the ACAT inhibitor significantly increased cell association of both apoA-I and Δ(187−243), which could contribute to the increased efflux observed, however, binding of Δ(187−243) to THP-1 cells remained well below that of apoA-I under all conditions tested (Table 1). Two important observations emerge from these data. First, the carboxy-terminal deletion mutant is perfectly able to promote cholesterol efflux from an available efflux competent pool of cholesterol. Second, the process of efflux to the lipid-free apolipoprotein does not appear to require binding of the recombinant protein to the cell surface and therefore may proceed by a purely diffusional mechanism. The deficiency on the part of this mutant in mediating cholesterol efflux in other circumstances could be due to a deficiency in bringing cholesterol into an efflux-competent pool. This process requires the binding of apoA-I, via the carboxy-terminal domain, to the cell surface. Significantly slower cholesterol ester metabolism has been noted in THP-1 cells compared to other macrophage cell lines, and it has been concluded that the slow hydrolysis of cholesterol esters explains their failure to show markedly enhanced efflux with ACAT inhibition (*38*). Therefore, the major alteration occurring in the absence of PMA may involve a change in the machinery that governs cholesterol mobilization to the cell surface or the concentration of cholesterol in microdomains of the cell surface.

In summary, we have demonstrated that the carboxyl-terminal domain of apoA-I is crucial for its ability to promote cellular lipid efflux from cholesterol-loaded macrophages. The reduced efflux to the C-terminal deletion mutant also appears related to a reduced ability to associate with the macrophage cell surface and to promote the translocation of intracellular cholesterol to an efflux-competent pool. Further studies will investigate the mechanisms by which the omission of PMA during cholesterol loading increases cholesterol efflux to apoA-I in THP-1 cells. It is of interest that a recent report of a mutation introducing a deletion of

Glu_{235} of apoA-I induced hypoalphalipoproteinemia in vivo and reduced cholesterol efflux from macrophages in vitro (*66*).

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REFERENCES

1. Johnson, W. J., Mahlberg, F. H., Rothblat, G. H., and Phillips, M. C. (1991) *Biochim. Biophys. Acta 1085*, 273−298.
2. Fielding, C. J., and Fielding, P. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3911−3914.
3. Czarnecka, H., and Yokoyama, S. (1996) *J. Biol. Chem. 271*, 2023−2028.
4. Davidson, W. S., Gillotte, C. L., Lund-Katz, S., Johnson, W. J., Rothblat, G. H., and Phillips, M. C. (1995) *J. Biol. Chem. 270*, 5882−5890.
5. Ji, Y., Jian, B., Wang, N., Sun, Y., Moya, M. D., Phillips, M. C., Rothblat, G. H., Swaney, J. B., and Tall, A. R. (1997) *J. Biol. Chem. 272*, 20982−20985.
6. Jian, B., de la Liera Moya, Yong, J., Wang, N., Phillips, M. C., Swaney, J. B., Tall, A. R., and Rothblat, G. H. (1998) *J. Biol. Chem. 273*, 5599−5606.
7. Oram, J. F., and Yokoyama, S. (1996) *J. Lipid Res. 37*, 2473−2491.
8. Yokoyama, S. (1998) *Biochim. Biophys. Acta 1392*, 1−15.
9. Mendez, A. J., Oram, J. F., and Bierman, E. L. (1991) *J. Biol. Chem. 266*, 10104−10111.
10. Garver, W. S., Deeg, M. A., Bowen, R. F., Culala, M. M., Bierman, E. L., and Oram, J. F. (1997) *Arterioscler. Thromb. Vasc. Biol. 17*, 2698−2706.
11. Li, Q., Tsujita, M., and Yokoyama, S. (1997) *Biochemistry 36*, 12045−12052.
12. Hokland, B. M., Slotte, J. P., Bierman, E. L., and Oram, J. F. (1993) *J. Biol. Chem. 268*, 25343−25349.
13. Li, W. H., Tanimura, M., Luo, C. C., Datta, S., and Chan, L. (1988) *J. Lipid Res. 29*, 245−271.
14. Segrest, J. P., Jones, M. K., De Loof, H., Brouillette, C. G., Venkatachalapathi, Y. V., and Anantharamaiah, G. M. (1992) *J. Lipid Res. 33*, 141−166.
15. Frank, P. G., Bergeron, J., Emmanuel, F., Lavigne, J. P., Sparks, D. L., Denèfle, P., Rassart, E., and Marcel, Y. L. (1997) *Biochemistry 36*, 1798−1806.
16. Holvoet, P., Zhao, Z. A., Vanloo, B., Vos, R., Deridder, E., Dhoest, A., Taveirne, J., Brouwers, E., Demarsin, E., Engelborghs, Y., Rosseneu, M., Collen, D., and Brasseur, R. (1995) *Biochemistry 34*, 13334−13342.
17. Schmidt, H. H., Remaley, A. T., Stonik, J. A., Ronan, R., Wellmann, A., Thomas, F., Zech, L. A., Brewer, H. B., Jr., and Hoeg, J. M. (1995) *J. Biol. Chem. 270*, 5469−5475.
18. Frank, P. G., N'Guyen, D., Franklin, V., Neville, T., Desforges, M., Rassart, E., Sparks, D. L., and Marcel, Y. L. (1998) *Biochemistry 37*, 13902−13909.
19. Brouillette, C. G., Jones, J. L., Ng, T. C., Kercret, H., Chung, B. H., and Segrest, J. P. (1984) *Biochemistry 23*, 359−367.
20. Fielding, P. E., Kawano, M., Catapano, A. L., Zoppo, A., Marcovina, S., and Fielding, C. J. (1994) *Biochemistry 33*, 6981−6985.
21. Banka, C. L., Black, A. S., and Curtiss, L. K. (1994) *J. Biol. Chem. 269*, 10288−10297.
22. Luchoomun, J., Theret, N., Clavey, V., Duchateau, P., Rosseneu, M., Brasseur, R., Denefle, P., Fruchart, J. C., and Castro, G. R. (1994) *Biochim. Biophys. Acta 1212*, 319−326.
23. Sviridov, D., Pyle, L., and Fidge, N. (1996) *Biochemistry 35*, 189−196.
24. Palgunachari, M. N., Mishra, V. K., Lund-Katz, S., Phillips, M. C., Adeyeye, S. O., Alluri, S., Anantharamaiah, G. M., and Segrest, J. P. (1996) *Arterioscler. Thromb. Vasc. Biol. 16*, 328−338.
25. Sviridov, D., Pyle, L. E., and Fidge, N. (1996) *J. Biol. Chem. 271*, 33277−33283.
26. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
27. Bergeron, J., Frank, P. G., Emmanuel, F., Latta, M., Zhao, Y. W., Sparks, D. L., Rassart, E., Denèfle, P., and Marcel, Y. L. (1997) *Biochim. Biophys. Acta 1344*, 139−152.
28. Arbogast, L. Y., Rothblat, G. H., Leslie, M. H., and Cooper, R. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3680−3684.
29. Radeau, T., Lau, P., Robb, M., McDonnell, M., Ailhaud, G., and McPherson, R. (1995) *J. Lipid Res. 36*, 2552−2561.
30. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. 37*, 911−917.
31. Calabresi, L., Meng, Q.-H., Castro, G. R., and Marcel, Y. L. (1993) *Biochemistry 32*, 6477−6484.
32. Basu, S. K., Goldstein, J. L., Anderson, G. W., and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3178−3182.
33. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206−210.
34. Marcel, Y. L., Jewer, D., Vezina, C., Milthorp, P., and Weech, P. K. (1987) *J. Lipid Res. 28*, 768−777.
35. Marcel, Y. L., Provost, P. R., Koa, H., Raffai, E., Dac, N. V., Fruchart, J. C., and Rassart, E. (1991) *J. Biol. Chem. 266*, 3644−3653.
36. Johnson, W. J., Kilsdonk, E. P., Van Tol, A., Phillips, M. C., and Rothblat, G. H. (1991) *J. Lipid Res. 32*, 1993−2000.
37. Bielicki, J. K., Johnson, W. J., Weinberg, R. B., Glick, J. M., and Rothblat, G. H. (1992) *J. Lipid Res. 33*, 1699−1709.
38. Kritharides, L., Christian, A., Stoudt, G., Morel, D., and Rothblat, G. H. (1998) *Arterioscler. Thromb. Vasc. Biol. 18*, 1589−1599.
39. Goode, N. T., and Hart, I. R. (1990) *J. Cell. Physiol. 142*, 480−487.
40. Gwilliam, D. J., Jones, P. M., Persaud, S. J., and Howell, S. L. (1993) *Acta Diabetol. 30*, 99−104.
41. Virdee, K., Brown, B. L., and Dobson, P. R. (1994) *Biochim. Biophys. Acta 1224*, 489−494.
42. Von Eckardstein, A. (1996) *Curr. Opin. Lipidol. 7*, 308−319.
43. Mendez, A. J. (1997) *J. Lipid Res. 38*, 1807−1821.
44. Brouillette, C. G., and Anatharamaiah, G. M. (1995) *Biochim. Biophys. Acta 1256*, 103−129.
45. Gillotte, K., Zaiou, M., Lund-Katz, S., Anantharamaiah, G. M., Holvoet, P., Dhoest, A., Palgunachari, M. N., Segrest, J. P., Weisgraber, K., Rothblat, G. H., and Phillips, M. C. (1999) *J. Biol. Chem. 274*, 2021−2028.
46. Graham, D. L., and Oram, J. F. (1987) *J. Biol. Chem. 262*, 7439−7442.
47. Matsumoto, A., Mitchell, A., Kurata, H., Pyle, L., Kondo, K., Itakura, H., and Fidge, N. (1997) *J. Biol. Chem. 272*, 16778−16782.
48. Murao, K., Terpstra, V., Green, S. R., Kondratenko, N., Steinberg, D., and Quehenberger, O. (1997) *J. Biol. Chem. 272*, 17551−17557.
49. Fluiter, K., van der Westhuijzen, D. R., and van Berkel, T. J. C. (1998) *J. Biol. Chem. 273*, 8434−8438.
50. Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., and Kreiger, M. (1996) *Science 271*, 518−520.
51. Xu, S. Z., Laccotripe, M., Huang, X. W., Rigotti, A., Zannis, V. I., and Kreiger, M. (1997) *J. Lipid Res. 38*, 1289−1298)
52. Morrison, J., Fidge, N. H., and Tozuka, M. (1991) *J. Biol. Chem. 266*, 18780−18785.
53. Rothblat, G. H., Mahlberg, F. H., Johnson, W. J., and Phillips, M. C. (1992) *J. Lipid Res. 33*, 1091−1097.
54. Minnich, A., Collet, X., Roghani, A., Cladaras, C., Hamilton, R. L., Fielding, C. J., and Zannis, V. I. (1992) *J. Biol. Chem. 267*, 16553−16560.
55. Phillips, M. C., Gillotte, K. L., Haynes, M. P., Johnson, W. J., Lund-Katz, S., and Rothblat, G. H. (1998) *Atherosclerosis 137* (Suppl.) S13−S17.
56. Li, Q., Czarnecka, H., and Yokoyama, S. (1995) *Biochim. Biophys. Acta 1259*, 227−234.
57. Li, Q., Komaba, A., and Yokoyama, S. (1993) *Biochemistry 32*, 4597−4603.

58. Li, Q., and Yokoyama, S. (1995) *J. Biol. Chem. 270*, 26216−26223.
59. Yancey, P. G., Bielicki, J. K., Johnson, W. J., Lund-Katz, S., Palgunachari, M. N., Anantharamaiah, G. M., Segrest, J. P., Phillips, M. C., and Rothblat, G. H. (1995) *Biochemistry 34*, 7955−7965.
60. Saito, H., Miyako, Y., Handa, T., and Miyajima, K. (1997) *J. Lipid Res. 38*, 287−294.
61. Fielding, C. J., and Fielding, P. E. (1997) *J. Lipid Res. 38*, 1503−1521.
62. Fielding, C. J., Bist, A., and Fielding, P. E. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3753−3758.
63. Mendez, A. J., Lin, G., and Oram, J. F. (1997) *Circulation 96*, 485 (Abstr.).
64. Kiss, A. L., and Geuze, H. J. (1997) *Eur. J. Cell Biol. 73*, 19−27.
65. Matveev, S. V., van der Westhuyzen, D. R., and Smart, E. J. (1998) *Circulation 98*, 384 (Abstr.).
66. Han, H., Sasaki, J., Matsunaga, A., Hakamata, H., Huang, W., Ageta, M., Taguchi, T., Koga, T., Kugi, M., Horiuchi, S., and Arakawa, K. (1999) *Arterioscler. Thromb. Vasc. Biol. 19*, 1447−1455.

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