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Influence of Domain Stability on the Properties of Human Apolipoprotein E3 and E4 and Mouse Apolipoprotein E

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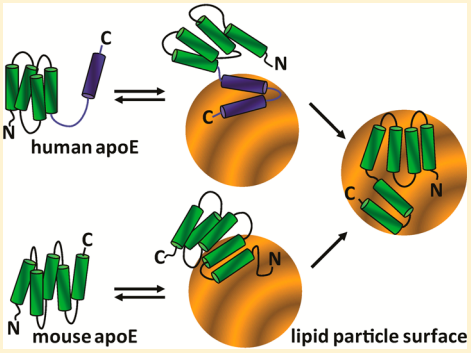
ABSTRACT: The human apolipoprotein (apo) E4 isoform, which differs from wild-type apoE3 by the single amino acid substitution C112R, is associated with elevated risk of cardiovascular and Alzheimer's diseases, but the molecular basis for this variation between isoforms is not understood. Human apoE is a two-domain protein comprising an N-terminal helix bundle and a separately folded C-terminal region. Here, we examine the concept that the ability of the protein to bind to lipid surfaces is influenced by the stability (or readiness to unfold) of these domains. The lipid-free structures and abilities to bind to lipid and lipoprotein particles of a series of human and mouse apoE variants with varying domain stabilities and domain−domain interactions are compared. As assessed by urea denaturation, the two domains are more unstable in apoE4 than in apoE3. To distinguish the contributions of the destabilization of each domain to the greater lipid-binding ability of apoE4, the properties of the apoE4 R61T and E255A variants, which have the same helix bundle stabilities but altered C-terminal domain stabilities, are compared. In these cases, the effects on lipid-binding properties are relatively minor, indicating that the destabilization of the helix bundle domain is primarily responsible for the enhanced lipid-binding ability of apoE4. Unlike human apoE, mouse apoE behaves essentially as a single domain, and its lipid-binding characteristics are more similar to those of apoE4. Together, the results show that the overall stability of the entire apoE molecule exerts a major influence on its lipid- and lipoprotein-binding properties.



Apolipoprotein (apo) E is a protein of major biological and clinical importance because it regulates lipid transport and cholesterol homeostasis in both the cardiovascular and central nervous systems.[1−4] Human apoE is a 299-residue molecule containing two independently folded domains: the N-terminal domain comprises a four-helix bundle encompassing residues 1−191 that is separated by a hinge region from the C-terminal domain, which spans residues 216−299.[5−8] The NMR structure of apoE3 indicates that there is extensive N- and C-terminal domain interaction involving salt bridges and hydrogen bonds and that the C-terminal domain presents a large exposed hydrophobic surface.[7] The protein is polymorphic and exists as three major isoforms, apoE2, apoE3, and apoE4, each differing by a single amino acid substitution. 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.[5] The isoforms are associated with different degrees of disease risk. Of particular note, relative to that for wild-type (WT) apoE3, apoE4 is associated with increased incidence of cardiovascular disease and Alzheimer's disease.[1,4] The single amino acid substitution C112R that distinguishes apoE4 from apoE3 modulates lipid- and lipoprotein-binding properties[9−11] and the ability to interact with amyloid beta peptide in the brain.[3,12] The molecular basis for these variations in properties is not understood completely.

It is established that the basis for the different effects of apoE3 and apoE4 on lipoprotein metabolism is due to the preferential binding of the latter isoform to very low-density lipoprotein (VLDL).[11,13−16] The interaction of apoE with a lipid surface involves conformational reorganization of the protein molecule so that amphipathic α-helices can insert between phospholipid (PL) molecules. Surface-bound apoE can assume two conformations: the helix bundle domain can adopt either open or closed conformations, depending upon the available surface area and the ease with which the helix bundle can unfold (i.e., its stability).[9,17] The enhanced binding of apoE4 to VLDL is a consequence of the greater lipid-binding ability of this isoform. This effect arises in some way from a reorganization of the C-terminal domain[16,18,19] induced by intramolecular interaction between the relatively unstable apoE4 N-terminal helix bundle domain (which contains the C112R substitution) and the C-terminal domain, which initiates lipid-binding.[8,14,15] The altered domain−domain

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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.[14,15] Weisgraber and colleagues have further validated this concept by observing corresponding effects in mouse apoE variants.[20,21] Although alterations in R61 and E255 in human apoE (and the corresponding positions in mouse apoE, which is six amino acids shorter[5]) clearly influence the domain−domain interaction, the effects may not be simply due to direct R61−E255 salt bridge formation but rather allosteric effects.[22] These possibilities are unresolved because the influence of R61 and E255 mutations on the structure of intact human and mouse apoE molecules has not been determined. In addition, the consequences of altering R61 and E255 on the stabilities of the N- and C-terminal domains are unknown. It is important to have this information because apoE4 differs from apoE3 not only in possessing (1) the R61−E255-mediated domain−domain interaction but also (2) a less stable helix bundle domain.[23,24] Without knowledge of the N- and C-terminal domain stabilities in the intact proteins, it is impossible to unambiguously attribute differences between the properties of apoE3 and apoE4 to one or the other of these parameters.

With a goal of resolving this problem, we examine the influence of disruption of the R61−E255 salt bridge by introduction of the mutations R61T and E255A into apoE4 and determining the effects on N- and C-terminal domain stability and lipid binding. The hypothesis being tested is that if this salt bridge disruption has no effect on lipid binding, then the enhanced lipid binding of apoE4 (relative to apoE3) is primarily due to the lower stability of the N-terminal helix bundle rather than altered domain−domain interaction. To see if these concepts apply more generally, we also study mouse apoE, which binds lipids better than apoE3, by introducing a domain−domain salt bridge via the T61R mutation and destabilizing the protein via introduction of the T61R/G83T/N113G triple mutation. The lipid-binding characteristics of the various apoE variants are evaluated by monitoring (1) binding to lipid emulsion particles, which model lipoprotein particles and present a stable lipid−water interface, (2) binding to VLDL and HDL particles, which are key events in lipoprotein metabolism, and (3) interaction with DMPC MLVs, which gives a measure of the kinetics of lipid association and ability of apoE to solubilize PL bilayers. The results show that the stabilities (ease of undergoing conformational change) of the N- and C-terminal domains have a major influence on the ability of apoE to interact with lipid and lipoprotein surfaces. This enhanced understanding of apoE structure−function relationships should provide a basis for manipulating the protein to ameliorate the pathological effects of the apoE4 isoform.

EXPERIMENTAL PROCEDURES

Materials. Human and mouse apoE variants were expressed in *Escherichia coli* as thioredoxin fusion proteins and isolated and purified as described.[9,25] Full-length human apoE3, apoE4, and the apoE4 E255A variant have been described previously.[9,16,26] The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was employed to introduce the R61T point mutation into apoE4. A pET32a expression vector containing the cDNA of mouse apoE was kindly provided by Dr. Karl Weisgraber.[20] The T61R and T61R/G83T/N113G mutations were introduced into mouse apoE as described above. The apoE preparations were at least 95% pure, as assessed by SDS-PAGE. The apoE variants were ^{14}C-trace-labeled by reductive methylation as described previously.[10,27] 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.[28] HDL_3 and VLDL were purified by sequential ultracentrifugation from a pool of normolipidemic human plasma as described previously.[16,29] 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-napthalenesulfonic acid (ANS) was purchased from Molecular Probes (Eugene, OR).

Gel Filtration Chromatography. ApoE samples were subjected to gel filtration chromatography on a calibrated Superdex 200 column using an Akta FPLC system to assess their degree of self-association, as described previously.[16,26,30,31]

Binding of ApoE to Emulsion Particles. Emulsion particles (86 ± 7 nm diameter) were prepared by sonication of a triolein/egg yolk PC mixture (3.5/1w/w) in pH 7.4 Tris buffer.[9−11] The binding of apoE was monitored by incubating ^{14}C-labeled apoE (50 μg/mL) with emulsion (300 μg PC/ml) for 1 h at room temperature and separating free and bound apoE by centrifugation, as described previously.[9]

VLDL/HDL Distribution of ApoE. The partitioning of the apoE variants between human HDL_3 and VLDL was monitored using a previously described assay.[11,16] 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 (MLV) by the apoE variants were measured by monitoring the decrease in absorbance at 325 nm, as described previously.[32] The 10 min decrease in absorbance was measured as a function of apoE concentration to obtain K_m and V_{max} values.[33]

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.[10] For chemical denaturation experiments, apoE samples at a concentration of 50 μg/mL were incubated overnight at 4 °C with urea at various concentrations. Trp emission fluorescence was then monitored at 25 °C as described previously.[34]

Circular Dichroism (CD) Spectroscopy. The average α-helix content of the apoE variants was determined by measuring CD spectra at room temperature using a Jasco J-810 spectropolarimeter.[35] The α-helix content was derived from the molar ellipticity at 222 nm, as described previously.[16]

RESULTS

Structural Characterization. *Primary Structures of Human and Mouse ApoE Variants.* The mature human and mouse apoE proteins contain 299 and 293 amino acids, respectively, and the sequences are 70% identical when aligned by either the Clustal or Blast programs. Hydropathy plots[6,36] of the two amino acid sequences are similar. As mentioned earlier, the R61 side chain is organized differently in human apoE3 and

apoE4;[14] standardizing the numbering system to the human apoE sequence (which has eight additional N-terminal residues) indicates that the equivalent mouse side chain is T61.[20,21] To investigate the consequences of having an arginine residue at position 61, we used the mouse apoE T61R variant, the isolated N-terminal domain fragment of which has been studied by Weisgraber and colleagues.[20,21] The mouse apoE variant containing the T61R/G83T/N113G triple mutation, which was shown to have a destabilized helix bundle,[21] was also utilized. To remove the putative salt bridge partner for R61 in human apoE4 and thereby alter the domain−domain interaction, we employed the apoE4 E255A variant.[10,15]

Secondary and Tertiary Structure of ApoE Variants. The data in Table 1 provides some insights into the effects of the above mutations on the structures of human and mouse apoE. Regarding the secondary structure, the α-helix content of all the variants lies in the range 59 ± 5%, indicating that major structural changes are not induced by the mutations. Prior investigations of the surface of the human apoE molecule using ANS binding to detect exposed hydrophobic surface showed that the C-terminal domain is largely responsible for creating this hydrophobic surface.[10,35] The data in Table 1 confirm that ANS binds more to apoE4 than to apoE3. The R61T mutation increases ANS binding a little, presumably because the packing in the four-helix bundle is altered, which exposes some hydrophobic surface. In contrast, the E255A mutation apparently somewhat reduces the amount of exposed hydrophobic surface. Consistent with rodent and human apoE, which exhibit similar structural features and biophysical characteristics,[21,37] ANS binding to mouse and human apoE3 is similar. Introducing the T61R single mutation into the helix bundle of mouse apoE has no effect on ANS binding, whereas the T61R/G83T/N113G triple mutation increases hydrophobic surface exposure by ∼50%, presumably as a result of destabilization and opening of the helix bundle (see below).

Table 1. α-Helix Content and ANS Binding for apoE Variants

apoE	% α-helix[a]	ANS fluorescence intensity[b]
Human		
E3	59	1.0
E4	59	1.15 ± 0.07
E4 R61T	62	1.30 ± 0.15
E4 E255A	54	1.08 ± 0.02
Mouse		
E	62	0.91 ± 0.07
T61R	64	1.02 ± 0.02
T61R/G83T/N113G	61	1.34 ± 0.02

[a]Mean ± standard deviation (1% in all cases) from four measurements. [b]Values are ratios to human apoE3. Mean ± SD; n = 4−8.

Self-association of ApoE Variants. Lipid-free human apoE molecules reversibly self-associate in a concentration-dependent manner to form tetramers in aqueous solution.[38] Previous gel filtration profiles showed that the tetramer/monomer ratio is higher for apoE3 than for apoE4 when freshly dialyzed from 6 M GdnHCl solution into Tris buffer.[16] The elution profile for apoE4 under the conditions described for Figure 1 has been published previously,[31] and the profiles for apoE4 R61T and apoE4 E255A are not significantly different (data not shown), indicating that apoE4 self-association is not affected by these mutations. Figure 1A demonstrates that, in contrast to that of human apoE, mouse apoE exists predominantly in the monomeric state under the same experimental conditions. The gel filtration profiles in Figure 1B,C indicate that introduction of the single T61R mutation and triple T61R/G83T/N113G mutations into mouse apoE promotes increased oligomerization. The increased surface hydrophobic exposure with the latter variant increases the self-association such that some larger aggregated material elutes in the void volume of the gel filtration column.

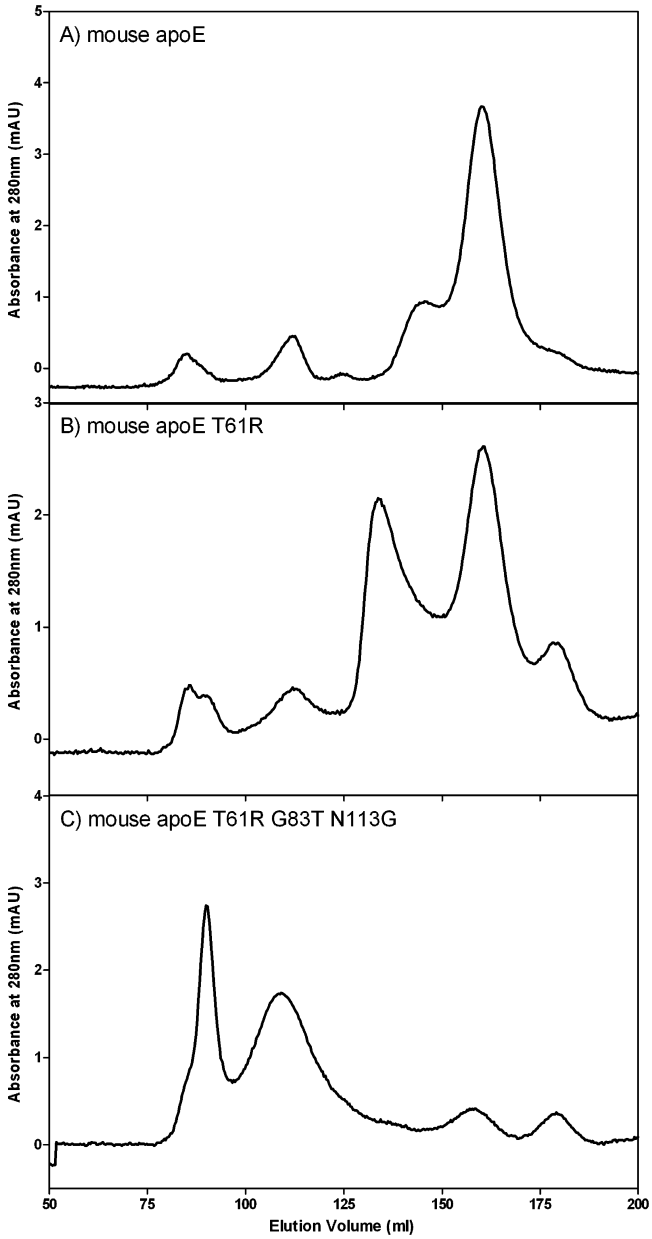

Figure 1. Elution profiles upon gel filtration of mouse apoE variants (0.2 mg/mL in Tris buffer, pH 7.4) on a Superdex 200 column: (A) WT, (B) T61R, and (C) T61R/G83T/N113G. The void and total volumes of the column are 92 and 236 mL, respectively. Monomeric apoE elutes at 159 mL, and the peaks at elution volumes in the range 110−150 mL correspond to self-associated states.

Stability of ApoE Variants. Because of the separate unfolding of the α-helices in the N- and C-terminal domains, human apoE3 and apoE4 exhibit biphasic denaturation curves, as measured by changes in molar ellipticity when exposed to increasing concentrations of either GdnHCl[23] or urea.[39] The

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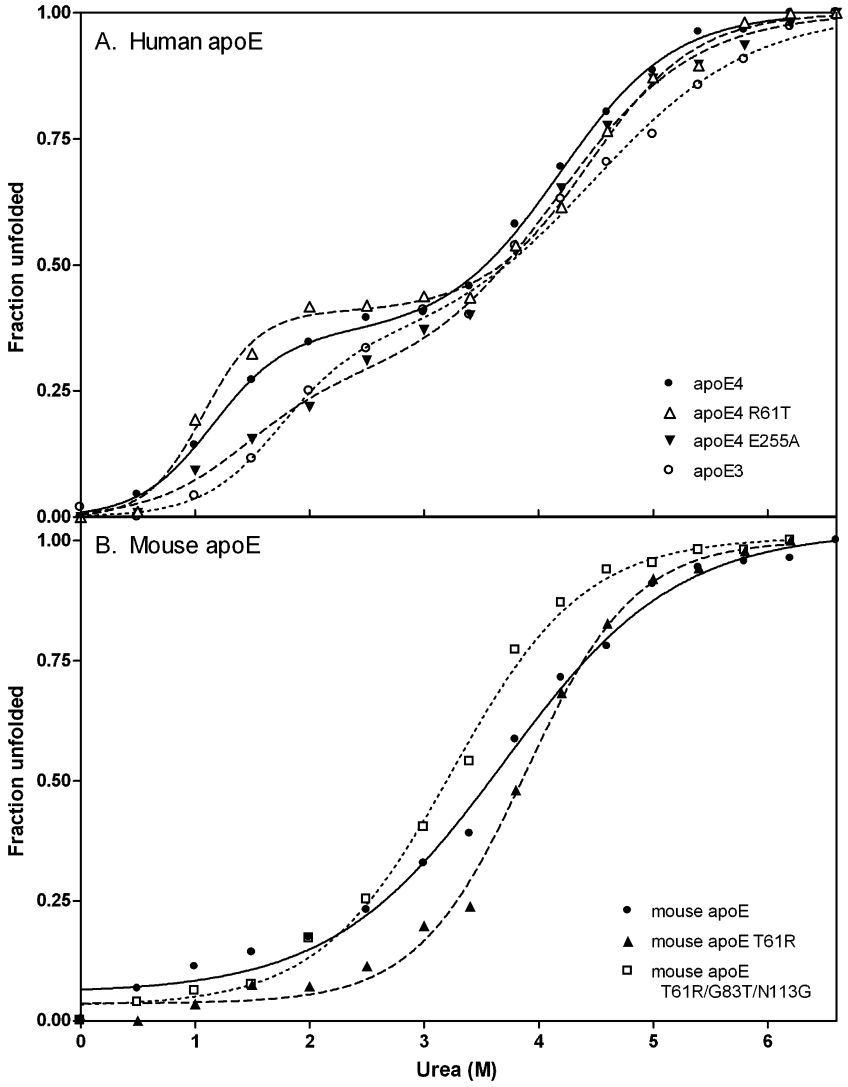

Figure 2. Urea denaturation of apoE variants monitored by Trp fluorescence. (A) Human apoE: apoE3 (○), apoE4 (●), apoE4 R61T (△), and apoE4 E255A (▼). (B) Mouse apoE: WT mouse apoE (●), mouse apoE T61R (▲), and mouse apoE T61R/G83T/N113G (□). The data points are averages from two independent experiments, which gave similar results.

data in Figure 2A confirm that biphasic denaturation curves are obtained when the Trp fluorescence of apoE3 and apoE4 is monitored as a function of urea concentration; the C-terminal domain unfolds over the range 0−2 M urea and the N-terminal helix bundle domain unfolds in the range 3−6 M urea. It is apparent that both domains are less stable in apoE4 than in apoE3. Because the mutations in the apoE variants involve alterations in charged amino acids, nonionic urea was used as a denaturant rather than the ionic GdnHCl denaturant so that the contributions of electrostatic interactions were not suppressed. Interestingly, the two mutations, R61T and E255A, have opposite effects on the stability of the C-terminal domain; the former mutation exerts a destabilizing effect and the latter exerts a stabilizing one such that the C-terminal domain stability of the apoE4 E255A variant is similar to that of apoE3 (Figure 2A). The N-terminal helix bundle domains are equally stable in these two apoE variants, with the stability being marginally less than that of the apoE4 helix bundle.

The denaturation curves in Figure 2B show that the unfolding behavior of the mouse apoE variants is strikingly different from that of the human apoE variants. The denaturation curves are not biphasic, indicating that separate unfolding of the N- and C-terminal domains is not evident; presumably, the stability across the whole mouse apoE molecule is uniform, so the unfolding is essentially a two-state process. It should be noted that mouse apoE contains one less Trp residue than the seven present in human apoE (human apoE contains W210, whereas the equivalent position in mouse apoE is F210), but W264 and W276 are present to report on the C-terminal region in experiments using Trp fluorescence. Monitoring the unfolding by CD rather than Trp fluorescence also does not reveal clear biphasic denaturation behavior (data not shown). It is apparent from the shapes of the denaturation curves in Figure 2B that introduction of the T61R point mutation into the mouse apoE molecule increases the cooperativity of unfolding. The two-state denaturation of this variant was analyzed as described previously[26,40] to determine its stability; the free energy of denaturation (ΔG_D) and midpoint of denaturation ($D_{1/2}$) are 3.8 ± 0.2 kcal/mol and 3.7 ± 0.4 M urea, respectively. The $D_{1/2}$ value for WT mouse apoE is 3.5 ± 0.5 M urea, indicating that the amino acid substitution does not significantly affect the overall stability of the protein. Consistent with the lack of effect of the T61R mutation on the overall stability of the mouse apoE molecule, this point

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mutation does not affect the stability of the isolated helix bundle domain of mouse apoE.[21] In contrast, the triple amino acid substitution in the mouse apoE, T61R/G83T/N113G, maintains the more cooperative unfolding seen with the R61T variant (Figure 2B) but destabilizes the protein (ΔG_D = 3.0 ± 0.1 kcal/mol and $D_{1/2}$ = 3.1 ± 0.2 M urea). It is apparent from the shape of the denaturation curves in Figure 2B that the cooperativity of unfolding of the two mouse apoE variants is similar and that the m values obtained from the analysis of the two-state denaturation are the same (1.0 ± 0.04 kcal/mol apoE/mol urea). In comparison, the denaturation curve for WT mouse apoE (Figure 2B) is less cooperative (m = 0.6 ± 0.1 kcal/mol/mol urea) because, although the data fit better to a monophasic curve, a trend to partial biphasic denaturation is apparent (preventing reliable determination of a ΔG_D value). Because the cooperativity of denaturation of the 22 kDa forms of WT and T61R mouse apoE is the same,[21] it follows that the lower cooperativity of unfolding of intact WT mouse apoE occurs because the C-terminal α-helices are relatively unstable and unfold at lower urea concentrations. This latter effect is attenuated by the presence of the T61R mutation.

Functional Characterization. *Interactions of ApoE with Lipid Emulsion Particles.* As we showed previously,[10,11] apoE4 binds more than apoE3 to lipid emulsion particles; the data in Figure 3A are consistent with this conclusion. Also, it is apparent that mouse apoE binds more than apoE3 and similarly to that of apoE4. Regarding apoE4, it is apparent that the N-terminal domain R61T mutation, which eliminates the putative interdomain R61−E255 salt bridge, has no effect on the amount of protein bound (Figure 3B). In contrast, the C-terminal domain E255A mutation increases the degree of apoE binding. The results in Figure 3C show that creation of a potential interdomain salt bridge via introduction of the T61R mutation in mouse apoE tends to increase the degree of binding to the emulsion particles. The T61R/G83T/N113G triple mutation, which destabilizes the protein molecule (Figure 2B), has no effect on binding relative to that of WT mouse apoE, but it significantly decreases the amount bound relative to that of the T61R variant (Figure 3C).

Solubilization of DMPC MLV by ApoE. Figure 4A confirms that apoE4 solubilizes DMPC MLV more rapidly than apoE3 does.[11,16,32] The reason for this effect is the lower degree of self-association of apoE4, which enhances the rate of lipid binding by the monomer and an increased rate of DMPC bilayer conversion to discoidal HDL particles.[39] The clearance rate for mouse apoE is the highest (Figure 3A), presumably because mouse apoE exists mostly in the monomeric state (Figure 1A). The effectiveness of mouse apoE is maintained over a range of protein concentrations (Figure 4B), and the calculated catalytic efficiencies (V_{max}/K_m) (Table 2) give the order of effectiveness of DMPC MLV solubilization as mouse apoE > apoE4 > apoE3. The apoE4 R61T and E255A variants have unaltered catalytic efficiencies (Table 2); the result for the latter variant contrasts with a previous study where we observed somewhat slower clearance relative to apoE4.[16] The T61R mutation in mouse apoE also has no effect on DMPC MLV clearance kinetics (Table 2). In contrast, destabilization of the mouse apoE molecule by the T61R/G83T/N113G triple mutant significantly increases the catalytic efficiency (Table 2), indicating that the rate of DMPC MLV clearance can be sensitive to helix bundle stability.

Interactions of ApoE with Lipoprotein Particles. Previously, we have examined the partitioning of apoE3 and apoE4 between VLDL and HDL_3 particles.[11,16] The data in Figure 5A confirm that apoE4 binds better than apoE3 to VLDL. It is apparent that mouse apoE binds more like that of apoE4 than apoE3, as also occurs with binding to lipid emulsion particles (Figure 3A). This concurrence is expected because apoE binding to VLDL and lipid emulsions occurs predominantly by interactions with PL molecules in the particle surface.[11] For this reason, more-or-less parallel effects of the R61T and E255A mutations in apoE4 occur with VLDL binding (Figure 5B) and emulsion binding (Figure 3B). The data in Figure 3C and 5C demonstrate that the T61R and T61R/G83T/N113G mutations also have similar effects on the binding of mouse apoE to lipid emulsions and VLDL.

In contrast to the interaction of apoE with VLDL particles, where protein−lipid interactions dominate, apoE−resident apolipoprotein interactions are also involved in the binding to HDL_3 particles.[11] As shown in Figure 6A, for this reason, apoE3 and apoE4 bind similarly to HDL_3. Strikingly, mouse apoE binds to HDL_3 particles to a much reduced degree, implying that mouse apoE−resident apolipoprotein interactions are less favorable in this case. The mutations in both human and mouse apoE that can effect emulsion and VLDL binding (Figures 3 and 5) have little or no effect on HDL_3 binding (Figure 6B,C).

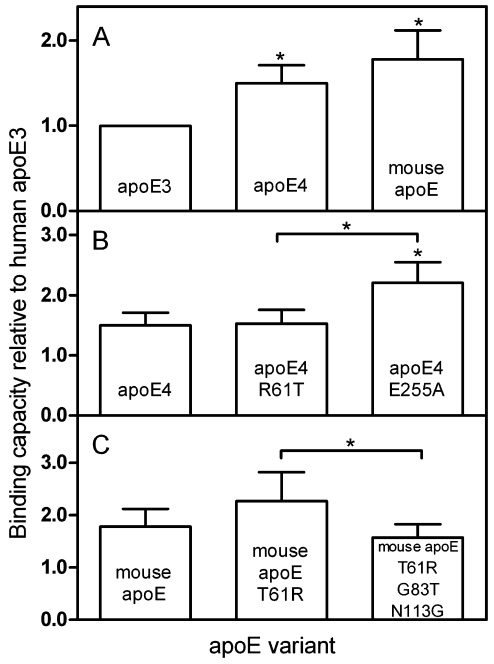

Figure 3. Binding of human and mouse apoE variants to triolein/PC emulsion particles. The binding assay described in Experimental Procedures was used to determine the fraction of each apoE variant that bound to the emulsion particles. This value is normalized to the fraction (17 ± 3%, n = 5) of human apoE3 bound. (A) Comparison of the binding of human apoE3, apoE4, and mouse apoE. (B) Influence of the R61T and E255A point mutations on the binding ability of apoE4. (C) Influence of the T61R and T61R/G83T/N113G mutations on the binding ability of mouse apoE. One-way analysis of variance (ANOVA) followed by a Tukey's multiple-comparison test using GraphPad Prism 4.0 was used to evaluate statistically significant differences. An asterisk above the error bar indicates that the variant value is significantly different (p < 0.05) from the reference apoE (apoE3, apoE4, and mouse apoE in panels A, B and C, respectively). An asterisk above a bar between the values for two apoE variants indicates that the values are statistically different (p < 0.05).

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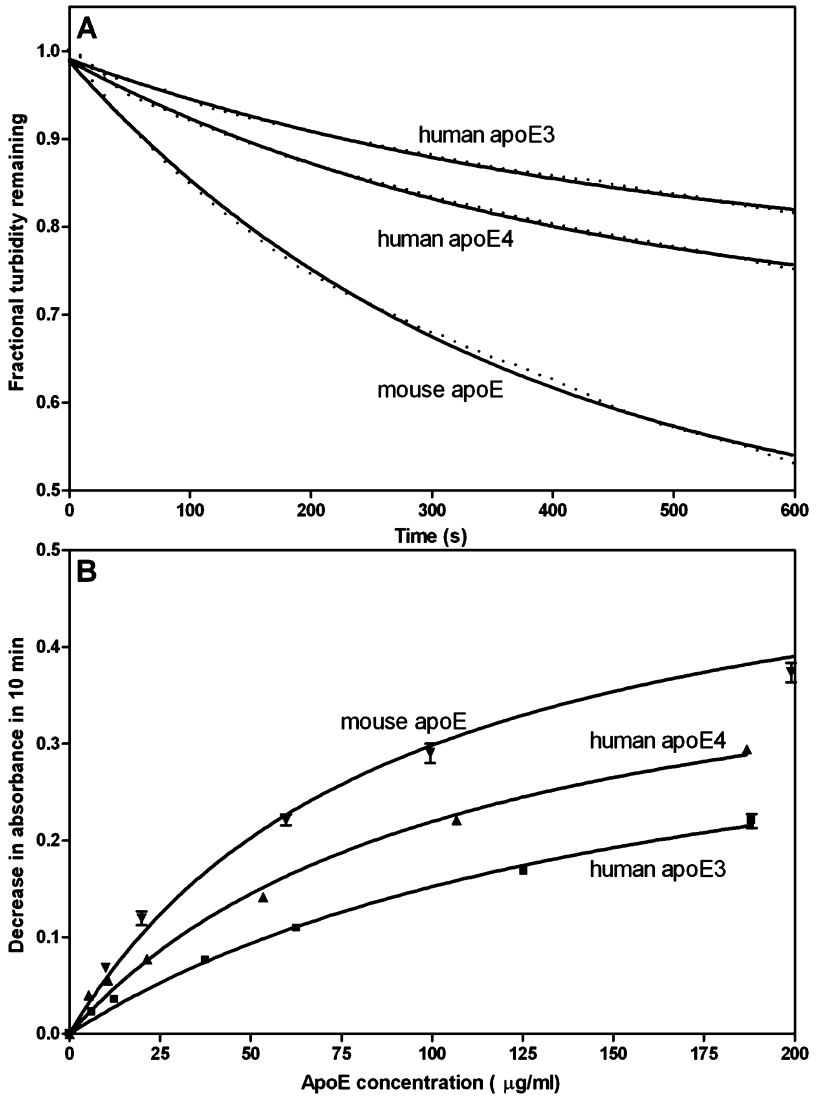

Figure 4. Solubilization of DMPC MLV by human and mouse apoE. (A) Representative time courses of the decrease in absorbance at 325 nm (recorded every 10 s) when DMPC MLV (0.25 mg/mL) was incubated with 60 μg/mL protein for 10 min at 24.0 ± 0.1 °C. The measured absorbance values (solid circles) are fitted to a monoexponential decay equation (continuous lines). (B) Effect of protein concentration on the decrease in absorbance in 10 min; the experimental data are fitted to the Michaelis–Menten equation.

Table 2. Comparison of the Ability of apoE Variants to Solubilize DMPC MLV[a]

apoE	V_{max} (absorbance decrease in 10 min)	K_m (μM)	relative catalytic efficiency (V_{max}/K_m)
Human			
E3	0.41 ± 0.03	4.9 ± 0.6	1.0
E4	0.45 ± 0.03	3.1 ± 0.3	1.8
E4 R61T	0.43 ± 0.04	3.4 ± 0.1	1.6
E4 E255A	0.50 ± 0.07	3.3 ± 0.8	1.8
Mouse			
E	0.56 ± 0.02	2.6 ± 0.2	2.7
T61R	0.50 ± 0.03	2.2 ± 0.3	2.8
T61R/G83T/N113G	0.57 ± 0.03	2.1 ± 0.3	3.4

[a]Kinetic parameters were obtained from data of the type shown in Figure 4B and fitting to the Michaelis–Menten equation. Values are mean ± SE (n = 21).

The partitioning of apoE between VLDL and HDL_3 has important consequences for clearance of triglyceride-rich lipoprotein remnants.[41] Indeed, the enhanced VLDL/HDL_3 distribution of apoE4 compared to that of apoE3 (Figure 7) is the underlying cause of the higher ratio of VLDL cholesterol/HDL_3 cholesterol seen in the plasma of mice expressing apoE4 rather than apoE3.[42] Introduction of the N-terminal domain R61T mutation into apoE4 reduces the VLDL/HDL_3 binding ratio to a value lower than that of apoE4 (cf. ref 14). The C-terminal domain E255A mutation in apoE4 has little or no effect on the VLDL/HDL_3 lipoprotein distribution; this is in contrast to a prior report that used a different assay involving whole plasma and where a decrease in the ratio was observed.[15] Primarily because of a reduced ability to bind to HDL_3, the three mouse apoE variants exhibit markedly enhanced VLDL/HDL_3 binding ratios relative to either apoE3 or apoE4 (Figure 7).

DISCUSSION

A major question regarding human apoE is what is the molecular basis for the different lipid and lipoprotein binding properties of the apoE3 and apoE4 isoforms, given that there is

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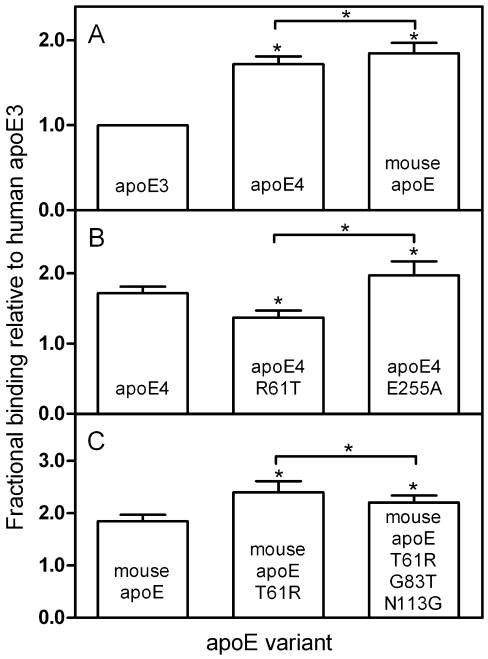

Figure 5. Binding of human and mouse apoE variants to human VLDL. The VLDL/HDL_3 distribution assay described in Experimental Procedures was used to determine the fraction of each apoE variant that bound to the VLDL particles, and this value is normalized to the fraction (13 ± 1%) of human apoE3 bound. The data in panels A–C correspond to the equivalent data in Figure 3. The fractional binding values are plotted as mean ± standard deviation (SD) (n = 9). The statistical test described for Figure 3 was applied, and the asterisk indicates differences significant at the $p < 0.05$ level.

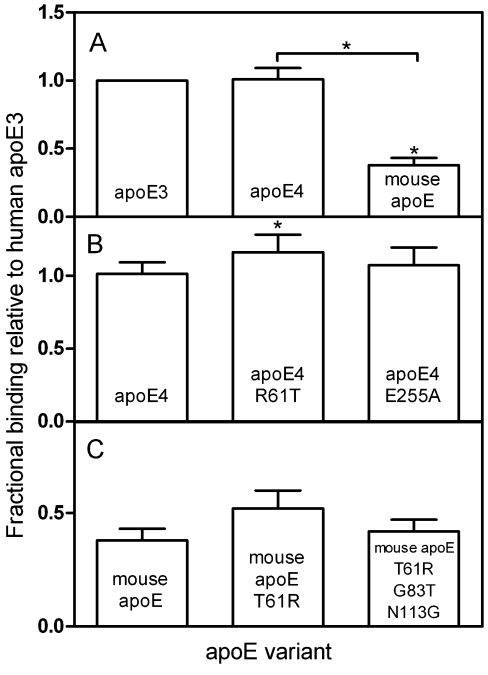

Figure 6. Binding of human and mouse apoE variants to human HDL_3. The fractional binding was determined as described in the legend to Figure 3, and the values are normalized to the value for human apoE3 (14 ± 1%). The data in panels A–C correspond to the equivalent data in Figure 3. The statistical test described for Figure 3 was applied, and the asterisk indicates differences significant at the $p <$ 0.05 level.

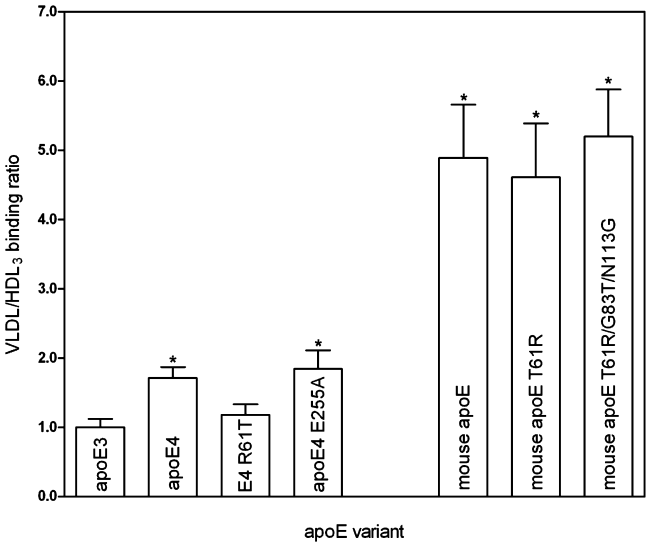

Figure 7. Relative binding of human and mouse apoE variants to VLDL and HDL_3. The VLDL/HDL_3 distribution of each protein was calculated from the values presented in Figures 5 and 6. The ratios are plotted as mean ± SD (n = 9), and the asterisk indicates significant difference by ANOVA ($p < 0.05$) from the ratio for human apoE3.

just a single amino acid substitution in the 299 amino acid protein molecule? Here, we examine the concept that a major requirement for effective interaction of apoE with the surfaces of lipid and lipoprotein particles is the ability to readily undergo conformational reorganization. Thus, the stability of an apoE molecule, which reflects its ability to unfold, is expected to modulate surface-binding events. The present results for a series of apoE variants with altered structural stabilities, domain properties, and binding interactions provide an experimental test of this concept.

Human ApoE3 and ApoE4 Domain Stability and Lipid- and Lipoprotein-Binding Properties. The urea denaturation data in Figure 2A show that the N- and C-terminal domains are less stable in apoE4 than in apoE3. The N-terminal helix bundle domain is destabilized by replacement of the small cysteine side chain at position 112 with the bulky and positively charged arginine side chain.[24] This destabilization of the helix bundle domain in apoE4 enhances lipid binding because the isolated 22 kDa N-terminal fragment of apoE4 binds better to lipid emulsion particles[10] and solubilizes DMPC MLV more effectively[32] than the 22 kDa apoE3 counterpart. Relative to the situation in apoE3, the C-terminal domain in apoE4 is destabilized by altered interaction with the helix bundle that is mediated, at least in part, by a R61–E255 salt bridge.[14,15] Consequently, the entire apoE4 molecule is less stable and unfolds more readily than the apoE3 molecule. As a result of this altered structural stability of the entire molecule, apoE4 exhibits enhanced lipid-binding activity relative to that of apoE3, as reflected by both DMPC MLV solubilization (Figure 4 and Table 2) and emulsion binding (Figure 3A). The relative contributions of the destabilizations of the N- and C-terminal domains to this altered functionality of apoE4 have been unknown heretofore.

The influences on lipid interaction of destabilization of the N-terminal helix bundle domain as compared to the C-terminal domain can be ascertained by comparison of the behavior of the apoE4 R61T and E255A variants. Formation of the putative R61–E255 salt bridge that mediates interaction between the N-

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and C-terminal domains is eliminated in these two proteins, and the helix bundle stability is the same in both proteins and is very similar to that of WT apoE4. However, the mutations have opposite effects on apoE4 C-terminal domain stability, with the R61T change causing some destabilization and the E255A change inducing stabilization (Figure 2A). This difference in effect presumably arises because the R61T mutation indirectly affects C-terminal domain stability by altering domain−domain interaction, whereas the E255A mutation has a direct local effect on the conformation of the C-terminal domain. As far as lipid binding ability goes, the R61T mutation has essentially no effect on emulsion binding ability (Figure 3B) and slightly decreases DMPC solubilization activity (Table 2). Conversely, the E255A mutation in apoE4 increases emulsion binding and has no effect on DMPC solubilization. Thus, by these criteria, destabilization of the C-terminal domain (at constant helix bundle stability) tends to decrease the lipid binding ability of apoE4, whereas stabilization of the C-terminal domain tends to increase it. However, it is clear that, unlike the situation with apoE4 where simultaneous destabilization of both domains leads to a marked enhancement of lipid binding (emulsion binding and DMPC MLV solubilization), a change in stability of the C-terminal domain alone has relatively minor consequences for such lipid interactions. Importantly, altering the stability of the C-terminal domain of apoE4 by the above mutations does not decrease lipid binding to that exhibited by apoE3 (Figure 3A,B and Table 2). Overall, the above data suggest that the stability of the entire apoE molecule is the feature that determines its lipid-binding activity.

Interaction of Mouse ApoE Variants with Lipids. As noted earlier, the denaturation data in Figure 2B indicate that, in contrast to that of human apoE3 and apoE4, the mouse apoE molecule unfolds essentially as a single entity. Consequently, mouse apoE provides a suitable system for exploring the role of the stability of the entire protein molecule. The performance of mouse apoE in binding to lipid emulsions (Figure 3A), solubilizing DMPC MLV (Figure 4A), and interacting with VLDL particles (Figure 5A) is closer to that of apoE4 than to that of apoE3. Because mouse apoE behaves as if it comprises a single folded domain, it is unlikely to mirror human apoE in being able to adopt two surface conformations (helix bundle open and closed).[9,29]

The VLDL/HDL ratio for mouse apoE is higher than the values for apoE3 and apoE4 (Figure 7) because binding to HDL_3 is a lot less than occurs with apoE3 and apoE4 (Figure 6). This difference in binding behavior is presumably the basis for the variation in susceptibility to dietary-induced hypercholesterolemia and atherosclerosis between mice expressing either apoE3 or mouse apoE.[43] The reduced interaction of mouse apoE with HDL is most likely due to altered protein−resident apolipoprotein interactions, given that the HDL particle surface is approximately 80% protein-covered.[11] The T61R mutation was introduced into mouse apoE to create R61−E255-mediated domain−domain interaction,[20] and this occurs without a change in overall protein stability (Figure 2). This variant has similar VLDL and HDL binding to WT mouse apoE (Figure 7). This observation indicates that the R61−E255-mediated domain−domain interaction has little effect on partitioning of the protein between lipoproteins; this effect occurs because the overall stability characteristics of the apoE molecule play a more important role. On this basis, one would expect the T61R mutation in mouse apoE to exert only a minor effect on lipoprotein metabolism in mice. In agreement with this idea, mice expressing either WT mouse apoE or the T61R variant have similar plasma lipid levels and lipoprotein cholesterol profiles when fed a chow diet;[44] the T61R variant is associated with slightly increased VLDL cholesterol levels for mice fed a high-fat−high-cholesterol diet.

In conclusion, this study of the structure−function relationships of some human and mouse apoE variants reveals that the overall stability of the protein exerts a major influence on its lipid- and lipoprotein-binding properties. The C112R substitution that distinguishes human apoE4 from apoE3 is located in the N-terminal helix bundle domain where it exerts a direct intradomain destabilizing effect. In addition, the presence of R112 in apoE4 has an indirect interdomain effect on the stability and organization of the C-terminal domain. The simultaneous destabilization of the N- and C-terminal domains in apoE4 enhances its lipid-binding capabilities relative to those of apoE3. The experimental results presented here indicate that the direct helix bundle destabilization induced by the presence of R112 is the major contributor to this outcome. The pathological properties of apoE4 perhaps could be offset by increasing the stability of the entire molecule to that of apoE3, which would primarily involve counteracting the effects of R112 on the stability of the helix bundle domain.

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Notes

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ABBREVIATIONS USED

ANS, 8-anilino-1-napthalenesulfonic acid; Apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine; GdnHCl, guanidine hydrochloride; HDL, high-density lipoprotein; MLVs, multilamellar vesicles; PC, phosphatidylcholine; PL, phospholipid; VLDL, very low-density lipoprotein; WT, wild type

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