The C-Terminal Lipid-Binding Domain of Apolipoprotein E Is a Highly Efficient Mediator of ABCA1-Dependent Cholesterol Efflux that Promotes the Assembly of High-Density Lipoproteins†

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ABSTRACT: This study was undertaken to identify the α-helical domains of human apoE that mediate cellular cholesterol efflux and HDL assembly via ATP-binding cassette transporter A1 (ABCA1). The C-terminal (CT) domain (residues 222−299) of apoE was found to stimulate ABCA1-dependent cholesterol efflux in a manner similar to that of intact apoE2, -E3, and -E4 in studies using J774 macrophages and HeLa cells. The N-terminal (NT) four-helix bundle domain (residues 1−191) was a relatively poor mediator of cholesterol efflux. On a per molecule basis, the CT domain stimulated cholesterol efflux with the same efficiency ($K_m \sim 0.2\ \mu M$) as intact apoA-I and apoE. Gel filtration chromatography of conditioned medium from ABCA1-expressing J774 cells revealed that, like the intact apoE isoforms, the CT domain promoted the assembly of HDL particles with diameters of 8 and 13 nm. Removal of the CT domain abolished the formation of HDL-sized particles, and only larger particles eluting in the void volume were formed. Studies with CT truncation mutants of apoE3 and peptides indicated that hydrophobic helical segments governed the efficiency of cellular cholesterol efflux and that conjoined class A and G amphipathic α-helices were required for optimal efflux activity. Collectively, the data suggest that the CT lipid-binding domain of apoE encompassing amino acids 222−299 is necessary and sufficient for mediating ABCA1 lipid efflux and HDL particle assembly.

In humans, apolipoprotein E (apoE)[1] exists as three major isoforms designated E2, E3, and E4 (*1*). ApoE3 is considered the normal isoform, while apoE2 is associated with type III hyperlipoproteinemia attributed to decreased LDL receptor binding activity and apoE4 with an increased risk of atherosclerosis and Alzheimer's disease (*1*−*5*). ApoE2 and -E3 display a binding preference for HDL, while apoE4 has a preference for VLDL-sized particles (*1*, *6*). At the protein level, the isoforms of apoE differ by a single amino acid substitution. ApoE3 is the most prevalent isoform having a cysteine (C) at position 112 and an arginine (R) at position 158, whereas apoE2 and -E4 are less common having C112 and C158, and R112 and R158, respectively (*1*, *6*, *7*).

Cholesterol loading of macrophages induces apoE production (*8*, *9*), and apoE has been localized with macrophages in human atherosclerotic lesions (*10*, *11*). Macrophage-specific expression of apoE can protect against atherosclerosis in animal models (*12*−*15*). The anti-atherogenic properties of apoE are related, in part, to its ability to facilitate efflux of cholesterol from peripheral cells to HDL and to its role in reverse cholesterol transport, whereby excess cholesterol is delivered to the liver for excretion in feces (*16*−*18*). The presence of exogenous apoE unbound to HDL as well as endogenous, lipid-poor apoE secreted from macrophages can promote cellular cholesterol efflux (*19*−*27*). This process of apolipoprotein-mediated cholesterol efflux results in the assembly of nascent HDL and involves ATP-binding cassette transporter A1 (ABCA1) that is defective in Tangier disease (*28*−*37*). In the brain, ABCA1 is thought to be critical for the lipidation of apoE, where the formation of the apoE−HDL complex plays a vital role in

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[1] Abbreviations: apoE, apolipoprotein E; CT, C-terminal; NT, N-terminal; apoA-I, apolipoprotein A-I; PL, phospholipids; FC, unesterified or free cholesterol; HDL, high-density lipoproteins; ABCA1, ATP-binding cassette transporter A1; aa, amino acid; DMPC, dimyristoylphosphatidylcholine; cpt-cAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; BSA, bovine serum albumin.

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intercellular cholesterol transport and nerve regeneration (*1*, *38*). The absence of ABCA1 activity reduces apoE concentrations in the brain, suggesting a functional interaction between the two proteins in vivo (*39*−*41*).

The domains of apoE that are involved in mediating cholesterol efflux via ABCA1 are not known. Consequently, we have investigated structure−function relationships of apoE related to its role in ABCA1-mediated cholesterol efflux. Recent studies suggest that apoE exhibits a tertiary structure similar to that of apoA-I (*42*−*45*). Both apoA-I and apoE possess an N-terminal four-helix bundle linked to hydrophobic C-terminal α-helices that bind phospholipids strongly (*43*, *45*−*48*). The C-terminal domain of apoA-I, including helices 9 and 10, is thought to be involved in mediating ABCA1-dependent cholesterol efflux (*49*−*52*). This prompted us to hypothesize that the CT lipid-binding domain of apoE may also be important for stimulating cellular cholesterol efflux. We found that the CT lipid-binding domain of apoE, lacking the hinge sequence and NT domain, stimulated ABCA1-dependent cholesterol efflux with high efficiency and promoted the assembly of HDL particles. Our results also suggest that a long span of native CT sequence involving conjoined class A and G helical segments is required to evoke an optimal cholesterol efflux response via ABCA1.

MATERIALS AND METHODS

Expression and Purification of Recombinant Apolipoproteins and the N- and C-Terminal Domain Fragments of ApoE. The following proteins were used in this study: intact apoE2, -E3, and -E4 (residues 1−299), N-terminal (NT) fragments of apoE2, -E3, and -E4 corresponding to amino acids (aa) 1−191, C-terminal (CT) truncation mutants of apoE3 (aa 1−272, 1−260, and 1−250), and CT fragments corresponding to aa 222−299 (10 kDa) and 192−299 (12 kDa) of apoE. The procedures for construction of plasmids, expression of the variant forms of apoE in *Escherichia coli*, and purification of proteins have been described previously (*53*, *54*). The isolated proteins were lyophilized and stored at −20 °C. Lyophilized proteins were routinely dissolved in 6 M guanidine-HCl and 0.1% BME and dialyzed into the appropriate buffer before being used. SDS−PAGE (8 to 25% gels) verified that the recombinant forms of apoE were pure (≥95%). Recombinant full-length apoA-I was used in some experiments for comparative purposes, prepared as described previously (*49*, *55*).

Synthesis and Preparation of Peptides. Peptides were based on native sequences of amino acids as found in the CT domain of apoE. Biosynthesis Inc. (Lewisville, TX) synthesized the peptides with N-terminal acetyl and C-terminal amide groups. Peptides were purified by HPLC and used at a purity of >95%; the mass and composition were verified by GC−mass spectroscopy. Lyophilized peptides were dissolved in PBS (pH 7.4) with 6 M guanidine-HCl and dialyzed extensively against PBS immediately before being used. Peptide concentrations were determined by UV absorption spectroscopy at 280 nm. The ability of peptides to bind phospholipids was assessed in DMPC clearance assays as described previously (*49*).

Cell Culture and Cholesterol Efflux. J774 mouse macrophages (which do not synthesize apoE) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). For efflux experiments, cells were plated on 24- or 12-well culture plates and labeled with [^{3}H]cholesterol (1 μCi/mL) in RPMI-1640 with 1% FBS for 48 h. A cAMP analogue (cpt-cAMP) was added (final concentration of 0.3 mM) to the extracellular medium of some cells to upregulate ABCA1 (*49*). 2-Propanol was used to extract lipids from cells at time zero. Parallel sets of cells were extensively rinsed with serum-free RPMI-1640 medium followed by an extended rinse (2 h) with RPMI-1640 containing 0.2% bovine serum albumin (BSA). The apolipoprotein acceptors were used in lipid-free form and added to cells in serum-free RPMI-1640 medium to initiate cholesterol efflux. The amount of [^{3}H]cholesterol appearing in the medium was expressed as a percentage of the radioactivity initially present in cells at time zero. The background release of [^{3}H]-cholesterol to serum-free medium alone was subtracted from the values obtained with apolipoprotein acceptors.

HeLa cells transfected with ABCA1 cDNA and "mock"-transfected (control) cells were used to assess ABCA1-dependent and -independent mechanisms of cholesterol efflux, respectively, as described previously (*29*). The two cell lines (HeLa+ABCA1 and HeLa−ABCA1) were grown in α-MEM supplemented with 10% FBS and 200 μg/mL hydromycin B and 150 μg/mL G418. Labeling of HeLa cells with [^{3}H]cholesterol and incubations with apolipoprotein acceptors were achieved essentially as described above for J774 cells.

Gel Filtration Chromatography. A calibrated Superdex 200 column was used to assess the formation of HDL particles during cellular lipid efflux to exogenous proteins, as previously described (*56*). Briefly, J774 cells grown to 80−90% confluence were either singly labeled with 3 μCi/mL [^{3}H]cholesterol for 24 h or doubly labeled with 20 μCi/mL [^{3}H]choline chloride and 3 μCi/mL [^{14}C]cholesterol for 48 h. The labeled cells were treated with 0.3 mM cpt-cAMP for 12 h to upregulate the expression of ABCA1 and then exposed to 5 μg/mL apoE for 6 h. In some experiments, apoE was labeled with ^{14}C by reductive methylation (*56*). The efflux medium was filtered through a 0.45 μm filter, loaded (2.5 mL) onto the Superdex 200 column, and eluted with 10 mM Tris-HCl (pH 7.4) at a flow rate of 1 mL/min. The ^{3}H- and ^{14}C-labeled lipid and [^{14}C]apoE radioactivity in 1 mL column fractions was determined by liquid scintillation counting. With doubly labeled cells, lipids were extracted from 1 mL fractions of the medium by the procedure of Bligh and Dyer (*57*), and the radioactivity for [^{3}H]choline phospholipid and [^{14}C]cholesterol was determined. The particle size (hydrodynamic diameter in nanometers) of the various fractions and the apparent molecular mass (in kilodaltons) were determined as described in detail previously (*56*). Cross-linking of apoE was performed as described previously (*58*) to determine the degree of self-association of the protein in the various column fractions. In brief, apoE (1 mg/mL) was dialyzed against phosphate buffer (0.1 M, pH 7.4) and incubated with BS^3 (10 mM) at a volume ratio of 2:1 for 3.5 h at room temperature. The cross-linking reaction was then quenched with ethanolamine (final concentration of 25 mM), and samples were concentrated and examined by SDS−PAGE using standards with known molecular masses.

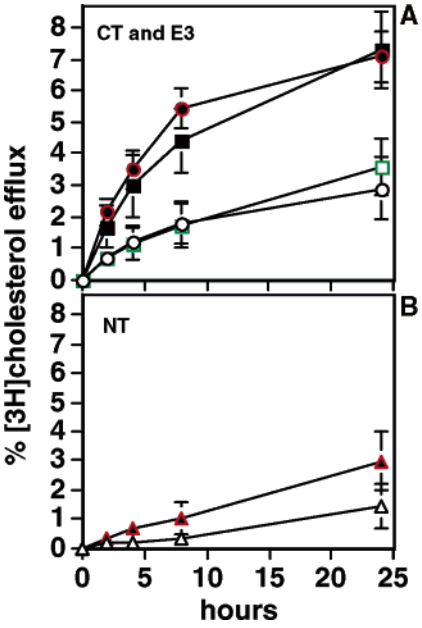

FIGURE 1: Cholesterol efflux activity of N- and C-terminal domain fragments derived from apoE3. J774 macrophages were labeled with [^{3}H]cholesterol and incubated (12 h) with (filled symbols) and without (empty symbols) a cAMP analogue to upregulate ABCA1 as described in Materials and Methods. (A) Kinetics of [^{3}H]-cholesterol efflux in response to the 12 kDa CT domain (circles) and intact apoE3 (squares). (B) Cholesterol efflux obtained with the NT domain (aa 1−191, triangles). Each acceptor was used in a lipid-free form at 50 μg/mL. Values are means ± the standard deviation: CT ($n = 5$), apoE3 ($n = 4$), and NT ($n = 3$). Error bars are smaller than symbols when not seen.

RESULTS

The CT Domain of ApoE Mediates Cholesterol Efflux in an ABCA1-Dependent Manner. Fragments of apoE corresponding to the N- and C-terminal domains were used in this study to identify the portion of the protein involved in mediating cellular cholesterol efflux. Initial experiments were conducted with J774 macrophages treated with and without a cAMP analogue (cpt-cAMP) to modulate ABCA1 protein expression. At high extracellular concentrations, the C-terminal (CT) domain of apoE stimulated cholesterol efflux from cpt-cAMP-treated J774 cells in a manner similar to that of intact apoE3 (Figure 1). Without cpt-cAMP, low levels of cholesterol efflux were observed with both the CT domain and apoE3. These results suggest that the CT domain stimulates cholesterol efflux in an ABCA1-dependent manner. In contrast, the N-terminal (NT) domain (aa 1−191) of apoE3 stimulated cholesterol efflux poorly (Figure 1). The rate of efflux of cholesterol to the NT domain increased slightly with cpt-cAMP treatment of J774 cells, suggesting weak activity toward ABCA1.

To verify that the CT domain of apoE stimulates cholesterol efflux in an ABCA1-dependent manner, experiments were conducted with HeLa cells expressing ABCA1 cDNA and mock-transfected control HeLa cells (Figure 2). The CT domain of apoE behaved like the isoforms of apoE, i.e., E2, E3, and E4, as well as full-length apoA-I in stimulating relatively high levels of efflux of cholesterol from ABCA1-expressing cells and low levels of efflux of cholesterol from control HeLa cells deficient in ABCA1. The NT domain of apoE was a poor acceptor of cellular [^{3}H]cholesterol (0.4 ± 0.03 and 1.6 ± 0.1% efflux/8 h from control and ABCA1-expressing HeLa cells, respectively), as found in studies using J774 macrophages (Figure 1). These data indicate that the CT domain of apoE is dependent on ABCA1 for stimulating cholesterol efflux, like the full-length isoforms of apoE.

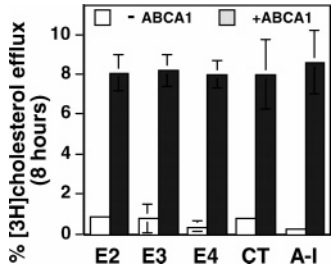

FIGURE 2: Dependence of apolipoproteins on ABCA1 for stimulating cholesterol efflux. Control HeLa cells deficient in ABCA1 and HeLa cells stably transfected with ABCA1 cDNA were labeled with [^{3}H]cholesterol as described in Materials and Methods. The percentage of cellular [^{3}H]cholesterol appearing in the medium after 8 h was determined using the lipid-free form of each acceptor at 25 μg/mL. Values are means ± the standard deviation. E2 refers to intact apoE2 ($n = 7$), E3 to intact apoE3 ($n = 4$), E4 to intact apoE4 ($n = 3$), CT to the 12 kDa fragment of apoE ($n = 3$), and A-I to intact apoA-I ($n = 3$).

The CT Domain of ApoE Is a Highly Efficient Mediator of Cellular Cholesterol Efflux. Figure 3A shows that the CT domain of apoE stimulated efflux of cholesterol from cpt-cAMP-treated J774 macrophages in a concentration-dependent manner. A hyperbolic relationship was obtained between cholesterol efflux and the concentration of the CT domain. The ability of the CT domain to stimulate cholesterol efflux was not dependent on the putative hinge sequence (aa 192−221) that links the C-terminal helices to the NT domain. Two molecular forms of the CT domain with (12 kDa) and without (10 kDa) the hinge sequence possessed the same high capacity and efficiency for stimulating cholesterol efflux, as determined by the detailed concentration dependence studies (Figure 3A) and the apparent K_m values for efflux activity (Table 1). The NT domain of apoE produced only a modest stimulation of cholesterol efflux when its concentration was increased from 1 to 20 μg/mL (Figure 3B). A linear relationship was obtained between cholesterol efflux and the extracellular concentration of the NT domain, which precluded accurate determinations of K_m values. The NT domains derived from apoE2, -E3, and -E4 behaved similarly, stimulating low levels of cholesterol efflux.

The concentration dependence curves showing the ability of intact apoE2, -E3, and -E4 to stimulate cellular cholesterol efflux are presented in Figure 3C. Each of the intact apolipoproteins promoted cholesterol efflux in a similar manner. In addition, the levels of phospholipid efflux were found to be similar between the apoE isoforms and apoA-I (data not shown). The apparent K_m values for stimulating cholesterol efflux were nearly identical for intact apoE3, -E4, and -A-I (Table 1). Slightly higher concentrations of apoE2 were required to stimulate efflux of cholesterol from J774 macrophages (cpt-cAMP-treated), as reflected by an increase in the K_m (Table 1). The CT domain of apoE consistently stimulated ABCA1-dependent cholesterol efflux with high efficiency. On a mass basis, i.e., micrograms per milliliter, the CT domain produced high levels of efflux of cholesterol from J774 cells at concentrations slightly lower than that of the intact apolipoproteins (Table 1). Correcting for differences in molecular mass revealed that the CT domain

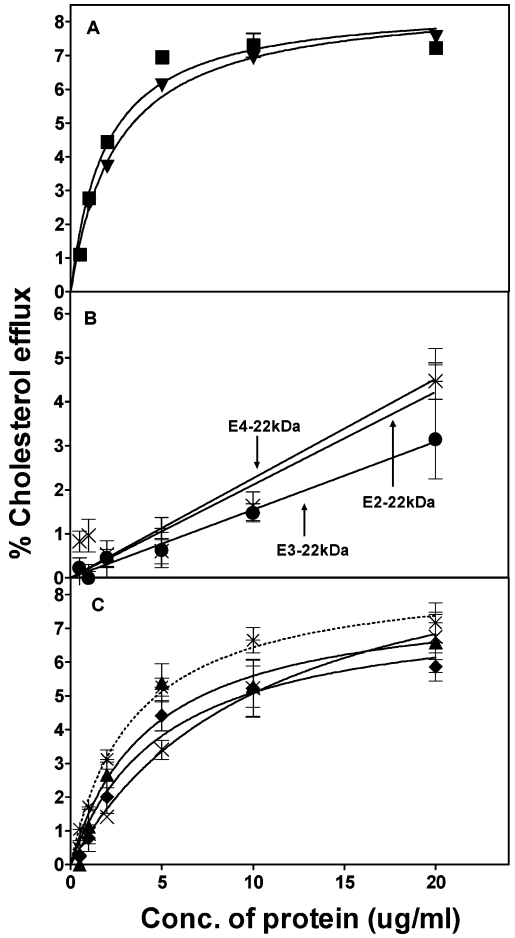

FIGURE 3: Dependence of cholesterol efflux on the concentrations of CT and NT domains and full-length apolipoproteins. J774 macrophages were labeled with [^{3}H]cholesterol (3 μCi/mL) and treated with cpt-cAMP to upregulate ABCA1. Cholesterol efflux was initiated by the addition of different concentrations (0−20 μg/mL) of either CT or NT domain or apoE. After 4 h, the medium was removed and filtered, lipids were extracted, and the ^{3}H radioactivity was determined. Curves were generated by fitting the fractional efflux at 4 h (subtracting background efflux to serum-free medium), measured at various concentrations of acceptors, to the Michaelis−Menten equation. (A) Cholesterol efflux curves generated for the 10 (■) and 12 kDa (▼) CT domain fragments of apoE. (B) Efflux to the NT domains (22 kDa) of apoE2 (×), apoE3 (●), and apoE4 (+) fitted by linear regression with the line forced through the origin. (C) Efflux curves obtained with intact apolipoproteins, i.e., apoA-I (∗), apoE2 (×), apoE3 (▲), or apoE4 (◆).

stimulated cholesterol efflux with the same efficiency (K_m ~ 0.2 μM) as apoA-I and -E on a per molecule basis; similar results were obtained using HeLa cells expressing ABCA1 (data not shown).

A Long Span of Native CT Sequence Is Required To Stimulate Cholesterol Efflux Efficiently. Figure 4 shows the strategy used to create truncation mutants of apoE3 (i.e., E3-1−272, E3-1−260, and E3-1−250) and synthetic peptides to map the sequences of amino acids within the CT domain that confer cholesterol efflux activity. The E3-1−272 truncation mutant lacks a large portion (Δ273−299) of the hydrophobic class G helix found at the C-terminal end of apoE3. The E3-1−272 truncation mutant retained efflux activity, but higher concentrations (K_m = 28 ± 4 μg/mL) were required to stimulate cholesterol efflux compared to intact apoE3 (Figure 5A). This suggests that hydrophobic sequences govern the efficiency with which the CT helices

Table 1: Kinetic Parameters for Efflux of Cholesterol to ApoE Variants

apolipoprotein	V_{max}[a] (% FC efflux/4 h)	K_m[b] μg of apo/mL	K_m[b] μM
E2 (n = 6)	10.5 ± 0.7	10.6 ± 1.5	0.31 ± 0.044
E3 (n = 6)	8.6 ± 0.5	4.5 ± 0.8	0.13 ± 0.023
E4 (n = 6)	7.6 ± 0.5	5.1 ± 0.8	0.15 ± 0.024
10 kDa CT domain (n = 3)	8.5 ± 0.4	1.9 ± 0.3	0.19 ± 0.030
12 kDa CT domain (n = 3)	8.5 ± 0.4	2.5 ± 0.2	0.21 ± 0.017
wild-type apoA-I (n = 6)	8.7 ± 0.5	3.4 ± 0.6	0.12 ± 0.021

[a] Means ± the standard deviation. Comparison of the V_{max} values by ANOVA followed by a Tukey's test showed that there are no significant differences between values. [b] The K_m for apoE2 is significantly higher (p < 0.05) than the remaining values which are not significantly different from one another.

of apoE mediate cellular cholesterol efflux. Truncation of a larger sequence of amino acids (Δ261−299) that endows hydrophobic character produced a dramatic decrease in the level of cholesterol efflux, as determined using an apoE3-1−260 mutant that was poorly active like apoE3-1−250 and the NT domain (aa 1−199). Taken together, these results indicate that deletion of aa 261−299 was sufficient to ablate the cholesterol efflux activity attributed to the CT domain of apoE3. Residues 261−272 seem particularly important because there is a large difference in cholesterol efflux between the E3-1−272 and E3-1−260 mutants (Figure 5A). The region of aa 261−272 is helical and hydrophobic, and critical for lipid binding (*6*, *61*). Substitution of proline 267 with alanine (P267A) in this region had a significant effect on the ability of apoE3 to stimulate cholesterol efflux (Figure 5A). Therefore, disruptions in the lipid-binding region of the CT domain via truncations or amino acid substitution compromise the ability of apoE3 to mediate cholesterol efflux via ABCA1.

Since aa 261−299 of the CT domain of apoE appeared to be critical for mediating cholesterol efflux, we tested whether this segment was sufficient to elicit an efflux response using peptides. A 40-mer peptide based on aa 260−299 stimulated a low level of cholesterol efflux that was only 26% of that obtained with intact apoE and the CT domain (Figure 5B). Variation of the amount of 40-mer peptide in the extracellular medium produced a small increase in the level of cholesterol efflux that displayed a linear dependence on concentration, which precluded determinations of K_m values (Figure 5B). The 40-mer peptide possessed nonpolar character having a calculated hydrophobicity of −0.15 kcal/mol, and it cleared a turbid solution of DMPC ($t_{1/2}$ ~ 4 min; peptide:DMPC mass ratio of 1:8), indicating the peptide was able to bind phospholipids. Despite its amphipathic nature and lipid binding activity, the 40-mer peptide failed to promote high-level cholesterol efflux in an efficient manner like the CT domain.

A 51-mer peptide (aa 216−266) based on the first portion of the CT domain stimulated a low level of cholesterol efflux that was ~20% of the maximum response obtained with the 10 kDa CT domain (Figure 6A). The 51-mer peptide displayed lipid binding activity in DMPC clearance assays ($t_{1/2}$ ~ 2 min; peptide:DMPC mass ratio of 1:2), but relatively

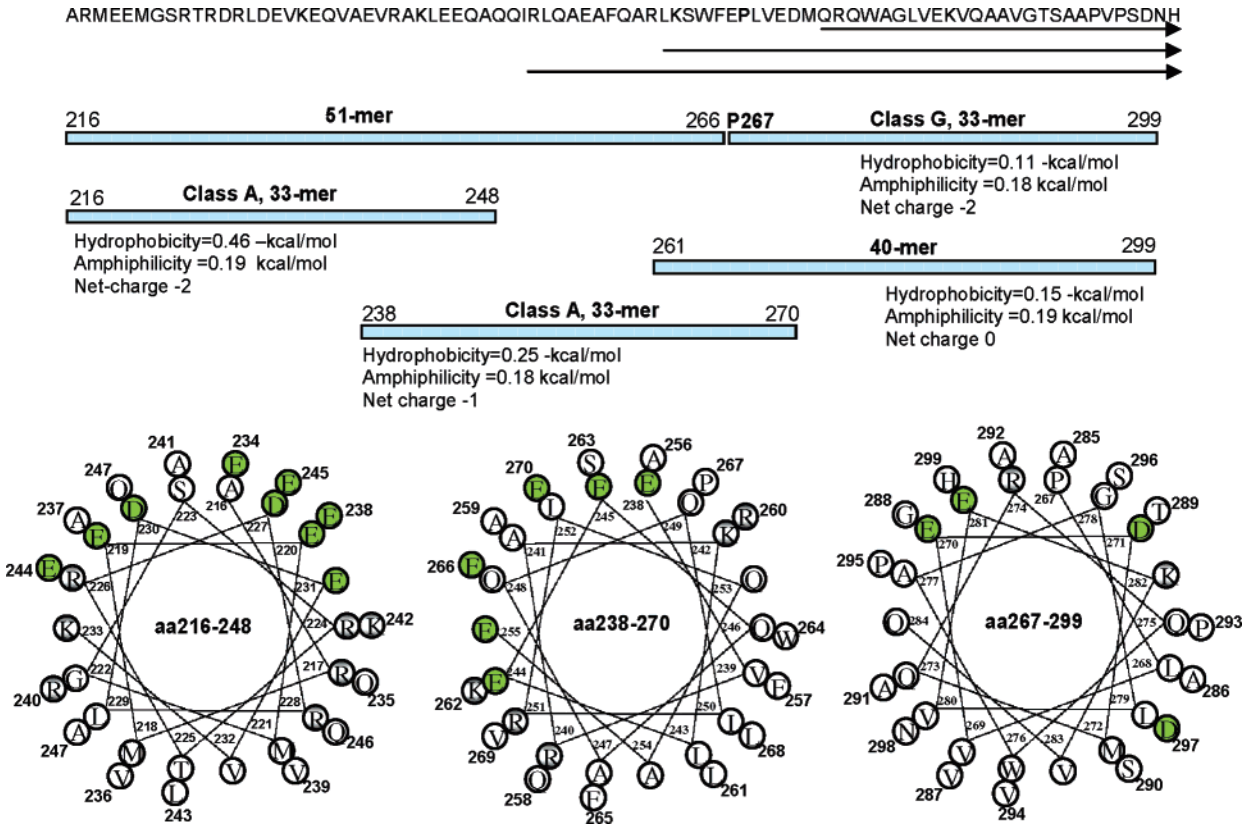

FIGURE 4: Diagram showing truncations of apoE3 and sequences used to create peptides. The top lists the sequence of amino acids (aa) corresponding to residues 216−299 of the apoE CT domain. The bold residue is P267 that links two distinct regions (51 and 33 residues) that comprise the CT domain (*59*, *60*). The arrows denote sequences that were deleted to create the apoE3 CT truncation mutants E3-1−272 (Δ273−299), E3-1−260 (Δ261−299), and E3-1−250 (Δ251−299). The shaded bars indicate the sequences used to create synthetic peptides. The 51-mer corresponds to the first portion of the CT domain. P267 was taken as the starting point for the synthesis of the 33-mer class G helix found at the end of apoE. The helical-wheel projections show equal 33-mer segments used to create peptides; the hydrophobic moment (i.e., amphiphilicity) and hydrophobicity of the segments were calculated as described previously (*49*). Shaded circles represent acidic residues and partially shaded circles positively charged amino acids.

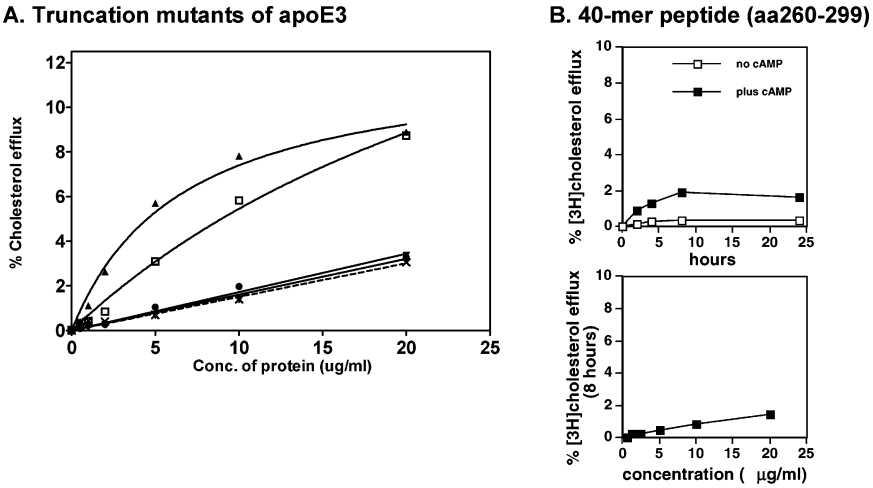

FIGURE 5: Cholesterol efflux properties of truncation mutants of apoE3 and the 40-mer peptide based on aa 260−299. J774 macrophages were treated with cpt-cAMP and labeled with [^{3}H]cholesterol as described in the legend of Figure 3. (A) Cholesterol efflux to increasing concentrations of either intact apoE3 (▲), E3-1−272 (□), E3-1−260 (▼), or E3-1−250 (×), shown as a percentage of ^{3}H label appearing in the medium at 4 h. Efflux to apoE3 (P267A) (●) is also depicted. Means ± the standard deviation (n = 3) are shown. (B) Kinetics of cholesterol efflux mediated by the 40-mer peptide (20 μg/mL) based on aa 260−299 of the apoE CT domain, using [^{3}H]cholesterol-labeled J774 cells treated with (filled symbols) and without (empty symbols) a cpt-cAMP analogue (top). Dependence of cholesterol efflux on the extracellular concentration of the 40-mer peptide, determined using cpt-cAMP-treated J774 cells (bottom). Results are representative of two experiments performed in triplicate. Error bars are smaller than symbols.

high concentrations were required to get the same rapid clearance as the 40-mer peptide based on the hydrophobic C-terminal class G helix (above). Both the 51- and 40-mer peptides cleared turbid solutions of DMPC more effectively than intact apoE3, consistent with the lipid binding activity of the CT domain (data not shown). A 33-mer peptide corresponding to the class G helix (aa 267−299) found at the C-terminal end of apoE failed to stimulate efflux of

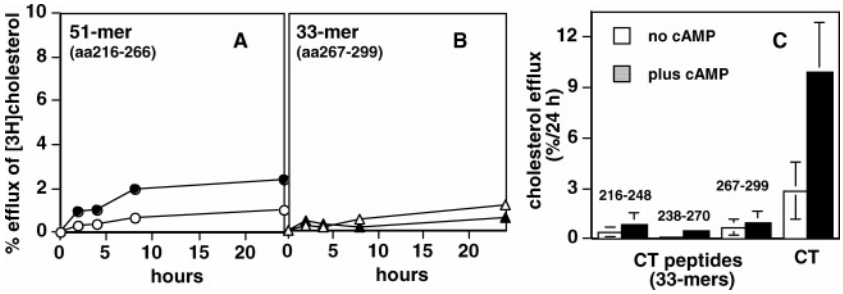

FIGURE 6: Cholesterol efflux activity of synthetic peptides derived from the CT domain of apoE. J774 macrophages were labeled with [^{3}H]cholesterol and treated with cpt-cAMP as described in the legend of Figure 1. Panels A and B show the kinetics of cholesterol efflux obtained using the 51- and 33-mer peptides corresponding to the class A and G helical segments of the CT domain of apoE, respectively (Figure 4). The peptides were used at a concentration of 30 μg/mL. The results are representative of two experiments; error bars are smaller than symbols. Panel C summarizes results of cholesterol efflux experiments using 33-mer peptides derived from different portions of the CT domain (shown as helical-wheel projections in Figure 4). CT corresponds to the 10 kDa domain fragment of apoE. Each acceptor was used at 50 μg/mL. Values correspond to the percentage efflux at 24 h; means $\pm$ the standard deviation ($n = 3$) are shown.

Table 2: Characteristics of Particles Released by J774 Cells Incubated with Different ApoE Domains

apolipoprotein	K_{av}	diameter (nm)	molecular mass (kDa)	PL counts per minute distribution across peaks (%)
E3 (34 kDa)	0	>19	–	64
	0.16	13	411	27
	0.41	8	90	9
E3 NT domain (22 kDa)	0	>19	–	95
	0.41	8	90	5
CT domain (10 kDa)	0	>19	–	21
	0.16	13	411	69
	0.41	8	90	10

cholesterol from cpt-cAMP-treated J774 cells (Figure 6B,C), even when its concentration was increased from 1 to 40 μg/mL (data not shown). Simultaneous addition of both the 51- and 33-mer (class G, aa 267−299) peptides to the medium of J774 cells (with cpt-cAMP) had no effect, indicating the two segments need to be joined via P267 to promote cholesterol efflux via ABCA1 (data not shown). The overall hydrophobicity (−0.11 kcal/mol) of the 33-mer class G peptide is greater than those of any of the other peptides we tested (Figure 4) and similar to that of the 9/10 segment of apoA-I (*49*). This suggests that although hydrophobicity may be necessary for efficient stimulation of cholesterol efflux in the context of the CT domain of apoE (Figure 5), it is not sufficient to evoke a cholesterol efflux response as we found with 40-mer peptide aa 260−299 (Figure 5) and hydrophobic helix 10 of apoA-I that was previously reported (*49*).

Fragmenting the CT domain into small 33-mer segments produced peptides with low activity, compared to the longer 51- and 40-mer peptides (Figure 6B,C). In general, the 33-mer peptides, including the class G peptide (aa 267−299), failed to stimulate efflux of cholesterol from cpt-cAMP-treated J774 cells above the levels obtained in the absence of cpt-cAMP. Peptide aa 216−248 was relatively polar, as judged by its low hydrophobicity (Figure 4), and it failed to clear a turbid solution of DMPC (data not shown). This probably accounts for its inability to stimulate cholesterol efflux. A 33-mer peptide based on the central portion (aa 238−270) of CT also failed to elicit a cholesterol efflux response from cpt-cAMP-treated J774 cells, despite having a lipid binding activity in DMPC clearance assays like the 51-mer based on aa 216−266 (not shown).

Overall, these results are consistent with our previous findings that not all hydrophobic peptides based on native amphipathic α-helices are able to stimulate ABCA1-dependent cholesterol efflux (*49*). The joining of two apoE α-helices that constitute the CT domain (aa 222−299) appears to be required for stimulation of ABCA1 cholesterol efflux with high efficiency.

The CT Domain of ApoE Promotes the Assembly of Nascent HDL Particles. To understand the structural contribution of the different domains of apoE to HDL particle formation during cholesterol efflux, cpt-cAMP-stimulated J774 cells labeled with [^{3}H]choline chloride and [^{14}C]-cholesterol were incubated with either CT, NT, or intact apoE3 (each at 5 μg/mL) for 6 h, and the particles present in the extracellular medium were characterized by gel filtration chromatography. Figure 7A shows that intact apoE3 formed a distinct HDL size particle (13 nm diameter) with a K_{av} of 0.16. The diameters of these apoE3-containing particles are in agreement with that reported previously (*30*). The occurrence of the small peak with a K_{av} of 0.41 is consistent with the limited formation of HDL particles with a diameter of ~8 nm. The majority of the [^{3}H]PL and [^{14}C]-FC released into the extracellular medium was contained in particles that eluted in the void volume ($K_{av} = 0$). Although the elution profiles of the HDL particles formed by CT and intact apoE3 were alike, as indicated by similar K_{av} values (Table 2), more HDL particles were formed by CT than by apoE3 (Figure 7C). The phospholipid (PL) content in the HDL peak was ~3 times greater than the void volume peak for CT, while a reverse PL distribution was observed for apoE3 (Table 2). In contrast, cells incubated with NT did not form HDL particles, and nearly all of the FC and PL released were present in the void volume (Figure 7B and Table 2). This result is consistent with the data obtained from the efflux studies showing that NT was a poor promoter of cholesterol efflux from ABCA1-upregulated cells (Figures 1 and 3).

The elution profiles of the particles that were present in the extracellular medium of ABCA1-upregulated J774 cells labeled with [^{3}H]cholesterol and exposed to 5 μg/mL [^{14}C]-apoE for 6 h are presented in Figure 8. While the [^{3}H]-cholesterol-containing HDL peak for cells treated with intact apoE3 was seen at a K_{av} of 0.16 consistent with earlier observations (Figure 7A), [^{14}C]apoE3 exhibited three peaks (I, II, and III, Figure 8A). Analysis of the apoE in these peaks by cross-linking with BS^3 and SDS−PAGE demonstrated that peak I was tetrameric apoE and peak II was a mixture

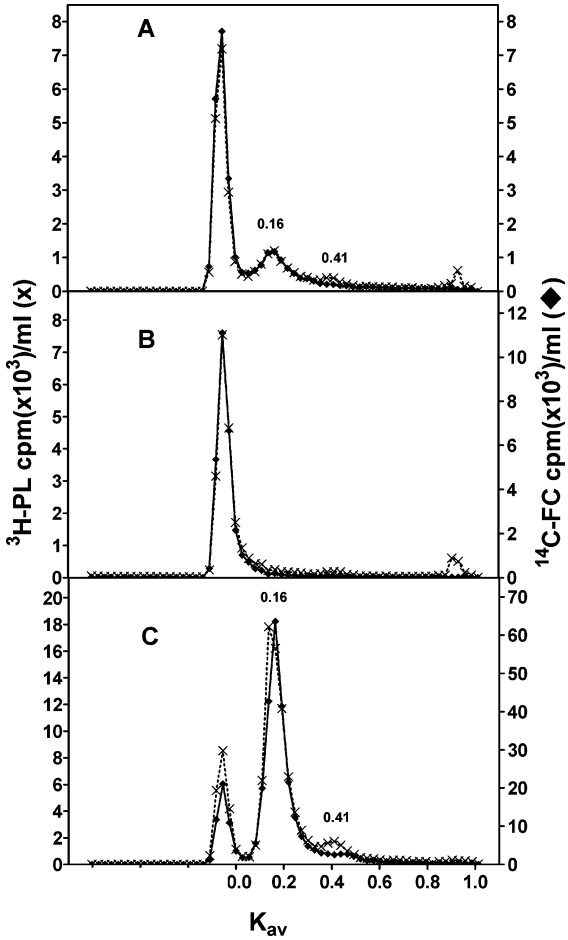

FIGURE 7: Gel filtration elution profiles of medium collected after incubation of cAMP-stimulated J774 macrophages with either apoE3 or its NT and CT domains. J774 macrophages were labeled with [^{3}H]choline chloride and [^{14}C]cholesterol, and these labeled cells were incubated with 0.3 mM cpt-cAMP overnight. Efflux was initiated by the addition of 5 μg/mL apoE3 (A), 5 μg/mL NT domain of apoE3 (B), or 5 μg/mL CT (10 kDa) domain of apoE (C). After 6 h, aliquots of the media were collected, filtered, and subjected to gel filtration chromatography on a calibrated Superdex 200 column. Fractions were extracted for lipids, and radioactivity was determined by liquid scintillation counting. The level of PL efflux for cells exposed to apoE was in the range of 1−3% of that of cell PL. It should be noted that the specific activities of the radiolabeled cellular lipids are not the same for the experiments in panels A−C.

of monomer and dimer forms. Peak III was not characterized, but this fraction contained a proteolytic fragment of apoE or apoE for which elution was delayed by interaction with the gel matrix. Comparison of Figures 7A and 8A demonstrates that only the tetrameric form of apoE3 coeluted with the HDL peak, and a similar observation was made for particles generated by CT (Figures 7C and 8C). However, removal of the C-terminal domain of apoE3 prevented it from self-associating which is evident from the fact that only a single major peak (II) (K_{av} = 0.56) was observed for this protein (Figure 8B). In contrast to the formation of a single large HDL by apoE3 and CT (10 kDa), apoA-I generated two HDL particles with diameters of ~9 and ~12 nm (Figure 8D) in concurrence with prior studies (*56*).

To determine the effect of apoE isoform structure on the nature of particles generated by ABCA1, upregulated single- or double-labeled J774 cells were treated with 5 μg/mL ^{14}C-

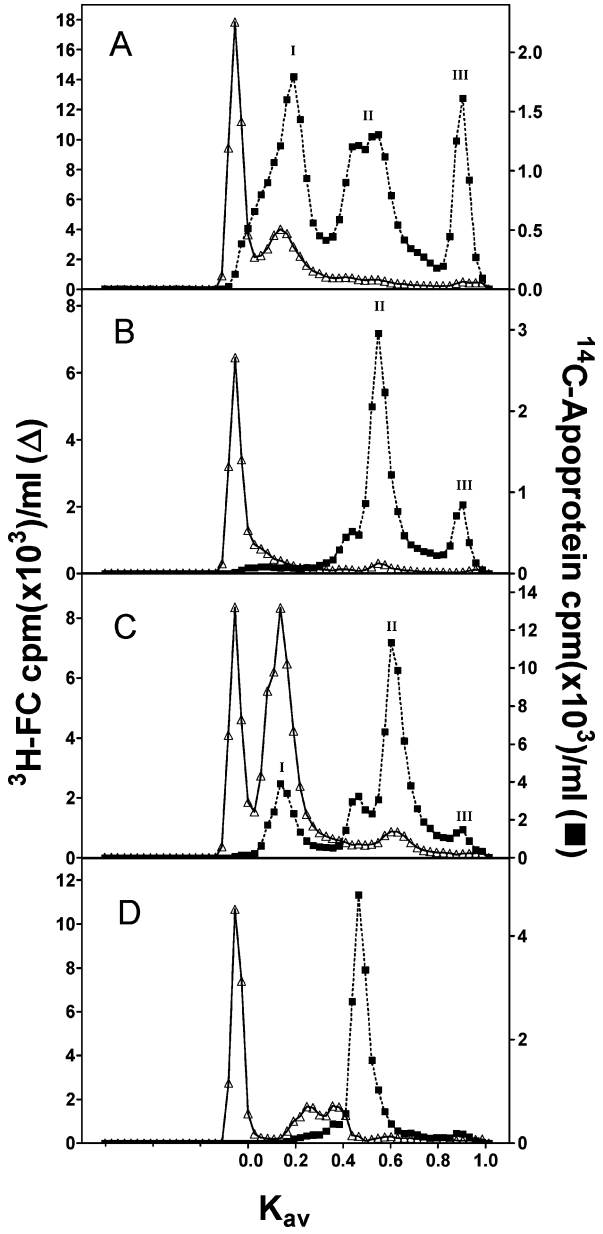

FIGURE 8: Gel filtration elution profiles of medium collected after incubation of cAMP-stimulated J774 macrophages with ^{14}C-labeled apoproteins. J774 macrophages were labeled with [^{3}H]cholesterol, and these labeled cells were incubated with 0.3 mM cpt-cAMP overnight. Efflux was initiated by the addition of 5 μg/mL [^{14}C]-apoE3 (A), 5 μg/mL NT domain of apoE3 (B), 5 μg/mL CT (10 kDa) domain of apoE (C), or 5 μg/mL wild-type apoA-I (D). After incubation for 6 h, aliquots of the media were collected, filtered, and subjected to gel filtration chromatography on a calibrated Superdex 200 column. It should be noted that the specific activities of the radiolabeled cellular lipids are not the same for the experiments in panels A−D.

labeled or unlabeled apoE2, -E3, or -E4 for 6 h, and the medium was subjected to gel filtration chromatography. As shown in Table 3, all the isoforms of apoE formed similar HDL particles with diameters ranging from 12 to 14 nm. There was no significant variation in the distribution of PL between extracellular particles formed in the presence of the three isoforms of apoE (Table 3).

DISCUSSION

ApoE is synthesized by macrophages and possesses anti-atherogenic properties (*18*, *62*−*64*). Extracellular accumula-

Table 3: Characteristics of Particles Released by J774 Cells Incubated with Different ApoE Isoforms

apolipoprotein	K_{av}	diameter (nm)	molecular mass (kDa)	PL counts per minute distribution across peaks (%)
E2 (34 kDa)	0	>19	−	68
	0.19	12	343	14
	0.41	8	90	18
E3 (34 kDa)	0	>19	−	64
	0.16	13	411	27
	0.41	8	90	9
E4 (34 kDa)	0	>19	−	55
	0.13	14	494	31
	0.41	8	90	14

tion of endogenously synthesized apoE promotes ABCA1-mediated cholesterol efflux, which contributes to its atheroprotective effects. The structural features of apoE that are essential for facilitating cholesterol efflux are not well understood. Therefore, we examined the efficiencies with which the major isoforms of apoE and their corresponding N- and C-terminal domains stimulate cellular cholesterol efflux. Our results indicate that each of the apoE isoforms stimulated ABCA1-dependent cholesterol efflux, as judged in studies using J774 macrophages and HeLa cells. These findings are consistent with earlier studies indicating that various apolipoproteins act as good acceptors of lipids from ABCA1-expressing cells (*29*, *65*).

ApoE contains two independently folded domains, a 22 kDa N-terminal (NT) domain and a 10 kDa C-terminal (CT) domain. Although apoE2, -E3, and -E4 differ by only single-amino acid substitutions, the mutations have significant effects on interactions of apoE with cellular proteins (*66*−*68*). In the study presented here, the CT lipid-binding domain (10 and 12 kDa) was found to be a highly efficient mediator of ABCA1-dependent cholesterol efflux. The cholesterol efflux activity of the CT domain did not require the NT four-helix bundle domain, since the CT domain stimulated cholesterol efflux with the same efficiency as intact apoE (Table 1). The latter supports the notion that NT and CT domain−domain interactions do not play a major role in modulating the interaction of the apoE CT domain with ABCA1. This is further supported by our findings that apoE2, -E3, and -E4 produced similarly sized HDL particles, consistent with their similar capacity to stimulate ABCA1-dependent cholesterol efflux and their comparable lipid binding affinities (*46*, *54*). These observations are in agreement with previous reports indicating the isoforms of apoE behave similarly in stimulating efflux of cholesterol from RAW macrophages (*24*) and binding to ABCA1 in studies employing fibroblasts (*30*). However, we did find that apoE2 was somewhat less efficient than E3 and E4 in stimulating efflux of cholesterol from cpt-cAMP-treated J774 cells. This effect may be dependent on the cell type and/or level of ABCA1 expression since we did not observe any differences in cholesterol efflux efficiency between apoE2 and the other apoE isoforms using ABCA1-expressing HeLa cells (data not shown).

Studies of human fibroblasts by Krimbou et al. (*30*) have shown that apoE3 forms HDL particles ranging from 9 to 15 nm in diameter, which is similar to our findings with the apoE isoforms and ABCA1-expressing cells. The CT domain forms particles similar to apoE3, whereas removal of the CT domain from apoE abolished the formation of HDL-sized particles. These observations suggest that the CT domain is vital for lipidation and subsequent formation of HDL via ABCA1. The CT domain exhibits lipid binding affinity greater than that of intact apoE3 (*46*), which may explain why it generated more 13 nm HDL particles, as shown in Figures 7 and 8. The HDL particles (≤13 nm) contain FC, PL, and apoE, while the larger material in the void volume consists of PL and FC. The size of these larger particles (>19 nm) suggests that they are probably microparticles (*69*). Formation of these microparticles is independent of the release of apoE-HDL and does not require apolipoprotein in the medium (*56*). However, since microparticle release is dependent on ABCA1 activity (*56*) and apolipoproteins stabilize ABCA1 at the cell surface, more microparticles are released when apoE is present.

The CT domain of apoE possesses a large hydrophobic surface (*54*, *59*, *60*). Our studies with CT truncation mutants suggest that hydrophobic segments of apoE are important for stimulating ABCA1-dependent cholesterol efflux. Removal of the class G helix (aa 273−299) from apoE3 reduced its efficiency for promoting cholesterol efflux. Deletion of an additional hydrophobic sequence (aa 260−272) in conjunction with removal of the class G segment almost completely ablated cholesterol efflux activity. The apoE3-1−260 truncation mutant stimulated cholesterol efflux poorly, like apoE3-1−250 and the NT four-helix bundle domain (apoE3-1−191). This suggests that a region of apoE encompassing aa 260−299 that contains the most hydrophobic segments within the CT domain is important for mediating cholesterol efflux. The region encompassing aa 260−299 is derived, at least in part, from the first helical segment of the CT domain of apoE linked to the class G helix via P267. Thus, a relatively long span of native sequence involving class A and G helices that make up the major lipid-binding region of the CT domain of apoE appears to be critical for promoting lipid efflux via ABCA1.

The CT domain of apoE is relatively long, possessing a hydrophobic nonpolar surface with a composition of amino acids that is different compared to the 9/10 helical segments of apoA-I. These observations are consistent with the idea that there is no strict stereospecific requirement by which helical apolipoproteins mediate cellular cholesterol efflux, as suggested in studies employing peptides composed of D-amino acids (*70*). Therefore, it is not likely that apolipoproteins interact in a specific fashion with a hydrophobic site to mediate cholesterol efflux via ABCA1. In our view, one plausible hypothesis evokes a membrane microsolubilization process driven by the hydrophobicity of amphipathic apoE α-helices as being important for promoting cholesterol efflux (*26*). Since the presence of the strongly hydrophobic, lipid-binding region of apoE is critical for efficient cholesterol efflux, lipid binding and microsolubilization seem to control the overall efficacy of the efflux process.

Although hydrophobicity seems to be important for mediating cellular cholesterol efflux, a 40-mer peptide based on aa 260−299 of the CT domain of apoE was only weakly active. This suggests that factors in addition to hydrophobicity are also important for mediating ABCA1-dependent cholesterol efflux, as we have previously suggested (*49*). Therefore, the helical segments encompassing aa 260−299 of apoE appear to be lacking in determinants required for

recapitulating the cholesterol efflux activity of apoE and the CT domain. Additional determinants may involve aligned acidic residues on the polar surface and/or other unique aspects of secondary structure (*49*, *71*). In support of this, a 33-mer peptide based on the C-terminal class G helix of apoE failed to elicit a cholesterol efflux response from cpt-cAMP-treated J774 cells. This class G segment possesses an overall hydrophobicity identical to that of the 9/10 segments of apoA-I that promoted cholesterol efflux via ABCA1 (*49*), but it lacks an alignment of acidic residues down the center of its polar surface. Thus, it is not surprising that this class G segment (i.e., 33-mer) is not able to stimulate ABCA1-dependent cholesterol efflux. Acidic residues align down the class A amphipathic α-helices formed by segments (aa 216−266) that comprise the first portion of the CT domain, yet a 51-mer peptide based on these segments alone poorly stimulated cholesterol efflux. The hydrophobicity of these segments is considerably lower than that of the lipid-binding region encompassing aa 260−299. This suggests that determinants of the ABCA1 reaction may be spatially dispersed throughout the apoE CT domain (aa 222−299), requiring two segments for optimal cholesterol efflux activity.

It is important to note that the cholesterol efflux activity of the CT domain of apoE was not simply an additive function of its individual segments, since the 33-, 40-, and 51-mer peptides were either inactive or only weakly active in stimulating cholesterol efflux. This suggests that multiple and cooperative interactions brought about by class A and G helices in the CT domain of apoE synergize to produce high-efficiency stimulation of cellular cholesterol efflux, the level of which far exceeds that based on hydrophobic segments alone. This effect perhaps involves protein−protein interactions with ABCA1, in addition to lipid interactions that may promote membrane microsolubilization. Studies of the mutated transporter in Tangier disease and cross-linking experiments suggest that apolipoproteins directly bind to ABCA1 and that this step is necessary for stimulating lipid efflux (*72*, *73*). Binding and lipid acquisition could occur via a two-step process, which is consistent with the studies presented here. It is possible that each of the two steps required for stimulating cholesterol efflux is mediated by different interaction sites within the apoE CT domain, i.e., a specific class A motif that mediates protein−protein interactions with ABCA1 and adjacent hydrophobic segments that mediate protein−lipid interactions and membrane microsolubilization.

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