Human apolipoprotein (apo) E contains an amino- and a carboxy-terminal domain, which are connected by a hinge region (approximately residues 165 to 215). The interaction of the two domains has been suggested to be responsible for the apoE4-binding preference for very low density lipoproteins (VLDL). In the absence of this interaction in apoE3, the preference is for high density lipoproteins (HDL). To exclude the possibility that the interaction of apoE with other apolipoproteins on the native particles may contribute to the isoform-specific preferences, VLDL-like emulsion particles were incubated with apoE, and the lipid-bound apoE was separated from free apoE on a Superose 6 column. The apoE4 bound more effectively to these particles than did apoE3, indicating that the apoE4 preference for VLDL is due not to interactions with other apolipoproteins but to an intrinsic property of apoE4, likely related to domain interaction. Previously, arginine 61 was shown to be critical for the isoform preferences, suggesting that it interacted with an acidic residue(s) in the carboxyl terminus. Substitution of arginine 61 with lysine did not alter the preference of apoE4 for VLDL, demonstrating that a positive charge rather than a specific requirement for arginine is critical for domain interaction. To identify the acidic residue(s) in the carboxyl terminus interacting with arginine 61, the six acidic residues (244, 245, 255, 266, 270, and 271) in a region known to be important for both lipoprotein association and isoform-specific preferences were substituted individually with alanine in apoE4. Only substitution of glutamic acid 255 altered the preference of apoE4 from VLDL to HDL, indicating that this was the sole residue in the carboxyl terminus that interacts with arginine 61. The participation of the hinge region in domain interaction was examined with internal deletion mutants. Deletion of the residues 186–202 or 186–223, representing major portions of the hinge region, had no effect on the apoE4 preference for VLDL. This suggests that the hinge region may act as a spacer that connects the two domains. Further deletion into the carboxy-terminal domain (to residue 244) results in a loss of apoE4 VLDL binding. These studies establish that interaction of arginine 61 and glutamic acid 255 mediates apoE4 domain interaction.

Apolipoprotein (apo) E (299 amino acids) plays an important role in lipoprotein metabolism through its interaction with the low density lipoprotein (LDL) receptor (1, 2). The three common isoforms of apoE (apoE2, apoE3, and apoE4) are distinguished from each other by their cysteine/arginine content at two polymorphic sites. Apolipoprotein E3 contains cysteine and arginine at positions 112 and 158, respectively, whereas apoE2 contains cysteine and apoE4 contains arginine at both sites (3).

Apolipoprotein E4 is associated with higher plasma cholesterol and LDL concentrations than apoE3 and apoE2 and, as a result, is a risk factor for cardiovascular disease (4–8). One of the distinct metabolic characteristics of apoE4 is its preferential association with very low density lipoproteins (VLDLs) (9–11), which may contribute to elevated cholesterol and LDL levels (4, 11). In addition to its role in lipoprotein metabolism, apoE plays an important role in neurobiology (12, 13), the apoE4 allele being a major risk factor for Alzheimer’s disease (14–16).

Apolipoprotein E contains two structural domains, which are connected by a hinge region (approximately residues 165 to 215) (17, 18). The amino-terminal domain (residues 1–191, 22-kDa fragment) contains the receptor-binding region (19–21), and the carboxyl-terminal domain (residues 216–299, 10-kDa fragment) contains the major lipid-binding determinants (11, 22–24). Although the two domains are independently folded, each can influence the properties of the other (11, 25, 26), a concept referred to as domain interaction (2). For example, the substitution of cysteine 112 by arginine in the amino-terminal domain of apoE4 influences the lipid-binding property of the carboxyl-terminal domain and results in the preference of apoE4 for VLDL (11). In the absence of this interaction, as is the case with apoE3, the preference is for high density lipoproteins (HDL).

The domain interaction in apoE4 requires the positive charge at position 112 (11). However, this charge does not interact directly with the carboxyl-terminal domain (22); rather, it changes the conformation of the arginine 61 side chain compared with that in apoE3 (22). Replacement of the arginine 61 with threonine shifts the lipoprotein preference of apoE4 from VLDL to HDL (22). This result suggests two possibilities: 1) the change in the conformation of the arginine 61 side chain results in an interaction of apoE4 with other apolipoproteins on VLDL particles, accounting for VLDL preference; or 2) arginine 61, as a key mediator of intramolecular domain interaction in apoE4, interacts with one or more acidic residues in the carboxyl terminus, probably by salt bridge formation.

To distinguish between these possibilities, VLDL-like emulsion particles were used to examine the relative affinities of apoE4 to various particles.
apoE3 and apoE4. Mutagenesis studies focused on three aspects of domain interaction: 1) the arginine 61 residue, 2) acidic residues in the carboxyl terminus that could potentially interact with arginine 61, and 3) the hinge region, whose role in domain interaction is unknown. The specific requirement of arginine 61 for domain interaction was examined by replacing this residue with lysine. To identify the acidic residue(s) in the carboxyl terminus that interacts with arginine 61, a series of apoE4 mutants were prepared with alanine replacing acidic residues 244–272, a region known to be critical for both lipoprotein association and the isoform-specific preferences (22). The role of the hinge region in apoE4 domain interaction was examined by generating internal deletions.

MATERIALS AND METHODS

Binding of ApoE Isoforms to VLDL-like Emulsion Particles—The VLDL-like emulsion particles were prepared as described previously (27, 28). Briefly, triolein (100 mg, Sigma) and egg yolk phosphatidylcholine (25 mg, Sigma) were mixed together and then dried under a stream of nitrogen. The pellets were resuspended in 5 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 1 mM EDTA and then sonicated as described previously (28). The triolein/phosphatidylcholine ratio of the resulting emulsion particles was 2.7:1 (w/w). The morphology and size of the particles were determined by negative staining electron microscopy. The emulsion particles were spherical with an average diameter (35.8 ± 14.9 nm) similar to that of native human VLDL (34.5 ± 18.7 nm) as previously shown (29). The apoE isoforms were reduced with β-mercaptoethanol (1% final concentration) and then incubated with the emulsion particles at 37 °C for 2 h. The emulsion particle-bound apoE was separated from unbound apoE on a Superose 6 column (10/50 HR, Pharmacia Fine Chemicals, Uppsala, Sweden) and then the emulsion particle-bound apoE was separated from unbound apoE on a Superose 6 column (10/50 HR, Pharmacia Fine Chemicals). The column was eluted with 20 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. The 125I content was determined in a Beckman 8000 counter (Beckman Instruments).

Expression and Purification of ApoE Mutants—The glutathione S-transferase (GST) fusion protein expression vector pGEX3X (30) was used for expression of apoE mutants. Site-directed mutagenesis or deletions within the hinge region were performed by polymerase chain reaction (22). The sequences of constructs were verified with an ABI373A Sequencer (Perkin-Elmer). The expression plasmids were transformed into the Escherichia coli strain DH1 (Invitrogen). Transforms with high expression of the GST-apoE fusion protein were grown in LB medium at 37 °C, and expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (0.01% final concentration) to the culture.

The GST-apoE fusion proteins were purified by glutathione-agarose affinity chromatography and complexed with dimyristoylphosphatidylcholine (22). After cleavage of the GST-apoE fusion protein with Factor Xa (Haematologica Technologies, Essex Junction, VT) at a ratio of 200:1 (w:w, GST-apoE fusion protein:Factor Xa) at 0°C overnight, the cleavage products were applied to a DEAE-ion exchange column (21.5 × 150 mm) to remove the Factor Xa. The bound materials were eluted with an NaCl gradient (0–0.75 M). After dialysis, the protein was loaded on a Sephacryl 300 column, which was equilibrated and eluted with Tris-HCl buffer (0.1 M) containing 4 M guanidine and 1 mM EDTA (pH 7.4). The deletion variants of apoE were purified using a combination of heparin-Sepharose affinity, DEAE-ion exchange, and Sephacryl 300 chromatography (22). Human apoE3 and apoE4 were isolated from d < 1.006 g/ml lipoproteins (31).

Distribution of ApoE among Plasma Lipoproteins—Apolipoprotein E was iodinated with the Bolton-Hunter reagent (DuPont NEN) (32); specific activities ranged from 150–900 dpm/μg. The iodinated protein was reduced with β-mercaptoethanol (0.1% final concentration) and incubated with normal human plasma at 37 °C for 2 h, as described previously (22). The plasma was fractionated into various lipoprotein classes by Superose 6 column chromatography (10/50 HR, Pharmacia Fine Chemicals). The column was eluted with 20 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. The 125I content was determined in a Beckman 8000 counter (Beckman Instruments).

RESULTS AND DISCUSSION

To determine if the preferential association of apoE4 for VLDL results from protein-protein interaction of apoE4 with other apolipoproteins on native particles or from intramolecular domain interaction, apoE3 and apoE4 were incubated with VLDL-like emulsion particles at protein concentrations of 25, 50, and 100 μg/50 μl of normal human plasma at 37 °C for 2 h. Sixty 0.5-ml fractions were collected, and the 125I content in each fraction was determined by γ counting. The elution positions for VLDL, LDL, and HDL are indicated by the bars. Iodination and column fractionations were carried out as described under "Materials and Methods."

To determine if the preferential association of apoE4 for VLDL results from protein-protein interaction of apoE4 with other apolipoproteins on native particles or from intramolecular domain interaction, apoE3 and apoE4 were incubated with VLDL-like emulsion particles at protein concentrations of 25, 50, and 100 μg/50 μl, and then the emulsion particle-bound apoE was separated from unbound apoE on a Superose 6 column. As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the bound and unbound fractions, more apoE4 than apoE3 bound to the VLDL-like particles at each concentration (Fig. 1). In the unbound fraction, there was a corresponding increase in apoE3 compared with apoE4. These results demonstrate that apoE4 binds more effectively to the emulsion particles than does apoE3. Thus, the higher affinity of apoE4 for VLDL-size particles is not dependent on protein-protein interactions with other apolipoproteins on native particles but is instead an intrinsic property of apoE4, likely due to domain interaction.

We have shown that arginine 61 is a key residue involved in the apoE4 preference for VLDL. To determine if this preference requires the presence of arginine, or merely a positive charge, arginine 61 was replaced with lysine. The lysine 61 variant also displayed a preference for VLDL (Fig. 2), which suggests that a positive charge at this position is the key determinant and that the arginine is not specifically required. In addition, the results also suggest that the domain interaction of apoE4 is mediated by a single salt bridge since lysine can interact with only one acidic residue (arginine can form bifurcating salt bridges).

To identify the acidic residue in the carboxyl terminus of apoE4 that interacts with arginine 61, the region between

FIG. 1. Association of apoE3 or apoE4 with VLDL-like emulsion particles. Apolipoprotein E3 and apoE4 (25, 50, and 100 μg) were incubated with emulsion particles at 37 °C for 2 h. The emulsion particle-bound apoE was separated from unbound apoE on a Superose 6 column. The bound and unbound fractions were pooled and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

FIG. 2. Effect of lysine or arginine at position 61 on apoE4 distribution among human plasma lipoproteins. After reduction with β-mercaptoethanol, 0.5–2 μg of iodinated apoE was incubated with 250 μl of normal human plasma at 37 °C for 2 h. Sixty 0.5-ml fractions were collected, and the 125I content in each fraction was determined by γ counting.

The elution positions for VLDL, LDL, and HDL are indicated by the bars. Iodination and column fractionations were carried out as described under "Materials and Methods."
residues 244 and 272 was targeted because it contains impor-
tant structural elements for both lipoprotein association and
the isoform-specific preferences (22, 24). Therefore, the six
acidic residues in this region (glutamic acid 244, glutamic acid
245, glutamic acid 255, glutamic acid 266, glutamic acid 270,
and aspartic acid 271) were individually replaced by alanine.
Replacement of the acidic residue at position 244, 245, 266,
270, or 271 had no effect on the apoE4 distribution (i.e. all
displayed a preference for VLDL) (Fig. 3). In marked contrast,
substitution of glutamic acid 255 altered the preference from
VLDL to HDL, resulting in a distribution identical to that of
apoE3 (Fig. 4 and Table I). These results demonstrate that
glutamic acid 255 is the acidic residue in the carboxyl terminus
that interacts with the arginine 61 to direct the binding of
apoE4 to VLDL.

Once the critical interacting residues in the two domains
were identified, focus was directed to the hinge region. Al-
though much is known about the functional roles of the amino-
and carboxyl-terminal domains, little is known about potential
functions of the hinge region. To investigate whether the hinge
region contains structural elements essential for domain inter-
action or whether it acts as a spacer connecting the two do-
 mains, an internal deletion approach was used. First, residues
186–202 in both apoE3 and apoE4 (Δ186–202) were deleted,
and the effects on lipoprotein distribution were determined.
Residue 186 was chosen as the starting point for the deletions
because previous studies with carboxyl-terminal truncations of
the 22-kDa fragment (residues 1–191) demonstrated that trun-
cation of the amino-terminal to residue 186 reduced binding
activity to the LDL receptor (33). Both the apoE3 and apoE4
Δ186–202 mutants displayed distribution patterns identical to
those of the respective intact isoforms (Fig. 5A). The next
deletion was extended from residue 186 to residue 223 (Δ186–
223), deleting a major portion of the hinge region. The apoE4
Δ186–223 mutant did not alter the preference for VLDL (Fig.

FIG. 3. Distribution of apoE4 variants among human plasma
lipoproteins. The distributions were determined as described in
the legend to Fig. 2.

FIG. 4. Distribution of apoE4 variant (Glu-255 → Ala) among
human plasma lipoproteins. The distributions were determined as
in Fig. 2.

TABLE I

| ApoE       | % Distribution |
|------------|----------------|
| n          | VLDL*          | LDL/LDL*       | HDL*          |
| ApoE3      | 4              | 24.3 ± 3.5     | 21.2 ± 3.0    | 54.2 ± 3.4    |
| ApoE4      | 2              | 36.5 ± 3.7b    | 27.4 ± 3.0    | 36.0 ± 2.3c   |
| ApoE4 (Glu-255 → Ala) | 5 | 25.8 ± 3.2b     | 21.6 ± 3.9    | 51.9 ± 2.5c   |

* The VLDL, intermediate density lipoprotein (IDL)/LDL, and HDL
fractions are indicated by the bars in Fig. 3.

b p < 0.01, apoE4 versus apoE4 (Glu-255 → Ala) (t-test).

c p < 0.01, apoE4 versus apoE4 (Glu-255 → Ala) (t-test).

5B), indicating that most of the hinge region could be deleted
without affecting the lipoprotein distribution. Deletion to resi-
due 244 (Δ186–244), which extends into the carboxyl-terminal
domain and eliminates portions of a long putative amphipathic
helix (residues 216–266) (34, 35), abolished the binding of
apoE4 to VLDL and increased binding to HDL (Fig. 5C). These
results suggest that the preference of apoE4 for VLDL can be
maintained without residues 186–223 but is lost when the
deletion is extended to residue 244.

Previously, we have demonstrated with carboxyl-terminal
truncation studies of intact apoE that the region between resi-
dues 244 and 272 contains elements for both VLDL and HDL
binding (22, 24). Truncation to residue 260 resulted in the loss
of VLDL binding. In the present studies, the deletion to residue
244 in apoE4 resulted in the loss of the VLDL binding although
the critical region between residues 244 and 272 was main-
tained. The inability of the Δ186–244 mutant to bind VLDL
suggests that deletion to residue 244 removes structural ele-
ments required for VLDL binding and/or that with removal of
residues 186–244, the two domains cannot attain proper posi-
tioning for effective interaction of arginine 61 with glutamic
acid 255, which is required for the VLDL preference. Clarifi-
cation of this point will require additional studies.

Based on our present and previous studies, the following
model is proposed to account for the higher affinity of apoE4 for
VLDL and, thus, the isoform-specific preferences. This model
results from the suggestion by Segrest et al. (34) that the length
of the amphipathic α-helix is an important determinant of
lipoprotein association. These investigators suggested that
long helices target larger particles with less curved surfaces
(i.e. VLDL) whereas shorter helices target smaller particles
with more curved surfaces (i.e. HDL) (34). Thus, in apoE4, the
interaction of arginine 61 with glutamic acid 255 may stabilize
an extended helical structure in the carboxyl terminus that is
best accommodated on a less curved VLDL surface. In the
absence of this interaction in apoE3, this stabilization would be
lost, and the resultant shorter helices would bind preferentially
to HDL.
The apoE4 preference for triglyceride-rich lipoproteins has been implicated in cholesterol metabolism. Subjects carrying the apoE4 allele have lower plasma concentrations of apoE and higher cholesterol and LDL concentrations than those with the apoE3 allele. The preferential association of apoE4 for chylomicron remnants and VLDL has been suggested to result in more rapid clearance of these particles and down-regulation of the hepatic LDL receptors (4). This would account for both higher LDL and lower plasma apoE concentrations. Consistent with this mechanism are the increased plasma clearance rates of retinol, a marker of chylomicrons and their remnants, in apoE4 subjects after a fatty meal (36). However, a recent study reported a prolonged postprandial hypertriglyceridemia in subjects with an apoE4/3 phenotype than in those with an apoE3/3 phenotype (37). In this case, the authors speculated that the increased residence time of VLDL and chylomycin remnants in apoE4 subjects resulted from the overaccumulation of apoE4 on these particles, which actually decreased the receptor-binding activity of apoE and increased the conversion of VLDL into LDL. Although the precise mechanism for the increased plasma concentration of LDL associated with apoE4 is not clear, the preferential association of apoE4 with triglyceride-rich lipoproteins appears to be a key component.

In addition, the underlying mechanism(s) of why apoE4 is a major risk factor for Alzheimer’s disease is not known. Several isoform-specific effects have been reported that may have an impact on Alzheimer’s disease (reviewed in Refs. 12, 13, and 46). Lipid-free apoE4 has been demonstrated to bind more effectively to the amyloid β-peptide (38) and to promote amyloid β-peptide fiber formation (39–41) more effectively than apoE4. Amyloid fiber deposition is a pathological marker of Alzheimer’s disease. Also, apoE has been suggested to play an important role in nerve regeneration (42–45) and in the maintenance and remodeling of synaptic connections (12, 13, 46). Interestingly, apoE4 blunts neurite extension in cultured neurons, whereas apoE3 promotes neurite extension (47). We suggest that apoE4 domain interaction extends beyond the apoE4 preference for VLDL and is a fundamental property of this isoform that accounts for its unique metabolic characteristics with respect to both lipoprotein metabolism and Alzheimer’s disease.

In summary, the present studies establish that the preferential association of apoE4 for VLDL is an intrinsic property of apoE4, likely related to domain interaction, and that domain interaction is probably mediated by a salt bridge between arginine 61 and glutamic acid 255. Greater understanding of apoE4 domain interactions may provide the basis for new insights into why apoE4 is a risk factor for both cardiovascular and Alzheimer’s disease.

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