Importance of Central α-Helices of Human Apolipoprotein A-I in the Maturation of High-Density Lipoproteins†

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ABSTRACT: We have studied the role of amphipathic α-helices in the ability of apoA-I to promote cholesterol efflux from human skin fibroblasts and activate lecithin:cholesterol acyltransferase (LCAT). Three apoA-I mutants were designed, each by deletion of a pair of predicted adjacent central α-helices [Δ(100−143), Δ(122−165), Δ(144−186)], and expressed in *Escherichia coli*. This strategy was used to minimize disruption of the predicted secondary structure of the resulting protein. These three central deletion mutants have been previously shown to be expressed as stable folded proteins but to exhibit altered phospholipid-binding properties. When recombined with phospholipids to form homogeneous LpA-I containing equivalent amounts of POPC and tested for their ability to promote diffusional cholesterol efflux from normal [^{3}H]cholesterol-labeled fibroblasts, each mutant and the wild-type recombinant protein (Rec.-apoA-I) promoted cholesterol efflux with very similar rates at all the concentrations tested. These experiments showed that all LpA-I could acquire cellular cholesterol with similar affinity and binding capacity. However, when the cell-incubated LpA-I were incubated with purified LCAT, two mutants, Δ(122−165) and Δ(144−186), appeared incapable of activating the enzyme. To directly determine their ability to activate LCAT, each mutant and the control were recombined with equivalent amounts of cholesterol and phospholipid and incubated with the purified enzyme. The results show that whereas deletion of residues 100−143 has little effect on LCAT activation, deletion of residues 122−165 or 144−186 results in an inability of the mutants to promote cholesterol esterification. In conclusion, our results show that no specific sequence in the central domain of apoA-I is required for efficient diffusional cholesterol efflux from normal fibroblasts; however, residues 144−186 appear critical for optimum LCAT activation and cholesteryl ester accumulation. Since deletion of residues 144−186 also perturbs phospholipid association and prevents the formation of large LpA-I particles [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], the data show that this pair of α-helices plays an important role in the maturation of HDL. Sequence analysis of these apoA-I helices further identifies specific residues that appear essential to this activity.

The importance of high-density lipoprotein (HDL)[1] in the protection against coronary artery disease (*1*) has been attributed to its role in the reverse cholesterol transport pathway, which allows the removal of excess cholesterol from peripheral cells and its transport to the liver for degradation or recycling. The exchangeable apolipoprotein A-I (apoA-I), the most abundant protein of HDL, is central to this process, specifically in the initial step of cholesterol efflux from peripheral cells and in the activation of lecithin: cholesterol acyltransferase (LCAT).

Several lines of evidence have directly implicated apoA-I as an initial acceptor of cellular cholesterol. Preβ1-HDL, which contain apoA-I as the only apoprotein, have been shown to be the initial acceptors of cellular cholesterol (*2*). The concentration of LpA-I (apoA-I-containing lipoproteins) is a better indicator of the ability of plasma to promote cholesterol efflux from cells than other subpopulations of HDL such as LpA-I:A-II (apoA-I and apoA-II-containing lipoproteins) (*3*). The displacement of apoA-I from HDL by apoA-II reduces its ability to promote cholesterol efflux from cells (*4*).

Initial work from Glomset (*5*) suggested that LCAT might play an important role in reverse cholesterol transport. Cholesterol esterification appears to promote the flux of cholesterol through the HDL pool by stimulating the forma-

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[1] Abbreviations: apoA-I, apolipoprotein A-I; CE, cholesteryl ester; DMS, dimethyl suberimidate; FBS, fetal bovine serum; GGE, gradient gel electrophoresis; HDL, high-density lipoprotein; LpA-I, apoA-I-containing lipoproteins; POPC, 1-palmitoyl-2-oleylphosphatidylcholine; Rec.-apoA-I, recombinant wild-type apoA-I expressed in *E. coli* with an N-terminal extension of 11 residues.

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tion of cholesterol esters that are transferred to apoB-containing lipoproteins by CETP, and then cleared via the LDL-receptor pathway in the liver (*6*). LCAT activation was previously shown to be affected by HDL lipid composition (*7*−*9*) and apoA-I conformation (*9*−*11*).

In the present work, we have analyzed the role of the central domain of apoA-I both in diffusional cholesterol efflux from [^{3}H]cholesterol-labeled fibroblasts and in LCAT activation. To this end, three apoA-I mutants were produced, each by deletion of two consecutive α-helices, Δ(100−143), Δ(122−165), and Δ(144−186). This strategy was selected to minimize the disruption of the predicted secondary structure and to conserve the periodicity of the protein. These mutant apoA-I molecules were previously shown to retain their ability to form lipoprotein particles, even though two of the mutants, Δ(122−165) and Δ(144−186), showed reduced kinetics of association with phospholipids (*12*).

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl-2-oleyl phosphatidylcholine (POPC) and cholesterol were obtained from Avanti Polar Lipids (Birmingham, AL). [1α,2α-^{3}H]Cholesterol was purchased from DuPont NEN (Boston, MA). PMA was obtained from Sigma. All other reagents were analytical grade. Human skin fibroblasts (GM00038B) were obtained from the Coriell Institute for medical research (Camden, NJ).

Production of the Mutant Proteins. Wild-type apoA-I with an N-terminal extension, Met-Arg-Gly-Ser-(His)$_6$-Met (Rec.-apoA-I), was expressed in a bacterial system as previously described. Three mutants, Δ(100−143), Δ(122−165), and Δ(144−186), were produced as previously described (*12*). After purification on nitriloacetic acid agarose (NTA, Qiagen), the purified samples were dialyzed against 5 mM NH_4HCO_3, 1 mM EDTA, 0.02% NaN_3 and lyophilized. Proteins were stored at −20 °C.

Preparation and Characterization of Reconstituted Lipoproteins. Reconstituted discoidal lipoproteins were produced using the cholate dispersion/Biobeads removal method as described by Sparks et al. (*13*). The initial POPC/A-I molar ratio used for all proteins was 25/1 (cholesterol was also added for LCAT activation studies so that the initial POPC/Chol/protein molar ratio was 35/5/1). All complexes were reisolated by gel filtration on a Superdex 200 column (Pharmacia). The final composition of the complexes was determined with phospholipid/cholesterol-specific enzymatic kits (Boehringer Mannheim) and Lowry assays using the modified method of Markwell et al. (*14*). The number of apoA-I molecules per complex was determined by cross-linking, using DMS as a cross-linker (*15*). Reconstituted LpA-I were analyzed on 0.5% agarose gels and nondenaturing polyacrylamide gradient gels to determine their surface charge, homogeneity, and size.

Cell Culture and Cholesterol Efflux. Human skin fibroblasts were cultured in a CO_2 incubator at 37 °C as previously described (*16*). They were maintained between passages 15 and 25 in DMEM low-glucose, 10% FBS, 4 mM glutamine, and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin). For the efflux experiments, cells were grown in 24-well plates (seeded at 2.75×10^4 cells per well in 500 μL of media) for 48 h. After two washes with PBS containing 0.2% fatty acid-free BSA (Sigma) and once with PBS only, cells were labeled with 20 μCi/mL [1α,2α-^{3}H]-cholesterol in DMEM (with supplements) containing 5% FBS for 48 h. After labeling, cells were incubated with DMEM (with supplements) containing 1 mg/mL fatty acid-free BSA for 24 h. The efflux experiment was started by washing the cells twice with DMEM, 0.2% BSA, and once with DMEM alone. Appropriate concentrations of lipoprotein complexes in media were added to each well, and the experiment was started. Aliquots were taken at different times and treated as previously described (*16*). At the end of the experiment, cells were solubilized in 1 N NaOH, and protein and radioactivity were determined. Results were expressed as the percentage of labeled cholesterol removed from the cells as a function of time.

Table 1: Properties of the Discoidal Lp2A-I Used in the Efflux with [^{3}H]Cholesterol-Labeled Fibroblasts

	protein	initial composition POPC/Chol/apoA-I (mol/mol/mol)	final composition POPC/Chol/A-I (mol/mol)
series 1	Rec.-apoA-I[a]	25/0/1	30/0/1
	Δ(100−143)[a]	25/0/1	27/0/1
	Δ(122−165)[a]	25/0/1	28/0/1
	Δ(144−186)[a]	25/0/1	29/0/1
series 2	Rec.-apoA-I[b]	35/5/1	43/1/1
	Δ(100−143)[b]	35/5/1	34/1/1
	Δ(122−165)[b]	35/5/1	26/1/1
	Δ(144−186)[b]	35/5/1	30/1/1

[a] Values represent the average of three different preparations (POPC ± 8). These complexes were used in all cholesterol efflux experiments (including the preparation LpA-I containing cell-derived [^{3}H]cholesterol used for LCAT assays described in Figure 3). [b] Values represent the average of two different preparations [POPC ± 15, 3, 9, and 3 for Rec.-apoA-I, Δ(100−143), Δ(122−165), Δ(144−186), respectively; Chol ± 1 for all apoproteins]. These complexes were used in the LCAT activation assays described in Figure 4.

Lecithin:Cholesterol Acyltransferase Assay. LCAT was purified and cholesterol esterification experiments were conducted as previously described (*10*). In these assays, two types of substrate were used. [1α,2α-^{3}H]Cholesterol-labeled LpA-I prepared by incubation of LpA-I (Table 1, series 1) with [1α,2α-^{3}H]cholesterol-labeled fibroblasts were reisolated by gel filtration on Superdex 200. A second series of [1α,2α-^{3}H]cholesterol-labeled LpA-I were prepared by the cholate dispersion/Biobeads removal methods with POPC and cholesterol. LpA-I prepared with [1α,2α-^{3}H]cholesterol and either Rec.-apoA-I or the mutant proteins were tested for their ability to stimulate the LCAT reaction. The LCAT reaction mixture consisted of varying amounts of Lp2A-I, 1.5 mg of fatty acid-free BSA, 5 mM β-mercaptoethanol, and reaction buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1 mM NaN_3, pH 8.0) to 450 μL final volume. Conditions for assay were generally as described before (*10*). Under these conditions, initial rates were estimated with minimal substrate conversion.

RESULTS

Preparation of Reconstituted Lp2A-I with Rec.-ApoA-I and the Mutant Proteins. To analyze the importance of apoA-I domains in diffusional efflux, we prepared reconstituted lipoproteins with very similar phospholipid to protein molar ratio. This experimental design eliminates compositional effects on efflux due to differing lipid content, an interpreta-

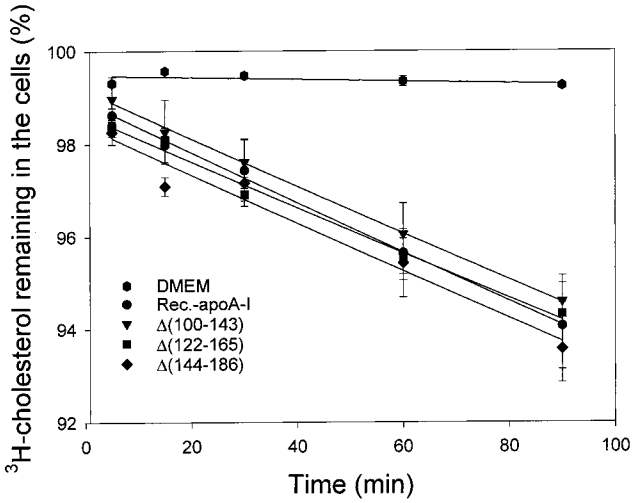

FIGURE 1: Cholesterol efflux from normal [^{3}H]cholesterol-labeled fibroblasts in the presence of Lp2A-I complexes. All complexes were incubated at the same concentration of apoA-I protein (1.70 μM). Aliquots of media were removed at different times of incubation and counted. The efflux was expressed as the percent of [^{3}H]cholesterol removed from the cells at different times of incubation. Experiments were performed in triplicate and are representative of three independent assays.

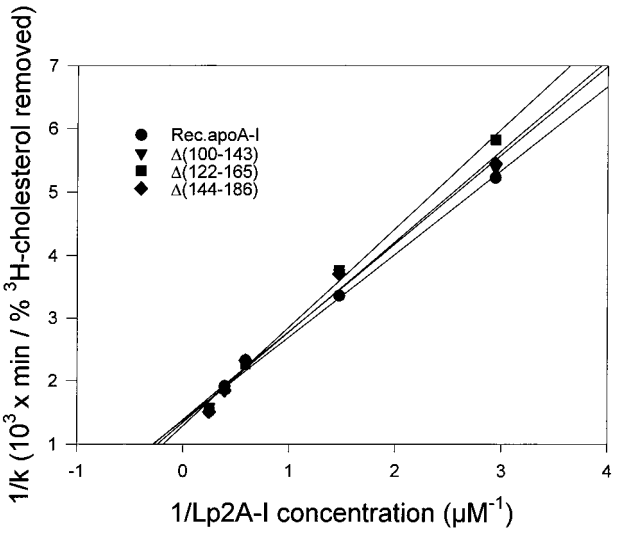

FIGURE 2: Double-reciprocal plot of the rate of cholesterol efflux versus the Lp2A-I concentration used for each protein. Efflux curves at every concentration were fitted with a monoexponential model [$y = A \exp(-kx) + B$], and the reciprocal of the rate (k) of efflux was plotted against the reciprocal of the protein concentration.

tive concern with other studies (*17*, *18*). Stable, homogeneous Lp2A-I were therefore produced using the cholate dispersion/Biobeads removal method (*13*). For each recombinant protein, we utilized the optimum POPC/apoA-I molar ratio of 25/1, which gives rise to completely homogeneous preparations of Lp2A-I for all recombinant proteins, including Rec.-apoA-I, as we have previously shown (*12*). Under these conditions, Rec.-apoA-I formed larger complexes as compared to the mutant proteins. After reisolation by gel filtration, the final composition of each Lp2A-I was essentially the same (Table 1, series 1). For LCAT studies, proteins were recombined with POPC and cholesterol (including [^{3}H]cholesterol) as described above. Reisolated complexes contained an average of one molecule of cholesterol and a comparable phospholipid content per apoprotein (Table 1, series 2). In these complexes, the recovery of cholesterol was low as compared to other studies, apparently due to the low phospholipid content (*10*). Electron microscopy showed that all complexes appear as rouleaux of stacked disks, indicative of a discoidal structure (data not shown).

Ability of ApoA-I Mutants To Promote Cholesterol Efflux from Fibroblasts. The ability of Rec.-apoA-I and the three central deletion mutants to promote cholesterol efflux from human skin fibroblasts was first tested at the same molar concentration (1.70 μM). As shown in Figure 1, the efflux measured between 5 and 90 min to the different LpA-I was linear and essentially identical. The effect of the acceptor concentration on efflux was determined using LpA-I concentrations varying between 0 and 4 μM. The rate of efflux was plotted as a function of LpA-I concentration with the efflux curves fitted using a single-compartment model as described by others (*19*). When the reciprocal of the rates of efflux (k) was plotted against the reciprocal of the concentrations of each LpA-I used (Figure 2), linear regressions demonstrated a correlation coefficient of 0.99. The plots obtained with Rec.-apoA-I and each of the mutant proteins had very similar slope and intercept, suggesting that all complexes had very similar "affinity" (K_d) and "binding capacity" for cellular cholesterol (B_{max}) (Table 2).

Table 2: Kinetic Parameters of Cholesterol Efflux to Lp2A-I Complexes Formed with the Different Proteins

protein in complex	B_{max} (10^{-4} × % of [^{3}H]cholesterol removed/min)[a]	K_d (μM)[a]
Rec.-apoA-I	7.26	0.96
Δ(100−143)	7.19	1.01
Δ(122−165)	7.73	1.21
Δ(144−186)	7.42	1.06

[a] K_d and B_{max} were estimated after determination of the x and y intercepts of a linear regression of the double-reciprocal plots presented in Figure 2.

Reaction of Lecithin:Cholesterol Acyltransferase with LpA-I Labeled with Cell-Derived [^{3}H]Cholesterol. To determine if LpA-I containing cell-derived [^{3}H]cholesterol could efficiently activate LCAT, reconstituted LpA-I (Table 1) were incubated with [^{3}H]cholesterol-labeled fibroblasts for 2 h. Cell-incubated LpA-I were repurified by gel filtration on Superdex 200 and then characterized for size and composition. No significant change in size and composition was observed after incubation with cells, other than enrichment in cholesterol (~0.8 μg of FC/mmol of protein). Assays were then performed to characterize their ability to activate LCAT (Figure 3). Rec.-ApoA-I and Δ(100−143) had very similar ability to promote cholesterol esterification whereas Δ(122−165) and Δ(144−186) could only activate LCAT at a level of ~10% of the control.

Activation of LCAT by ApoA-I Mutants. Reconstituted LpA-I were prepared with [^{3}H]cholesterol (composition shown in Table 1, series 2) and were then incubated with purified LCAT. Saturable substrate curves were obtained for all LpA-I particles (Figure 4). These plots indicate that Rec.-apoA-I and Δ(100−143) have a very similar ability to activate LCAT whereas Δ(122−165) and Δ(144−186) show markedly reduced activation properties. Double-reciprocal plots were derived from these graphs (Figure 4, inset), and appK_m and appV_{max} values were estimated (Table 3) and

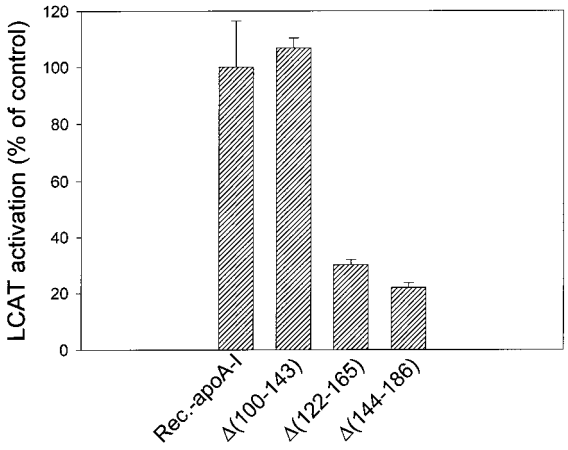

FIGURE 3: Reaction of LCAT with LpA-I reisolated after incubation with [^{3}H]cholesterol-labeled fibroblasts. LpA-I were prepared as indicated in the cholesterol efflux experiments (composition indicated in Table 1, series 1). After a 2 h incubation with [^{3}H]-cholesterol-labeled fibroblasts, LpA-I were reisolated by gel filtration on Superdex 200. After analysis of the purified LpA-I, they were used in an LCAT activation experiment as described under Experimental Procedures. Percentage of LCAT activation (as compared to control Rec.-apoA-I) is indicated at a concentration of 1.21 μM for all proteins.

shown to be similar to previously reported values for similar discoidal Lp2A-I (*10*). Kinetic values are consistent with the curves shown in Figure 3 and indicate markedly reduced rates of cholesterol esterification (appV_{max} and V_{max}/K_m) and increased appK_m for both Δ(122−165) and Δ(144−186) whereas Δ(100−143) was hardly affected by the deletion of residues 100−143.

Table 3: LCAT Activation Properties of ApoA-I and Central Deletion Mutants

protein	K_m (μM apoA-I)[a]	V_{max} (nmol of CE/h)[a]	V_{max}/K_m[a]
Rec.-apoA-I	0.91 (0.10)	1.05 (0.04)	1.15
Δ(100−143)	1.03 (0.71)	0.76 (0.19)	0.74
Δ(122−165)	1.33 (0.30)	0.20 (0.03)	0.15
Δ(144−186)	2.77 (0.91)	0.27 (0.06)	0.10

[a] Estimated values (±STDV) are representative of three separate experiments.

DISCUSSION

In this study, we have examined the ability of LpA-I formed with central deletion mutants of apoA-I to promote cholesterol efflux from normal fibroblasts. This efflux is thought to be mediated through a passive aqueous diffusion process and is dependent on the lipoprotein properties (*20*). Previous studies have demonstrated the importance of LpA-I-associated phospholipid in diffusional efflux (*21*, *22*). The cholesterol content of lipoproteins has also been shown to influence efflux by modifying the cholesterol gradient between cells and lipoproteins in the medium (*23*, *24*). To eliminate compositional effects, we have used homogeneous Lp2A-I complexes with similar POPC/A-I ratios (Table 1, series 1). Despite changes in the apoprotein stability (*12*), apoA-I mutant-containing lipoproteins have maintained their ability to interact with and retain cellular cholesterol. Other studies, in which efflux was determined on the basis of the phospholipid content of the LpA-I, have also reported that the deletion of selected domains of apoA-I has no effect on

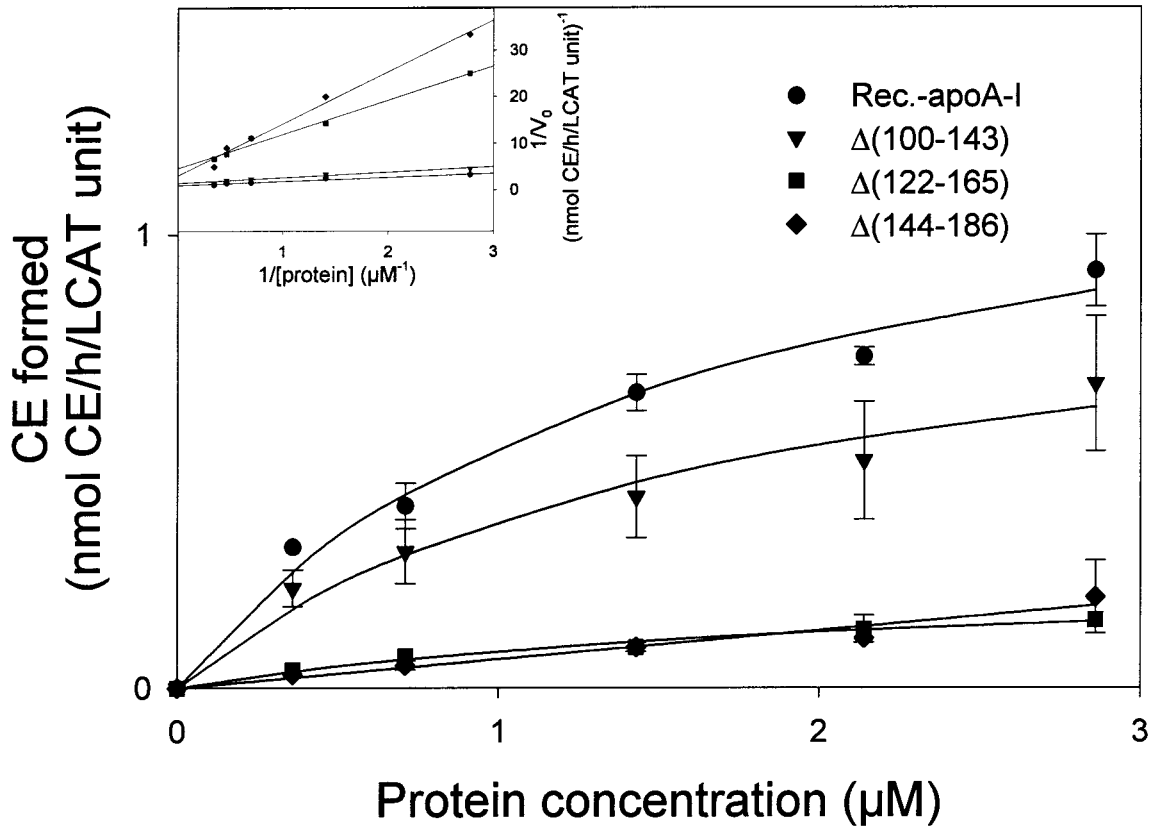

FIGURE 4: Effect of apoA-I central domain deletions on LCAT activation. Experiments were performed with LpA-I (composition indicated in Table 1, series 2) as described under Experimental Procedures. Inset: reciprocals of initial velocities are plotted against reciprocal of protein concentrations.

Table 4: Effect of ApoA-I Mutation or Neutralizing Antibodies on Cellular Cholesterol Efflux

domain examined	cell type	basis of comparison[a]	results[b] (% control)	reference
		Mutagenesis Studies		
44−126	L-fibroblasts	phospholipid	77 (NS)	*(17)*
139−170	L-fibroblasts	phospholipid	95 (NS)	*(17)*
190−243	L-fibroblasts	phospholipid	87 (NS)	*(17)*
223−243	HepG2 cells	phospholipid	∼80−90 (NS)	*(18)*
		particle number	∼90 (NS)	*(18)*
151−243	HepG2 cells	phospholipid	∼70 (S)	*(18)*
		particle number	∼ 100 (NS)	*(18)*
136−243	HepG2 cells	phospholipid	∼65 (S)	*(18)*
		particle number	130 (S)	*(18)*
		Antibody Inhibition Studies		
74−105	THP-1 monocytes	NA	<50 (S)	*(27)*
96−111	THP-1 monocytes	NA	<50 (S)	*(27)*
137−144	human skin fibroblasts	NA	∼60 (S)	*(30)*
28−82	Ob1771 adipocytes	NA	∼75 (S)	*(28)*
149−186	Ob1771 adipocytes	NA	∼75 (S)	*(28)*
140−147	HepG2 cells	NA	∼60 (S)	*(29)*
149−150	HepG2 cells	NA	∼60 (S)	*(29)*

[a] For mutagenesis studies. Apo-A-I-containing lipoproteins were compared based on phospholipid concentration or particle number. NA: Not applicable. [b] S: Efflux significantly different from that of control. NS: Efflux not different from control.

diffusional efflux of cholesterol from mouse L-cell fibroblasts (*17*) or from HepG2 cells (*18*) (Table 4). It should be noted, however, that in the study of Gillotte et al. (*17*), reconstituted lipoproteins that were compared differed in their lipid content, and contained between two and four apoA-I molecules per complex. Likewise, in the study of Sviridov et al. (*18*), evaluation of apoA-I mutants for their relative abilities to promote cholesterol efflux from HepG2 cells was confounded by the comparison of LpA-I that differed in lipid and apolipoprotein composition. As both the phospholipid and cholesterol contents of LpA-I can influence the ability to mediate cellular cholesterol efflux, it is difficult, in these two studies, to dissociate the effects that can be specifically attributed to deletions in apoA-I primary structure from those that are due to the lipid composition of the LpA-I particles. Furthermore, in each study, very different types of mutations have been examined. The three mutants that were evaluated by Gillotte et al. (*17*) have deletions that are distributed throughout apoA-I primary structure (residues 44−126, 139−170, and 190−243, respectively) whereas Svirodov et al. (*18*) have used a series of carboxy-terminally truncated apoA-I variants (deletion of residues 222−243, 210−243, 150−243, and 135−243, respectively). However, despite these interpretative concerns, our data appear consistent with the conclusion derived from these two studies; the deletion of any helices within apoA-I has little effect on the ability of the lipidated protein to promote cellular diffusional cholesterol efflux.

Several studies using mAbs against apoA-I have implicated the central region of apoA-I as being important in cholesterol efflux (summarized in Table 4). Binding of mAbs to apoA-I has, however, been shown to induce conformational changes in apoA-I that can affect its ability to interact with lipids (*25*). In addition, amino acid residues that are widely separated within the primary structure of a protein may be close together in the native protein. Thus, the ability of an anti-apoA-I mAb to block LpA-I-mediated cellular cholesterol efflux may reflect steric hindrance rather than the proximity in apoA-I primary structure of the corresponding epitope and an apoA-I functional domain. Indeed, we have reported in an earlier study with discoidal Lp2A-I that the mAb 3G10 (epitope 98−121) could compete with mAb 4A12 (epitope 173−205) for binding to apoA-I (*26*). This observation is of interest since mAbs binding close to the epitope recognized by 3G10 have been shown to affect cellular cholesterol efflux (*27*−*30*). Therefore, the observed effects of anti-apoA-I mAbs on cholesterol efflux may not be related to the function of specific domains in the apoA-I molecule, but may be due to secondary indirect effects resulting from the mAb−apoA-I interaction.

The K_d values of Rec.-apoA-I and other mutants in Lp2A-I for cellular cholesterol with normal fibroblasts are in the range of 1 μM (Table 2). Lower values were reported for lipid-free apoA-I in the case of cholesterol-loaded fibroblasts (0.04 μM), in contrast to a K_d of 1.6 μM obtained for HDL. Thus, our reconstituted Lp2A-I and HDL interact similarly with cellular cholesterol (*31*). In our experiments, the calculated K_d values are slightly lower than the dissociation constant of apoA-I for lipid (*32*). This indicates that, under these conditions, the efflux to Lp2A-I reflects association of apoA-I with directly accessible membrane lipids, with little or no contribution of intracellular pools. Therefore, central deletion mutants identified in this study can still interact properly with cholesterol from the plasma membrane of normal [^{3}H]cholesterol-labeled fibroblasts.

Several groups have attempted to determine the structural requirements for efficient activation of LCAT by apoA-I. We have summarized these complex and, in some cases, contradictory results in Table 5. Anti-apoA-I mAbs have been tested for their ability to inhibit the interaction between enzyme and substrate. These studies identified a region of apoA-I composed of residues 95−186 that may participate in LCAT activation. As discussed above and elsewhere (*33*), anti-apoA-I mAbs could potentially modulate LCAT activation by several different mechanisms. ApoA-I deletion mutants have also been used to identify domains of apoA-I that are responsible for activation of LCAT. Some of the earlier studies identified not only the same large central domain but also the C-terminal domain of apoA-I as being important for LCAT activation. However, many studies used vesicles incubated with lipid-free apoA-I mutants as substrates. With this approach, a decreased LCAT reaction may

Table 5: Effect of ApoA-I Mutations or Neutralizing Antibodies on LCAT Activation

technique	domain examined	substrate	results[a] (% control)	reference
mutagenesis	146−186	reconstituted LpA-I	90 (NS)	(52)
	113−124	unilamellar vesicle	47 (S)	(40)
	148−186	unilamellar vesicle	0.5 (S)	(40)
	Pro_{99}→His	unilamellar vesicle	93 (NS)	(40)
	Pro_{121}→His	unilamellar vesicle	77 (NS)	(40)
	99−120	unilamellar vesicle	15 (S)	(41)
	121−142	unilamellar vesicle	25 (S)	(41)
	143−164	unilamellar vesicle	2 (S)	(41)
	165−186	unilamellar vesicle	2 (S)	(41)
	44−126	reconstituted LpA-I	45 (S)	(34)
	139−170	reconstituted LpA-I	9 (S)	(34)
	190−243	reconstituted LpA-I	11 (S)	(34)
	123−166	reconstituted LpA-I	5 (S)	(42)
	209−243	reconstituted LpA-I	58 (S)	(53)
	1−43	reconstituted LpA-I	43 (S)	(54)
	143−164[b]	reconstituted LpA-I	19 (S)	(43)
	143−164[c]	reconstituted LpA-I	20 (S)	(44)
proteolysis	193−243	reconstituted LpA-I	100 (NS)	(55)
antibody inhibition	95−121	reconstituted LpA-I	20 (S)	(56)
	96−122	reconstituted LpA-I	2−25 (S)	(33)
	135−148	reconstituted LpA-I	10−45 (S)	(33)
	149−186	reconstituted LpA-I	10−60 (S)	(33)
	96−174	reconstituted LpA-I	20 (S)	(57)

[a] S: Esterification rates significantly different from that of control. NS: Esterification rates not different from control. [b] Residues 143−164 replaced with residues 220−241. [c] Primary amino acid sequence reversed from its normal orientation (rotation of the hydrophobic face by ∼80°).

reflect decreased lipid-binding properties of apoA-I mutants with C-terminal deletions (*34*, *35*).

To determine the LCAT activation properties of the three central deletion mutants, homogeneous reconstituted LpA-I were prepared with very similar POPC/cholesterol/A-I molar ratio. This ensures that we are primarily studying a protein effect and not secondary effects due to differing lipid composition that may affect apoA-I conformation (*9*). Two of the mutants examined, Δ(122−165) and Δ(144−186), were found to have significantly lower LCAT activation. This would suggest that helix 144−164, which is common to the two deletion mutants, may participate in the activation of LCAT. Importantly, helix 144−164 of apoA-I has been the most conserved of the apoA-I helices during evolution, which would be consistent with this domain having a functional role. As previously hypothesized (*6*, *9*), this helix may interact with residues 151−174 of LCAT and allow access to free cholesterol in discoidal LpA-I. This model of interaction, however, remains to be confirmed. Immunoreactivity and NMR studies suggested a possible involvement of this apoA-I domain in the binding of cholesterol (*26*, *36*).

Interestingly, deletion of residues 100−143 has little effect on the ability of apoA-I to activate LCAT, a finding that is in contrast with the results of others (*37*). In this work, a peptide corresponding to a dimer of the consensus amino acid sequence obtained for the eight 22-mer predicted α-helices of apoA-I gave maximal LCAT activation only when residue 13 of the helix was replaced with a glutamic acid. Since only helices within residues 66−121 contain a Glu at position 13 of each helix (residues 78 and 111 of apoA-I), it was concluded that this region is the LCAT-activating domain. Sequence alignment of this activator peptide with apoA-I helices indicates that region 143−164 has only 27.3% identity and shows that Glu at position 13 of an α-helix is not essential for LCAT activation. Surprisingly, when helix 165−186 is aligned with the activator peptide, an identity of 45.5% is observed (Figure 5). Such

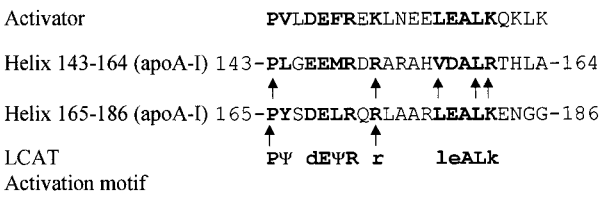

FIGURE 5: Alignement of the LCAT activator peptide with amino acids 143−164 and 165−186 of apoA-I. The residues highlighted "in bold" reflect common or conserved amino acids that may be required for LCAT activation. An LCAT activation motif, which reflects common or conserved residues between the three aligned sequences, is proposed. Lower case letters (d, r, l, e, and k) indicate conserved residues (the preponderant residue is shown), and the symbol Ψ denotes the presence of hydrophobic residues in the three aligned sequences. Mutations of residues identified by arrows have been identified in patients with reduced HDL levels and may result in lower LCAT activity (see text for details).

homology could suggest that helix 165−186 may be important in LCAT activation. We propose that residues of the activator peptide that are identical or homologous to residues of helix 143−164 and 165−186 may be essential for LCAT interaction. Alignment of helices 143−164 and 165−186 and the LCAT activator peptide indeed reveals a common motif, particularly with respect to hydrophobic and charged residues, that may be critical to the activation of LCAT by apoA-I (identified as an LCAT activating domain in Figure 5).

NMR and X-ray crystallography suggest that an interaction between helices 143−164 and 165−186 is unlikely (*38*, *39*). It therefore appears that rather than interacting together, these two helices may facilitate the association of LCAT with LpA-I, possibly through electrostatic interactions between these two helices (charged residues identified) and residues 151−174 of LCAT. Hydrophobic residues identified in Figure 5 could stabilize this interaction with the lipid interface. The N-terminal domain of apoA-I, which has been shown to affect LCAT activation (*33*, *9*) and to interact with the central domain of apoA-I (*26*), may also affect this interaction. In

small discoidal LpA-I, interactions between the N-terminus and central domain of apoA-I may prevent the interaction of LCAT with apoA-I and lipid. In large LpA-I, this access is facilitated since the interaction between these two apoA-I domains is reduced. Other studies from Minnich et al. (*40*), Sorci-Thomas et al. (*41*), Holvoet et al. (*34*), and Dhoest et al. (*42*) have also suggested an important role for residues 144−186 of apoA-I in the activation of LCAT. Sorci-Thomas et al. have recently produced apoA-I variants in which residues 143−164 and 220−241 were exchanged (*43*) or in which the sequence of helix 143−164 was altered so that the hydrophobic face of this domain was rotated by 80° (*44*). Both variants are defective in their ability to activate LCAT. Therefore, helix 143−164 of apoA-I not only appears to be essential for LCAT activation but also may require adjacent domains such as residues 164−186. This is supported by the present study and others (*40*−*42*).

In support of this view, mutations of the essential amino acids shown in Figure 5 have been associated with reduced LCAT activation properties of the resulting variants [Pro_{143}→Arg (*45*), Δ(146−160) (*46*), Leu_{151}→Cys (*47*), Val_{156}→Glu (*48*), Leu_{159}→Arg (*49*), Arg_{160}→Leu (*50*), Arg_{173}→Cys (*51*)]. A recent report from Miettinen et al. showed that a Leu_{159}→Arg substitution in apoA-I results in a 40% reduction of LCAT activation (*49*) and also the absence of large plasma HDL_2 in patients carrying this mutation. Other mutations [Arg_{160}→Leu (*50*); Val_{156}→Glu (*48*)] are associated with reduced HDL-cholesterol levels, HDL size, and LCAT activation for Val_{156}→Glu (not demonstrated for Arg_{160}→Leu but suggested by the authors). Mutations of residues within the domain 144−186 of apoA-I appear to affect both its interaction with lipid and its ability to activate LCAT. The reduced HDL size that is associated with inheritance of these mutant alleles may reflect a reduced ability of the variants to activate LCAT. The region of apoA-I composed of residues 144−186 may help HDL to accumulate CE and form large HDL and could therefore be an important regulator of HDL maturation.

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REFERENCES

1. Gordon, D. J., and Rifkind, B. M. (1989) *N. Engl. J. Med. 321*, 1311−1316.
2. Castro, G. R., and Fielding, C. J. (1988) *Biochemistry 27*, 25−29.
3. De la Llera Moya, M., Atger, V., Paul, J. L., Fournier, N., Moatti, N., Giral, P., Friday, K. E., and Rothblat, G. (1994) *Arterioscler. Thromb. 14*, 1056−1065.
4. Lagrost, L., Dengremont, C., Athias, A., De Geitere, C., Fruchart, J.-C., Lallemant, C., Gambert, P., and Castro, G. (1995) *J. Biol. Chem. 270*, 13004−13009.
5. Glomset, J. A. (1968) *J. Lipid Res. 9*, 155−167.
6. Fielding, C. J., and Fielding, P. E. (1995) *J. Lipid Res. 36*, 211−228.
7. Bolin, D. J., and Jonas, A. (1994) *J. Biol. Chem. 269*, 7429−7434.
8. Rye, K. A., Hime, N. J., and Barter, P. J. (1996) *J. Biol. Chem. 271*, 4243−4250.
9. Sparks, D. L., Frank, P. G., and Neville, T. (1998) *Biochim. Biophys. Acta 1390*, 160−172.
10. Sparks, D. L., Anantharamaiah, G. M., Segrest, J. P., and Phillips, M. C. (1995) *J. Biol. Chem. 270*, 5151−5157.
11. Durbin, D. M., and Jonas, A. (1997) *J. Biol. Chem. 272*, 31333−31339.
12. 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.
13. Sparks, D. L., Lund-Katz, S., and Phillips, M. C. (1992) *J. Biol. Chem. 267*, 25839−25847.
14. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206−210.
15. Swaney, J. B., and O'Brien, K. (1978) *J. Biol. Chem. 253*, 7069−7077.
16. 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.
17. Gillotte, K. L., Davidson, W. S., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (1996) *J. Biol. Chem. 271*, 23792−23798.
18. Sviridov, D., Pyle, L. E., and Fidge, N. (1996) *J. Biol. Chem. 271*, 33277−33283.
19. Mahlberg, F. H., and Rothblat, G. H. (1992) *J. Biol. Chem. 267*, 4541−4550.
20. Johnson, W. J., Mahlberg, F. H., Rothblat, G. H., and Phillips, M. C. (1991) *Biochim. Biophys. Acta 1085*, 273−298.
21. Zhao, Y. W., Sparks, D. L., and Marcel, Y. L. (1996) *J. Biol. Chem. 271*, 25145−25151.
22. Fournier, N., Moya, M. D., Burkey, B. F., Swaney, J. B., Paterniti, J., Jr., Moatti, N., Atger, V., and Rothblat, G. H. (1996) *J. Lipid Res. 37*, 1704−1711.
23. Johnson, W. J., Bamberger, M. J., Latta, R. A., Rapp, P. E., Phillips, M. C., and Rothblat, G. H. (1986) *J. Biol. Chem. 261*, 5766−5776.
24. Johnson, W. J., Mahlberg, F. H., Chacko, G. K., Phillips, M. C., and Rothblat, G. H. (1988) *J. Biol. Chem. 263*, 14099−14106.
25. Meng, Q. H., Bergeron, J., Sparks, D. L., and Marcel, Y. L. (1995) *J. Biol. Chem. 270*, 8588−8596.
26. Bergeron, J., Frank, P. G., Scales, D., Meng, Q. H., Castro, G., and Marcel, Y. L. (1995) *J. Biol. Chem. 270*, 27429−27438.
27. Banka, C. L., Black, A. S., and Curtiss, L. K. (1994) *J. Biol. Chem. 269*, 10288−10297.
28. 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.
29. Sviridov, D., Pyle, L., and Fidge, N. (1996) *Biochemistry 35*, 189−196.
30. Fielding, P. E., Kawano, M., Catapano, A. L., Zoppo, A., Marcovina, S., and Fielding, C. J. (1994) *Biochemistry 33*, 6981−6985.
31. Komaba, A., Li, Q., Hara, H., and Yokoyama, S. (1992) *J. Biol. Chem. 267*, 17560−17566.
32. Tajima, S., Yokoyama, S., and Yamamoto, A. (1983) *J. Biol. Chem. 258*, 10073−10082.
33. Meng, Q. H., Calabresi, L., Fruchart, J. C., and Marcel, Y. L. (1993) *J. Biol. Chem. 268*, 16966−16973.
34. 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.
35. 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.
36. Sparks, D. L., Davidson, W. S., Lund-Katz, S., and Phillips, M. C. (1993) *J. Biol. Chem. 268*, 23250−23257.
37. Anantharamaiah, G. M., Venkatachalapathi, Y. V., Brouillette, C. G., and Segrest, J. P. (1990) *Arteriosclerosis 10*, 95−105.
38. Borhani, D. W., Rogers, D. P., Engler, J. A., and Brouillette, C. G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12291−12296.
39. Wang, G., Sparrow, J. T., and Cushley, R. J. (1997) *Biochemistry 36*, 13657−13666.

40. 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.
41. Sorci-Thomas, M., Kearns, M. W., and Lee, J. P. (1993) *J. Biol. Chem. 268*, 21403−21409.
42. Dhoest, A., Zhao, Z. A., De Geest, B., Deridder, E., Sillen, A., Engelborghs, Y., Collen, D., and Holvoet, P. (1997) *J. Biol. Chem. 272*, 15967−15972.
43. Sorci-Thomas, M. G., Curtiss, L., Parks, J. S., Thomas, M. J., and Kearns, M. W. (1997) *J. Biol. Chem. 272*, 7278−7284.
44. Sorci-Thomas, M. G., Curtiss, L., Parks, J. S., Thomas, M. J., Kearns, M. W., and Landrum, M. (1998) *J. Biol. Chem. 273*, 11776−11782.
45. Utermann, G., Haas, J., Steinmetz, A., Paetzold, R., Rall, S. C. J., Weisgraber, K. H., and Mahley, R. W. (1984) *Eur. J. Biochem. 144*, 325−331.
46. Lindholm, E. M., Bielicki, J. K., Curtiss, L. K., Rubin, E. M., and Forte, T. M. (1998) *Biochemistry 37*, 4863−4868.
47. Bruckert, E., Von Eckardstein, A., Funke, H., Beucler, I., Wiebusch, H., Turpin, G., and Assmann, G. (1997) *Atherosclerosis 128*, 121−128.
48. Huang, W., Sasaki, J., Matsunaga, A., Nanimatsu, H., Moriyama, K., Han, H., Kugi, Koga, T., Yamaguchi, K., and Arakawa, K. (1998) *Arterioscler. Thromb. Vasc. Biol. 18*, 389−396.
49. Miettinen, H. E., Jauhiainen, M., Gylling, H., Ehnholm, S., Palomaki, A., Miettinen, T. A., and Kontula, K. (1997) *Arterioscler. Thromb. Vasc. Biol. 17*, 3021−3032.
50. Leren, T. P., Bakken, K. S., Daum, U., Ose, L., Berg, K., Assmann, G., and Von Eckardstein, A. (1997) *J. Lipid Res. 38*, 121−131.
51. Calabresi, L., Franceschini, G., Burkybile, A., and Jonas, A. (1997) *Biochem. Biophys. Res. Commun. 232*, 345−349.
52. Bruhn, H., and Stoffel, W. (1991) *Biol. Chem. Hoppe Seyler 372*, 225−234.
53. Laccotripe, M., Makrides, S. C., Jonas, A., and Zannis, V. I. (1997) *J. Biol. Chem. 272*, 17511−17522.
54. Rogers, D. P., Brouillette, C. G., Engler, J. A., Tendian, S. W., Roberts, L., Mishra, V. K., Anantharamaiah, G. M., Lund-Katz, S., Phillips, M. C., and Ray, M. J. (1997) *Biochemistry 36*, 288−300.
55. Ji, Y., and Jonas, A. (1995) *J. Biol. Chem. 270*, 11290−11297.
56. Banka, C. L., Bonnet, D. J., Black, A. S., Smith, R. S., and Curtiss, L. K. (1991) *J. Biol. Chem. 266*, 23886−23892.
57. Uboldi, P., Spoladore, M., Fantappiè, S., Marcovina, S., and Catapano, A. L. (1996) *J. Lipid Res. 37*, 2557−2568.

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