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Molecular Basis for the Differences in Lipid and Lipoprotein Binding Properties of Human Apolipoproteins E3 and E4[†]

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ABSTRACT: Human apolipoprotein (apo) E4 binds preferentially to very low-density lipoproteins (VLDLs), whereas apoE3 binds preferentially to high-density lipoproteins (HDLs), resulting in different plasma cholesterol levels for the two isoforms. To understand the molecular basis for this effect, we engineered the isolated apoE N-terminal domain (residues 1−191) and C-terminal domain (residues 192−299) together with a series of variants containing deletions in the C-terminal domain and assessed their lipid and lipoprotein binding properties. Both isoforms can bind to a phospholipid (PL)-stabilized triolein emulsion, and residues 261−299 are primarily responsible for this activity. ApoE4 exhibits better lipid binding ability than apoE3 as a consequence of a rearrangement involving the segment spanning residues 261−272 in the C-terminal domain. The strong lipid binding ability of apoE4 coupled with the VLDL particle surface being ∼60% PL-covered is the basis for its preference for binding VLDL rather than HDL. ApoE4 binds much more strongly than apoE3 to VLDL but less strongly than apoE3 to HDL_3, consistent with apoE−lipid interactions being relatively unimportant for binding to HDL. The preference of apoE3 for binding to HDL_3 arises because binding is mediated primarily by interaction of the N-terminal helix bundle domain with the resident apolipoproteins that cover ∼80% of the HDL_3 particle surface. Thus, the selectivity in the binding of apoE3 and apoE4 to HDL_3 and VLDL is dependent upon two factors: (1) the stronger lipid binding ability of apoE4 relative to that of apoE3 and (2) the differences in the nature of the surfaces of VLDL and HDL_3 particles, with the former being largely covered with PL and the latter with protein.

Apolipoprotein (apo)[1] E regulates lipid transport and cholesterol homeostasis in the cardiovascular and central nervous systems (*1*, *2*) and is therefore a protein of major biological and clinical importance. In particular, there is great interest in understanding the structure−function relationships of apoE because of its pronounced anti-atherogenic properties (*3*). A complication in understanding the structure−function relationships of human apoE is the occurrence of three major isoforms (apoE2, apoE3, and apoE4), each differing by a single amino acid substitution (*4*). ApoE3, the most common isoform, contains cysteine at position 112 and arginine at position 158, whereas apoE2 and apoE4 contain cysteine and arginine, respectively, at both sites. ApoE2 displays defective binding to the low-density lipoprotein (LDL) receptor and is associated with type III hyperlipoproteinemia (*5*). ApoE3 and apoE4 are also associated with different lipoprotein profiles; the presence of the latter isoform leads to higher plasma cholesterol levels and an increased risk of cardiovascular disease relative to individuals with apoE3 (*6*, *7*). Unlike the situation with apoE2, the different effects of apoE3 and apoE4 on the plasma cholesterol level are not due to variations in interactions with the LDL receptor because both of these isoforms bind similarly to the receptor (*8*). Rather, the difference is apparently a consequence of altered lipoprotein binding preferences of apoE3 and apoE4 (*9*, *10*). The C112R substitution that distinguishes apoE4 from apoE3 occurs in the N-terminal helix bundle domain of the molecule where it destabilizes the helix bundle (*11*) and loosens its structure (*12*). As a consequence, interactions between the N- and C-terminal domains of the protein molecule are modified so that, unlike apoE3, apoE4 binds preferentially to very low-density lipoprotein (VLDL) compared to high-density lipoprotein (HDL) in plasma (*10*, *13*, *14*). The altered domain−domain interaction in apoE4 is a result of a rearrangement of the R61 side chain in the helix bundle induced by the presence of R112 that allows R61 to interact with E255 in the C-terminal domain (*13*, *14*). This interaction causes the C-terminal domain to be organized differently in the two isoforms. For example, the spacing between the N-terminal and C-terminal domains is altered (*15*, *16*), and the C-terminal domain in apoE4 around position 264 is less organized and more exposed to the aqueous environment (*17*). As a result of such alterations in the C-terminal domain, apoE4 binds better than apoE3 to lipid surfaces (*14*, *18*). Studies of apoE3 and apoE4 variants containing a progressively truncated C-terminal domain have demonstrated that the region spanning residues 260−299 is important for determining the structure of apoE, its

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[1]Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MLVs, multilamellar vesicles; PC, phosphatidylcholine; PL, phospholipid; VLDL, very low-density lipoprotein; WT, wild type.

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self-association, and its ability to bind to lipoprotein particles (*13*, *17*, *19*−*21*).

Despite the insights provided by the investigations described above, the molecular basis for why apoE3 and apoE4 partition differently between VLDL and HDL remains to be explained. It is important to understand this effect because it underlies the altered catabolism of triglyceride-rich lipoproteins induced by apoE4 compared to apoE3 (*5*) and the greater risk of cardiovascular disease associated with apoE4 (*6*). Here we address this question by systematically investigating the lipid and lipoprotein binding properties of a series of apoE3 and apoE4 variants with altered C-terminal domains. The results demonstrate that the region spanning residues 261−272 contributes differently to lipid binding in apoE3 and apoE4 such that apoE4 binds better to lipids. The preferential association of apoE4 with VLDL occurs because apoE−lipid interactions control binding to the surface of this lipoprotein particle. ApoE4 does not bind better than apoE3 to HDL because interactions of apoE with other apolipoproteins resident in the HDL particle surface are dominant in this case.

EXPERIMENTAL PROCEDURES

Materials. Human apoE variants were expressed in *Escherichia coli* as thioredoxin fusion proteins and isolated and purified as described previously (*22*, *23*). Full-length apoE3 and apoE4 (residues 1−299), their 22 kDa N-terminal fragments (residues 1−191) and 12 kDa C-terminal fragments (residues 192−299), and the C-terminally truncated forms (residues 1−260 and 1−272) have been described previously (*17*, *20*, *23*). The variants containing point mutations (apoE3 K146E, apoE3 K146Q, apoE3 P267A, and apoE4 E255A) have also been described (*18*, *24*). The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was employed to introduce deletions (Δ192−260 and Δ261−272) as well as the G278P point mutation into apoE3 and apoE4. The apoE preparations were at least 95% pure as assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The apoE variants were ^{14}C trace labeled by reductive methylation as described previously (*18*). In all experiments, the apoE sample was freshly dialyzed from a 6 M GdnHCl and 10 mM DTT solution into a buffer solution before use. ApoE concentrations were determined either by a measurement of the absorbance at 280 nm or by the Lowry procedure (*25*). HDL_3 and VLDL were purified by sequential ultracentrifugation from a pool of normolipidemic human plasma as described previously (*17*, *26*). Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Pelham, AL), and egg yolk phosphatidylcholine (PC) and triolein were purchased from Sigma (St. Louis, MO). 8-Anilino-1-naphthalenesulfonic acid (ANS) was purchased from Molecular Probes (Eugene, OR).

Binding of ApoE to Emulsion Particles. Emulsion particles were prepared by sonication of a triolein/egg yolk PC mixture (3.5/1, w/w) in pH 7.4 Tris buffer (*18*, *23*). The triolein/PC molar ratio in the emulsion after isolation by ultracentrifugation was 4.6 ± 0.3/1 (mean ± standard error of the mean; $n = 6$), and the average particle diameter determined by quasi-elastic light scattering was 86 ± 7 nm. The binding of apoE was monitored by incubation of ^{14}C-labeled protein with emulsion for 1 h at room temperature and separation of free and bound apoE by centrifugation, as described previously (*23*).

VLDL versus HDL Distribution of ApoE. The partitioning of the apoE variants between human HDL_3 and VLDL was monitored using a previously described, competitive binding, assay (*17*) except that the apoE variants were added individually rather than in pairs. In brief, [^{14}C]apoE (5 μg) was incubated at 4 °C for 30 min with 0.45 mg of VLDL protein and 0.9 mg of HDL_3 protein (these concentrations give approximately equal total VLDL and HDL_3 particle surface areas available for apoE binding) in a total volume of 1 mL of Tris buffer (pH 7.4). VLDL, HDL_3, and unbound apoE were then separated by sequential ultracentrifugation.

DMPC Clearance Assay. The kinetics of solubilization of DMPC multilamellar vesicles (MLVs) by the apoE variants were measured by monitoring the decrease in absorbance at 325 nm, as described previously (*27*).

ANS Fluorescence Measurements. A Hitachi F-4500 fluorescence spectrophotometer was used to measure the fluorescence (400−600 nm) from 250 μM ANS in Tris buffer (pH 7.4) in the presence of 50 μg/mL apoE variant at an excitation wavelength of 395 nm (*18*).

Circular Dichroism (CD) Spectroscopy. The average α-helix contents of the apoE variants were determined by measuring CD spectra at room temperature using a Jasco J-810 spectropolarimeter (*28*). The α-helix content was derived from the molar ellipticity at 222 nm, as described previously (*17*).

RESULTS

Influence of the Manipulation of the C-Terminal Domain on ApoE Structure. Because we know that the C-terminal domain (residues 192−299) is primarily responsible for the lipid affinity of apoE, to improve our understanding of the molecular basis for this effect, we pursued a mutagenesis strategy aimed at systematically dissecting the contributions of different segments of this domain to lipid binding. The contributions of the N- and C-terminal ends of the domain were evaluated by deleting residues 192−260 [variant apoE(Δ192−260)] and residues 261−299 [variant apoE(1−260)], respectively. The influence of the latter segment was examined in further detail by deleting either residues 261−272 [variant apoE(Δ261−272)] or residues 273−299 [variant apoE(1−272)]. Contributions of the entire C-terminal domain were examined by employing the isolated N- and C-terminal domains (residues 1−191 and 192−299, respectively). The secondary structures of the apoE variants described above were determined by far-UV CD spectroscopy, while hydrophobic surface exposure was monitored by ANS binding using fluorescence spectroscopy. The results of these experiments are summarized in Table 1.

With regard to secondary structure, in agreement with prior reports (*11*, *17*, *20*, *29*), the human apoE molecule (residues 1−299) is ~50% α-helical, so that some 150 residues are located in α-helices (Table 1). The exact α-helix content is dependent upon sample history, which can affect the degree of self-association of the protein. The isolated N-terminal domain (residues 1−191) contains a bundle of amphipathic α-helices (*4*, *30*) comprising ~60% of the residues (Table 1). The separately folded C-terminal domain (residues 192−299) contains a similar level of α-helix. The sum of the numbers of helical residues in the isolated N- and C-terminal domains is greater than that in the intact apoE molecule (Table 1), suggesting that interactions between the two domains when covalently attached prevent some folding into α-helix.

As reported previously (*17*, *20*, *21*), C-terminal truncation of apoE3 and apoE4 to either residue 272 or 260 leads to reductions in α-helix content (Table 1); this is consistent with the region

Table 1: α-Helix Contents and ANS Binding for ApoE3 and ApoE4 C-Terminal Variants

	% α-helix[a]		no. of amino acid residues in α-helix		ANS fluorescence intensity[b]	
apoE variant	apoE3	apoE4	apoE3	apoE4	apoE3	apoE4
1−299	50 ± 3	56 ± 2	150	169	1.0	1.2
1−191	57 ± 1	61 ± 1	110	118	0.3	0.4
192−299	59 ± 1		65		1.1	
Δ192−260	49 ± 1	38 ± 2	114	88	0.8	0.7
1−260	45 ± 1	38 ± 1	118	100	0.5	0.5
Δ261−272	27 ± 1	46 ± 1	78	133	0.8	1.3
1−272	42 ± 1	39 ± 1	115	107	0.8	0.9

[a]Means ± standard deviation from at least three measurements. [b]Values are ratios to apoE3(1−299). Estimated error of ±0.1.

spanning residues 261−299 containing some α-helix. At this stage, we are not able to analyze the helix content data in Table 1 to locate helical residues precisely because the elimination of certain segments can induce changes in secondary structure elsewhere in the protein molecule. For instance, removal of the entire C-terminal domain (residues 192−299) from apoE3 reduces the number of helical residues by 40, while deletion of either residues 192−260 or residues 261−299 causes a reduction of 36 or 32 residues, respectively (Table 1). This inconsistency indicates that any decrease in the number of helical residues arising from deletion of a segment of the apoE molecule cannot necessarily be attributed to direct removal of α-helix. The CD results summarized in Table 1 demonstrate that the C-terminal domain exerts different structural effects in apoE3 and apoE4. Thus, apart from the case of the Δ261−272 mutation, the C-terminal domain manipulations induce significantly larger losses of α-helical residues in the latter isoform. The exceptional behavior of the Δ261−272 variants in this regard indicates that the segment spanning residues 261−272 has a greater helix stabilizing effect in apoE3 than in apoE4. Consistent with this observation, the segment of residues 261−272 (as monitored by the fluorescence of W264) is packed differently in the two isoforms (*17*).

The ANS binding data in Table 1 confirm that the segments spanning residues 261−272 influence the overall protein structure differently in apoE3 and apoE4. Thus, this deletion in apoE3 reduces the level of ANS binding but increases it in apoE4. This is in contrast to the other variants of apoE3 and apoE4 (Table 1) containing alterations of the C-terminal domain in which, relative to that in the intact protein, the level of ANS binding is reduced similarly for both isoforms. The comparisons in Table 1 of ANS binding to the isolated N- and C-terminal domains confirm prior reports (*17*, *18*, *20*) showing that binding is primarily to the latter domain. It follows that the C-terminal domain has much more exposed hydrophobic surface than the N-terminal helix bundle domain, presumably underlying the stronger lipid binding ability of the C-terminal domain (see below).

DMPC Clearance Assay. We have shown previously (*17*, *27*) that the ability of apoE to solubilize DMPC MLV resides largely in the C-terminal domain, and the results depicted in Figure 1 for apoE(1−299), apoE(1−191), and apoE(Δ192−299) are consistent with this concept. Removal of the N-terminal end of the C-terminal domain (residues 192−260) has no effect on the activity of apoE3 and decreases the activity of apoE4 somewhat (Figure 1). In contrast, removal of the C-terminal end of the domain (residues 261−299) weakens the ability of both apoE3 and apoE4 to solubilize DMPC [see the results for variant apoE(1−260) in Figure 1]. This finding shows that residues 261−299 play a key role in the solubilization process. Further dissection

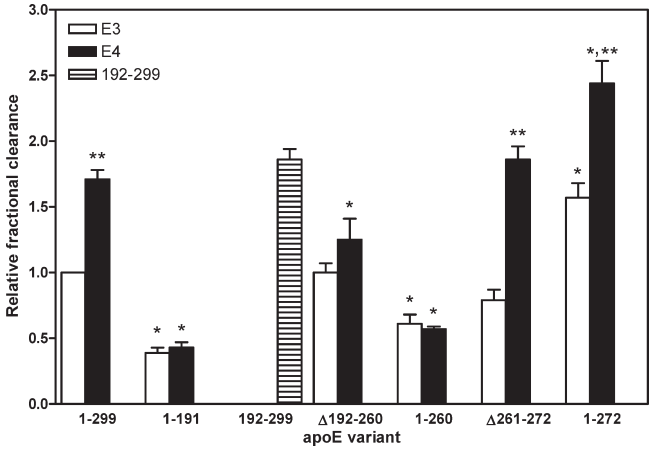

FIGURE 1: Influence of truncation of the C-terminal domain of apoE3 and apoE4 on the ability to solubilize DMPC multilamellar vesicles. Mixtures of DMPC (0.25 mg/mL) and apoE variants (0.1 mg/mL) were incubated at 24 °C, and the fraction of turbidity cleared in 10 min was measured. The fractional decreases in turbidity are normalized to the value observed with WT apoE3(1−299). The results are from three to five independent experiments. The empty and filled bars are for apoE3 and apoE4 variants, respectively. One asterisk indicates a value significantly different ($p < 0.001$) from that for the respective WT apoE(1−299) (one-way analysis of variance followed by the Tukey multiple comparison test using Graphpad Prism 4.0). Two asterisks indicate the value for the apoE4 variant is significantly different ($p < 0.001$) from that of the equivalent apoE3 variant.

of the contribution of this segment of the apoE molecule is complicated by alterations in the degree of self-association when residues in the range of residues 261−299 are deleted. Thus, removal of residues 261−272 (variant Δ261−272) has no effect on DMPC clearance, whereas removal of residues 273−299 enhances the rate of solubilization because the extent of oligomerization is reduced with this variant (*17*).

The results in Figure 1 demonstrate that the DMPC clearance abilities of the 1−299, Δ192−260, Δ261−272, and 1−272 variants of apoE4 are greater than those of their apoE3 counterparts and that this effect is not apparent for the 1−191 and 1−260 variants. It follows that the presence of residues in the range of residues 261−299 is required for the effect of the isoform on the DMPC solubilization rate to be evident, and that the presence of either residues 261−272 or residues 273−299 is sufficient.

Binding to Lipid Emulsion. The DMPC clearance results presented in the preceding section give a measure of how alteration of the C-terminal domain of apoE affects the rate of lipid interaction and breakdown of the DMPC MLV. To obtain a complementary measure of how the apoE variants affect steady

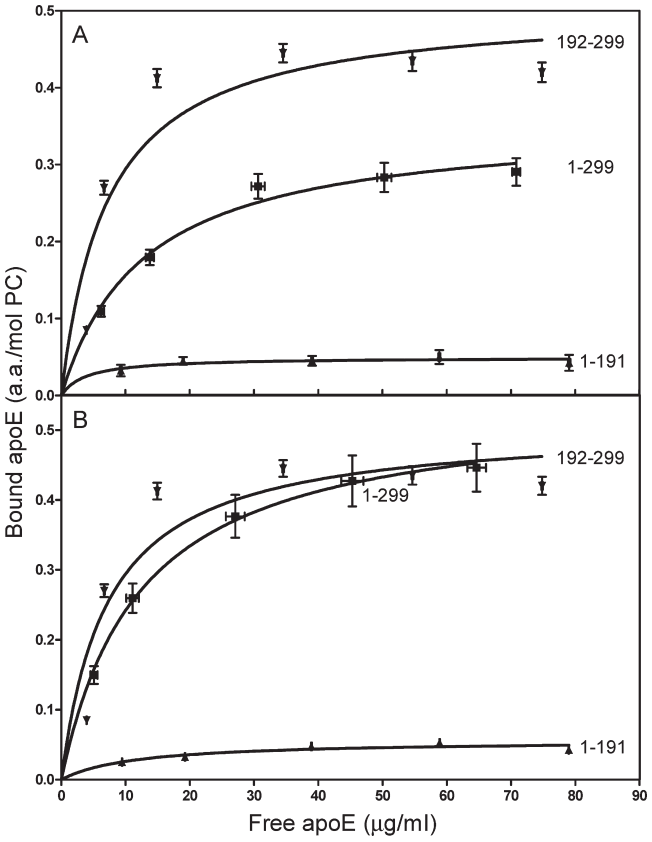

FIGURE 2: Binding of apoE N- and C-terminal domains to triolein/PC emulsion particles. (A) Binding isotherms for apoE3 (residues 1−299) (■), 22 kDa apoE3 (residues 1−191) (▲), and 12 kDa apoE3 (residues 192−299) (▼). (B) Equivalent binding isotherms for the apoE4 counterparts.

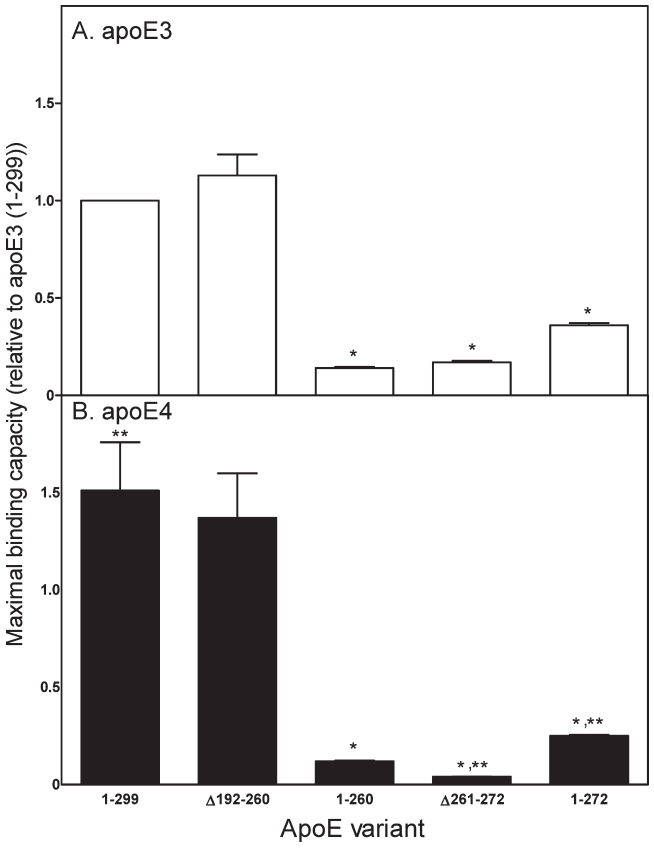

FIGURE 3: Binding of apoE3 and apoE4 C-terminal variants to triolein/PC emulsion particles. The binding parameters were derived from isotherms of the type shown in Figure 2 using a one-binding site model. (A) Relative maximal binding capacities of apoE3(1−299), apoE3(Δ192−260), apoE3(1−260), apoE3(Δ261−272), and apoE3-(1−272). (B) Relative maximal binding capacities of the equivalent apoE4 variants. One asterisk indicates a value significantly different ($p < 0.001$) from that of WT apoE(1−299) by one-way analysis of variance. Two asterisks indicate a significant difference between apoE3 and apoE4 variants.

state binding to a stable lipid−water interface, we employed triolein/egg PC emulsion particles as the lipid substrate in an apoE binding assay. The isotherms in Figure 2 describing the binding of apoE3 and apoE4 to such emulsion particles are consistent with prior reports (*18*, *20*, *23*) in showing that the isolated N-terminal domain (residues 1−191) binds much less well than intact apoE (residues 1−299). This observation indicates that the C-terminal domain (residues 192−299) plays a key role in the emulsion binding ability of apoE, and the binding isotherm for the isolated C-terminal domain confirms that it binds very well. Interestingly, the isolated C-terminal domain binds better than intact apoE3 (Figure 2A) but the same as intact apoE4 (Figure 2B). This result implies that the presence of the N-terminal helix bundle in apoE4 does not interfere with the ability of the C-terminal domain to interact with the emulsion whereas in apoE3 it does.

Comparison of the data for intact apoE3 and apoE4 in Figure 2A,B confirms that the latter isoform binds better to lipid emulsion, which has been reported previously (*14*, *18*). It is also apparent from Figure 3 that deletion of residues 192−260 from either isoform has no significant effect on emulsion binding. In marked contrast, removal of residues 261−299 [as in the apoE-(1−260) variants] drastically weakens its ability to bind to the lipid emulsion (with the reduction being greater for apoE4), indicating that residues 261−299 are critical for the process (cf. ref *20*). Furthermore, the poor binding exhibited by the Δ261−272 and 1−272 variants (Figure 3A,B) indicates that the entire segment spanning residues 261−299 is required for full binding activity. Interestingly, removal of residues 261−272 has a more damaging effect on the lipid binding activity relative to the effect of removing residues 273−299. No isoform difference in emulsion binding is evident with apoE3(1−260) and apoE4(1−260) that lack residues 261−299. Importantly, an isoform effect is seen when either residues 261−272 or 273−299 within the segment of residues 261−299 are present, but in a manner opposite from the situation for the full-length proteins, apoE4(1−272) and apoE4-(Δ261−272) bind less well to lipid emulsion than their apoE3 counterparts (cf. panels A and B of Figure 3). It follows that these residues contribute more to the emulsion binding of apoE4 than to the emulsion binding of apoE3.

As a basis for understanding the effects of point mutations in different domains of the apoE molecule on the ability to interact with lipoprotein particles (see below), we investigated the ways in which such mutations influence binding to a lipid emulsion (Table 2). Consistent with the results showing that residues 261−299 are critical for lipid binding, mutations in this region (P267A and G278P) weaken binding. The particular importance of the segment spanning residues 261−272 is exemplified by the fact the perturbation via the P267A mutation in apoE3 drastically weakens binding (cf. ref *18*). In comparison, the E255A mutation that modifies the interaction between the N- and C-terminal domains in apoE4 (*14*) has a smaller effect on the level of binding to the emulsion. Consistent with the minor contribution of the N-terminal helix bundle domain in lipid binding, the

Table 2: Influence of Point Mutations in ApoE3 and ApoE4 on Binding to Triolein/PC Emulsion Particles

	relative binding to emulsion[a]
apoE3	
WT	1.0
K146E	0.9 ± 0.1
K146Q	1.2 ± 0.2
P267A	0.2 ± 0.1
G278P	0.7 ± 0.1
apoE4	
WT	1.0
E255A	0.9 ± 0.2
G278P	0.7 ± 0.2

[a]Maximal binding of the apoE variants was determined from binding isotherms of the type shown in Figure 2 and is expressed relative to the value for the respective WT protein. The values are means ± the standard deviation.

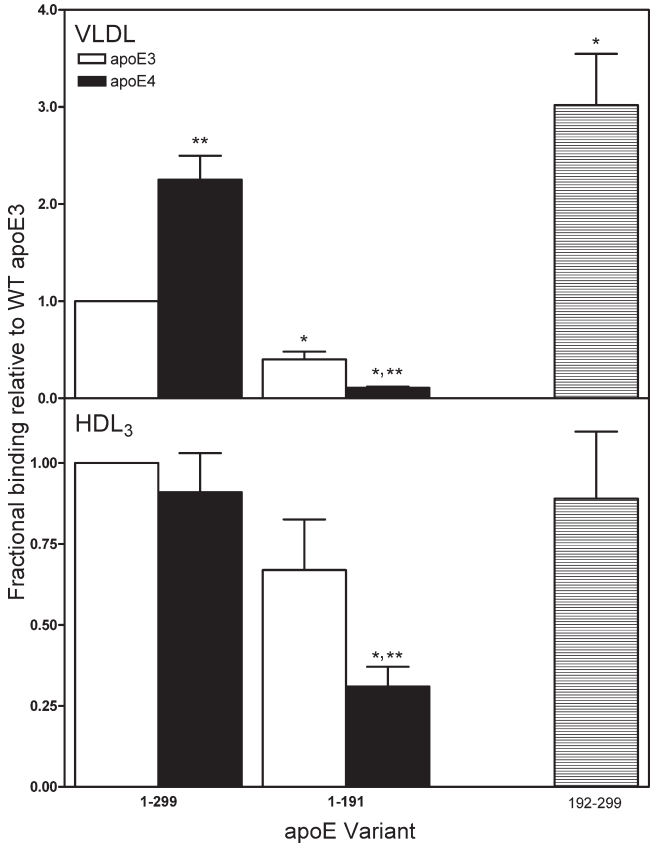

FIGURE 4: Binding of apoE N- and C-terminal domains to VLDL and HDL_3. The fractional binding of ^{14}C-labeled apoE3 and apoE4 domains was determined using the VLDL/HDL_3 distribution assay (see Experimental Procedures): fractional binding to VLDL normalized to the value for apoE3 (top) and fractional binding to HDL_3 normalized to the value for apoE3 (bottom). The empty and filled bars are for apoE3 and apoE4 domains, respectively. One asterisk indicates $p < 0.01$ compared to full-length apoE. Two asterisks indicate the apoE4 variant is significantly different ($p < 0.01$) from the equivalent apoE3 variant.

K146E and K146Q mutations in this domain have little or no effect on the ability of apoE3 to bind to the emulsion (Table 2).

Distributions of ApoE Variants between VLDL and HDL. The results in Figure 4 compare the distributions of full-length (residues 1−299) apoE3 and apoE4 between human VLDL and HDL_3; experimental conditions were such that approximately equal total VLDL and HDL_3 particle surface areas were available for apoE binding and little or no particle remodeling occurred during the binding (*17*). The ratio of apoE3 to apoE4 bound to VLDL is ~0.45, and the same ratio for HDL_3 is ~1.1. The higher apoE3/apoE4 ratio for HDL_3 is consistent with the lipoprotein distribution seen when the isoforms are added to human plasma (*10*, *13*, *19*). The relatively high apoE3/apoE4 ratio in HDL_3 compared to that in VLDL is also consistent with our prior findings using the same assay but under competitive binding conditions (i.e., apoE3 and apoE4 were added together rather than individually to the lipoprotein mixture) (*17*). However, in the competitive binding situation, the ability of apoE4 to bind better than apoE3 to VLDL was not apparent; in this case, the isoforms partitioned approximately equally to VLDL. This difference is apparently due to the complication of mixed oligomer formation when the two isoforms are present together; the distribution of a given apoE3 variant between VLDL and HDL_3 is dependent upon the particular competitor with which it is paired. To avoid the confounding issue of the existence of mixed apoE3 and apoE4 oligomers, the single-component VLDL/HDL_3 distribution assay was employed in this study. Under these conditions, apoE4 binds more to VLDL than to HDL_3 and apoE3 binds similarly to both lipoproteins (a trend of binding more to HDL_3 did not reach statistical significance) (Figure 4).

As is seen with apoE binding to lipid emulsion (Figure 2), deletion of residues 192−299 in apoE3 and apoE4 to give the isolated N-terminal domains (residues 1−191) weakens binding to VLDL (Figure 4) (cf. refs *19* and *26*). The decrease in the level of binding is greater for apoE4 than for apoE3, so that the isoform preference is reversed with apoE4(1−191) binding less well than for its apoE3 counterpart (Figure 4). Consistent with the C-terminal domain (residues 192−299) being primarily responsible for apoE binding to VLDL, the isolated C-terminal domain binds extremely well to VLDL (Figure 4) as is seen with emulsion binding (Figure 2). The strong binding of apoE(192−299) to VLDL has also been observed using surface plasmon resonance (*26*). Significantly, removal of the C-terminal domain has a relatively minor effect on binding of apoE to HDL_3 (Figure 4); the level of apoE3 and apoE4 binding is reduced by approximately one-third and two-thirds, respectively. It is also apparent from Figure 4 that the isolated N-terminal helix bundle domains (residues 1−191) of both isoforms bind better to HDL_3 than to VLDL. In contrast, the isolated C-terminal domain binds much less well to HDL_3 than to VLDL (Figure 4).

Previous work has shown that residues 261−299 play a key role in mediating the binding of apoE to lipoprotein particles (*13*, *17*, *19*, *26*). To be able to systematically compare the effects of C-terminal truncation of the apoE3 and apoE4 molecules on lipid binding ability (Figures 1−3) and lipoprotein binding (VLDL/HDL_3 distribution), we obtained the results summarized in Figure 5. It is apparent that removal of the N-terminal part of the C-terminal domain (i.e., residues 192−260) does not affect binding of either apoE3 or apoE4 to VLDL; furthermore, the enhanced binding of the apoE4 variant is maintained. In marked contrast, removal of residues 261−299 (to give the 1−260 variant) greatly reduced the level of binding to VLDL, with the effect being greater for apoE4. This observation confirms that the presence of residues 261−299 is critical for effective VLDL binding. As was seen with emulsion binding (Figure 3), removal of either residues 261−272 or 273−299 is sufficient to disrupt binding to VLDL (Figure 5). No isoform specificity in VLDL binding is evident for either of these apoE variants. Broadly similar effects of altering the C-terminal domain are evident for

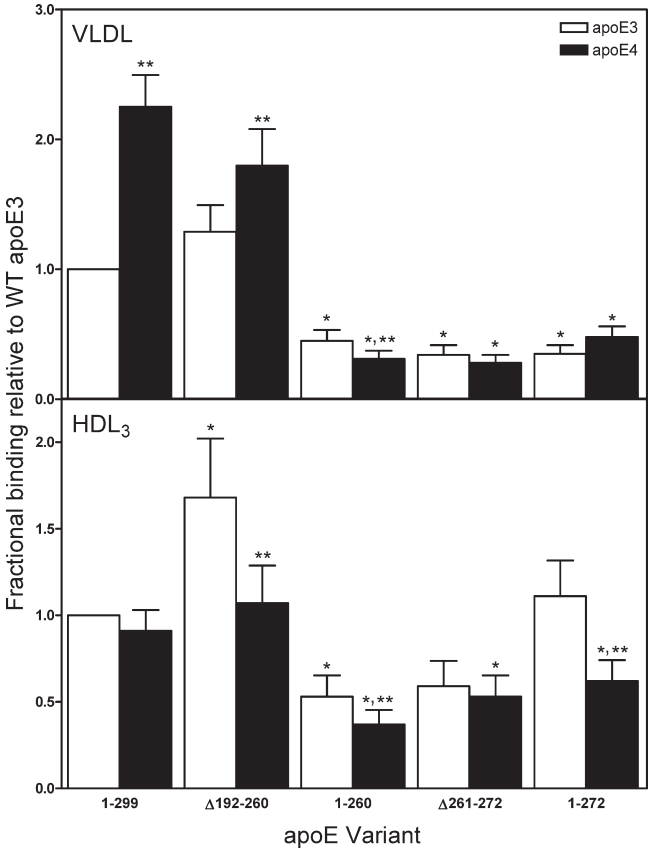

FIGURE 5: Binding of apoE3 and apoE4 C-terminal variants to VLDL and HDL_3. The relative fractional binding data for VLDL (top) and HDL_3 (bottom) were derived as described in the legend of Figure 4. The empty and filled bars are for apoE3 and apoE4 variants, respectively. One asterisk indicates $p < 0.05$ compared to full-length apoE. Two asterisks indicate the apoE4 variant is significantly different ($p < 0.01$) from the equivalent apoE3 variant.

the binding of the apoE variants to HDL_3, except that the alterations in binding are smaller than those seen with binding to VLDL (Figure 5); this effect has been observed previously using surface plasmon resonance to monitor binding of apoE to these lipoproteins (*26*). As was seen with binding to VLDL, removal of residues 192−260 from apoE does not impair binding and actually enhances binding of apoE3 to HDL_3. Another significant difference in the effects of C-terminal truncation on binding of apoE to VLDL and HDL is that deletion of residues 273−299 in apoE3 does not impair binding to HDL_3. Inspection of the data in Figure 5 also reveals that the apoE3 variants bind as well or better to HDL_3 than to VLDL. In contrast, the apoE4 molecules containing residues 261−299 (i.e., variants 1−299 and Δ192−260) bind much better to VLDL than to HDL_3, in marked contrast to the variants lacking the complete segment of residues 261−299 (i.e., variants 1−260, Δ261−272 and 1−272) that bind better to HDL_3 than to VLDL. Overall, the data in Figure 5 demonstrate that residues 261−299 play a key role in mediating binding of apoE to HDL_3 and VLDL particles; their contribution to VLDL binding is larger, and they are required for the ability of apoE4 to bind to VLDL better than apoE3 does.

The similarity in the effects of altering the C-terminal domain on binding of apoE to a lipid emulsion (Figures 2 and 3) and to VLDL (Figures 4 and 5) is consistent with apoE−lipid interactions being dominant in both cases. The fact that modifying the C-terminal domain has different effects on binding of apoE to VLDL and HDL is consistent with different interactions mediating

Table 3: Influence of Point Mutations in ApoE3 and ApoE4 on Binding to Lipoprotein Particles

	relative binding[a]	
	VLDL	HDL_3
apoE3		
WT	1.0	1.0
K146E	2.1 ± 0.5	1.2 ± 0.3
K146Q	2.6 ± 0.5	1.0 ± 0.2
P267A	0.5 ± 0.1	0.5 ± 0.1
G278P	0.7 ± 0.2	0.6 ± 0.2
apoE4		
WT	1.0	1.0
E255A	0.4 ± 0.1	0.5 ± 0.1
G278P	0.8 ± 0.1	0.6 ± 0.1

[a]Fractional binding of the apoE variants was determined using a VLDL/HDL_3 distribution assay under defined conditions (see Experimental Procedures) and is expressed relative to the value for the respective WT protein. The values are means ± the standard error of the mean.

the binding to HDL. As we have suggested previously, protein−protein interactions may also contribute to the binding of apoE to HDL (*26*). To further explore this concept, we employed in the VLDL/HDL_3 distribution assay the apoE variants containing point mutations that were investigated for their abilities to bind to lipid emulsions (Table 2). Most of the point mutations have similar consequences for binding of apoE to lipid emulsion and VLDL. Notable exceptions are the K146E and K146Q mutations in the helix bundle domain that greatly enhance binding of apoE3 to VLDL but not to the lipid emulsion (Table 3). A possible explanation for this effect is that, in addition to apoE−lipid interaction, some apoE−apoB interaction contributes to binding of apoE to VLDL. Interestingly, the same mutations do not affect binding of apoE3 to HDL_3 (Table 3), suggesting that electrostatic interactions between this region of the apoE molecule and apolipoproteins resident in the HDL_3 particle surface are not important for apoE binding.

DISCUSSION

The goal of this study is to understand the mechanisms responsible for the preferences in VLDL and HDL binding exhibited by apoE3 and apoE4. To this end, we have pursued a mutagenesis strategy designed to identify the particular region of the apoE molecule that confers lipid binding ability and compare the properties of this region in the two isoforms. Also, by comparing binding of apoE3 and apoE4 to a lipid emulsion on the one hand and VLDL or HDL on the other, we addressed the question of how the lipid binding capabilities of the two isoforms influence binding to the two types of lipoprotein particles.

Lipid Interactions of ApoE3 and ApoE4. The results in Figures 1 and 2 showing that the C-terminal domain (residues 192−299) binds lipids much better than the N-terminal domain (residues 1−191) confirm results found in the literature (for reviews, see refs *4* and *31*). Furthermore, the region spanning residues 261−299 is primarily responsible for this lipid binding ability because deletion of this segment from either apoE3 or apoE4 leads to the same weakening in the ability to bind to a lipid emulsion as seen when the entire C-terminal domain is removed (cf. Figures 2 and 3). The idea that residues 261−299 control the binding of apoE to lipid is confirmed by the observation that removal of residues 192−260 from the C-terminal domain does not weaken emulsion binding in either apoE3 or apoE4 (Figure 3).

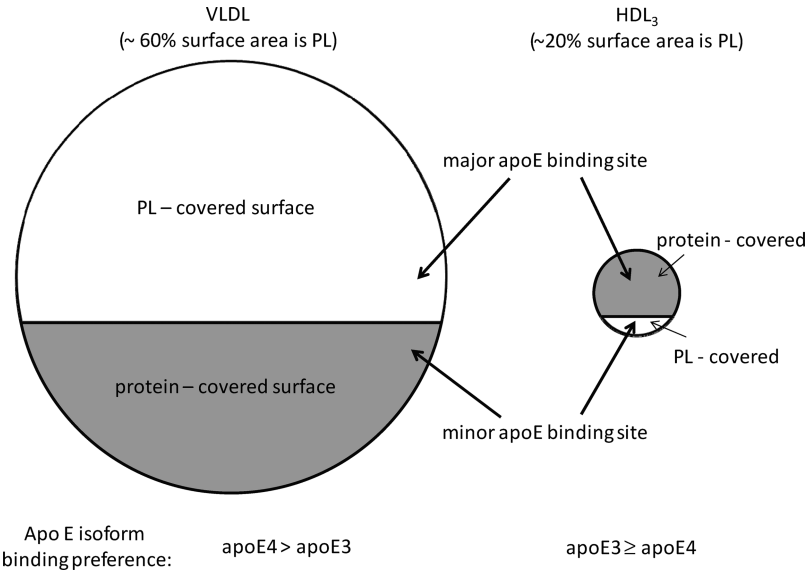

FIGURE 6: Model for isoform specificity of human apoE binding to human VLDL and HDL_3. ApoE4 binds better than apoE3 to lipid surfaces such as that of phospholipid (PL)-stabilized triolein emulsion particles because of C-terminal domain structural differences induced by the C112R substitution that distinguishes apoE4 from apoE3. The lipid binding ability of apoE is particularly mediated by the C-terminal segment spanning residues 261−299, and the better lipid binding ability of apoE4 is a consequence of the region encompassing residues 261−272 being organized differently in apoE4 and apoE3. As shown in the diagram, the surface area of a VLDL particle (~40 nm diameter) is ~60% PL-covered (see the text), so that the stronger lipid binding ability of apoE4 causes it to bind more than apoE3. ApoE-resident apolipoprotein (primarily apoB-100) interactions play a relatively minor role in the binding of apoE to VLDL. In contrast, apoE-resident apolipoprotein (especially apoA-I and apoA-II) interactions are significant for binding of apoE to HDL_3 (8 nm diameter) because these proteins cover ~80% of the particle surface. In this case, the protein−protein interaction is mediated primarily by the apoE N-terminal helix bundle domain (residues 1−191) and apoE3 tends to bind better than apoE4 to HDL_3. In the diagram, the relative sizes of the VLDL and HDL_3 particles are shown approximately to scale.

Thus, the entire C-terminal domain is not required for efficient lipid binding. Importantly, relative to apoE3, the emulsion binding capability of apoE4 is stronger and more sensitive to removal of residues 261−299; the latter point is reflected by the fact that the reduction in the level of binding relative to that of intact apoE4 is larger than the equivalent reduction for apoE3 (Figure 3) (cf, refs *17* and *26*). While both ends of the C-terminal segment spanning residues 261−299 contribute to lipid binding (cf. the results for the Δ261−272 and 1−272 variants in Figure 3), the fact that deletion of residues 261−272 induces the greater reduction in the level of emulsion binding (Figure 3) is consistent with this segment exerting the dominant effect on lipid binding. The contribution of this segment to lipid binding is greater in apoE4 than in apoE3, and this is true apparently because residues 261−272 exert different structural effects in apoE3 and apoE4. The α-helix contents and ANS binding results listed in Table 1 show that, while elimination of residues 273−299 [to give the apoE(1−272) variant] has similar effects in apoE3 and apoE4, elimination of residues 261−272 [to give the apoE(Δ261−272) variant] exerts different effects in the two isoforms. Namely, the reduction in α-helix content is much larger in apoE3, and the degree of exposure of the hydrophobic surface is decreased in apoE3 but increased in apoE4 (Table 1). At this point, it is apparent that residues 261−272 are most involved in the variations in C-terminal domain properties seen between apoE3 and apoE4, as well as in the enhanced lipid binding capability of the latter isoform. However, understanding of the exact structural basis for the effects of C-terminal domain modification on apoE lipid binding properties must await elucidation of the secondary and tertiary structures of the C-terminal domain, and more knowledge of how it interacts with the N-terminal helix bundle domain.

Lipoprotein Interactions of ApoE3 and ApoE4. The fact that apoE4 binds better than apoE3 to VLDL (Figure 4) raises the question of whether this effect is a consequence of the enhanced lipid binding ability of apoE4. This seems to be the case because mutations that alter the emulsion binding properties of apoE3 and apoE4 (Figures 2 and 3) induce parallel effects on VLDL binding (Figures 4 and 5). The idea that apoE−lipid interactions dominate binding to a VLDL particle is reasonable given that most of the particle surface is covered by PL. Knowing the amounts of PL and protein present in VLDL (diameter of 40 nm) and HDL_3 (diameter of 8 nm) particles (*32*) and assuming that the PL occupies 0.65 nm^2/molecule (*33*) and the proteins occupy an average molecular area of 0.15 nm^2/amino acid residue (*34*), we can estimate that ~60% of the VLDL particle surface is covered by PL. The stronger lipid binding ability of apoE4 compared to that of apoE3 promotes more binding to this PL surface, explaining the preferential VLDL binding of apoE4 (see the model in Figure 6). In addition, in apoE4 the interaction of R61 with E255 may stabilize an extended helical structure in the C-terminal domain that is better accommodated on the less curved VLDL surface (*14*). The apoE-PL surface interaction occurs by a two-state mechanism in which the C-terminal domain binds first followed by opening of the N-terminal helix bundle domain (*4*, *26*, *28*, *31*); the degree of helix bundle opening probably depends upon the available surface area (*23*, *35*).

As far as binding of apoE to HDL_3 is concerned, lipid interactions apparently play some role (more obvious with apoE4) because deletion of either the entire C-terminal domain (Figures 2 and 4) or residues 261−299 (Figures 3 and 5) has parallel effects on lipid emulsion and VLDL binding on one hand and HDL binding on the other. However, the effects of the deletions are smaller with binding to HDL (Figure 5), indicating that apoE−lipid interactions play a lesser role in this case. This conclusion is perhaps unsurprising given that the HDL_3 surface is predominantly covered with protein; surface area calculations of the type

described above for VLDL indicate that ~80% of the HDL_3 particle surface is protein-covered. Interactions with this protein surface are important for apoE binding, and because apoE−lipid interactions play an only minor role, the enhanced lipid binding capability of apoE4 does not promote more binding to HDL (Figure 6). The fact that apoE3 tends to bind better than apoE4 suggests that apoE3 participates in stronger protein−protein interactions in the HDL particle surface. By analogy to the situation with structurally related apoA-I whose interaction with the HDL particle surface has been monitored by fluorescence spectroscopy (*36*), the N-terminal helix bundle domain of apoE is likely to be involved in interactions with proteins resident on the HDL particle surface.

How are the apoE N- and C-terminal domains involved in the interactions with the apolipoproteins resident on the HDL surface? The apoE C-terminal domain is less important for binding to HDL than for binding to VLDL because the isolated N-terminal domain binds relatively well to HDL_3 but poorly to VLDL (Figure 4) (cf. ref *26*). Also, unlike the case with lipid emulsion (Figure 2) and VLDL binding (Figure 4) where the isolated C-terminal domain binds more than the isolated apoE3 N-terminal domain, the C-terminal and apoE3 N-terminal domains bind similarly to HDL_3 (Figure 4). The isolated apoE3 N-terminal domain binds better than its apoE4 counterpart to HDL_3 (Figure 4), implying that the more stable apoE3 helix bundle participates in stronger protein−protein interactions at the HDL surface. These interactions apparently do not involve electrostatic interactions with basic residues in the LDL receptor binding region of apoE3 (*4*) because the K146E and K146Q mutations do not inhibit binding to HDL_3 (Table 3).

Additional evidence of the different nature of apoE interactions with the surfaces of VLDL and HDL_3 particles comes from the observation that alterations in the C-terminal domain can have different effects on binding to the two types of lipoprotein particles. For example, deletion of residues 192−260 promotes binding of apoE3 to HDL_3 but not to VLDL (Figure 5). Along the same lines, deletion of residues 273−299 does not alter binding of apoE3 to HDL_3 but weakens binding to VLDL (Figure 5); the fact that the same deletion in apoE4 does weaken binding to HDL_3 implies that lipid interactions play a larger role with this isoform.

As summarized in the model depicted in Figure 6, there are two factors that contribute to the selectivity in the binding of apoE3 and apoE4 to VLDL and HDL_3: (1) the stronger lipid binding ability of apoE4 that arises from the reorganized C-terminal domain (especially around the segment spanning residues 261−272) in this isoform and (2) the differences in the nature of the surfaces of VLDL and HDL_3 particles. The former is largely PL-covered, whereas the latter is mostly covered by the resident apolipoproteins. In combination, these factors cause apoE4 to bind better than apoE3 to VLDL predominantly via apoE−lipid interactions and apoE3 to bind better to HDL_3 predominantly via apoE−protein interactions. These differential apoE binding effects underlie the variations in plasma VLDL−cholesterol and HDL−cholesterol levels seen in carriers of the apoE3 and apoE4 isoforms and are responsible for the different cardiovascular disease risk associated with this apoE polymorphism.

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