Effect of Arginine 172 on the Binding of Apolipoprotein E to the Low Density Lipoprotein Receptor*

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The region of apolipoprotein E (apoE) that interacts directly with the low density lipoprotein (LDL) receptor lies in the vicinity of residues 136–150, where lysine and arginine residues are crucial for full binding activity. However, defective binding of carboxy-terminal truncations of apoE3 has suggested that residues in the vicinity of 170–183 are also important. To characterize and define the role of this region in LDL receptor binding, we created either mutants of apoE in which this region was deleted or in which arginine residues within this region were sequentially changed to alanine. Deletion of residues 167–185 reduced binding activity (15% of apoE3), and elimination of arginines at positions 167, 172, 178, and 180 revealed that only position 172 affected binding activity (2% of apoE3). Substitution of lysine for Arg172 reduced binding activity to 6%, indicating a specific requirement for arginine at this position. The higher binding activity of the Δ167–185 mutant relative to the Arg172 mutant (15% versus 2%) is explained by the fact that arginine residues at positions 189 and 191 are shifted in the deletion mutant into positions equivalent to 170 and 172 in the intact protein. Mutation of these residues and modeling the region around these residues suggested that the influence of Arg172 on receptor binding activity may be determined by its orientation at a lipid surface. Thus, the association of apoE with phospholipids allows Arg172 to interact directly with the LDL receptor or with other residues in apoE to promote its receptor-active conformation.

Apolipoprotein E (apoE) is a 34-kDa, 299-amino acid protein (1) that is associated with several classes of lipoproteins in plasma and cerebrospinal fluid (2–5). ApoE is important in the transport and metabolism of cholesterol and triglyceride throughout the body through its interaction with receptors in the low density lipoprotein (LDL) receptor family (2, 6–9). There are three common isoforms of apoE (apoE2, apoE3, and apoE4), which are genetically determined by three corresponding alleles at a single gene locus on chromosome 19 (allelic frequency: e2 = 0.08, e3 = 0.77, and e4 = 0.15) (10–13). The isoforms differ at positions 112 and 158. The most common form, apoE3, has cysteine and arginine at these positions, respectively; apoE2 has cysteine and apoE4 arginine at both positions (1, 14).

There are two major structural and functional domains in apoE. (15, 16). The 22-kDa carboxy-terminal domain, shown by x-ray crystallographic studies to be a four-helix bundle (17), contains the receptor-binding region (2, 18–20). The 10-kDa carboxy-terminal domain contains important lipoprotein-binding regions, although the amino-terminal domain is capable of binding phospholipids and remodeling them into discoidal lipoprotein particles (15, 21–24). The two domains are joined by a hinge region (approximately residues 165–215) of unknown function (15, 16). To date, this hinge region has been considered simply a linker between the two domains of apoE, and significant portions of it can be deleted (residues 186–223) without affecting the binding of apoE to lipoproteins (25).

The original characterization of the receptor binding activity of apoE showed that arginine and lysine residues were critical (26, 27). The LDL receptor-binding region was narrowed down to residues in the vicinity of 136–150 by examining naturally occurring and genetically engineered point mutations (2, 18) and proteolytic fragments of apoE (19) and by using monoclonal antibodies to inhibit apoE binding to the LDL receptor (20). However, residues outside the receptor-binding region can also affect receptor binding activity. ApoE2 (Cys158) has only 1% of the LDL receptor binding activity of apoE3 (Arg158) and apoE4 (Arg158) (28). Studies involving chemical modification of cysteine groups (29), mutagenesis, and x-ray crystallography of the apoE2 22-kDa fragment (30, 31) demonstrated that the presence of a cysteine at position 158 in apoE2, instead of an arginine, indirectly affected receptor binding activity by rearranging crucial salt bridges, reducing the positive electrostatic potential of the receptor-binding region.

Studies of carboxy-terminal truncations of the 22-kDa fragment of apoE3 showed that residues 171–183 are also important for receptor binding activity (32). It was hypothesized that arginines in this region may interact directly with the LDL receptor, like Arg158, indirectly affect the receptor-binding region of apoE. Since the electron density of residues 163–191 is uninterpretable in the x-ray structure of the 22-kDa fragment of apoE (17, 23, 31), we have taken a mutagenesis approach to determine which residues in this region are important in receptor binding activity. An internal deletion in apoE3 of residues 167–185 (apoE3Δ167–185) confirmed that this region had an effect on receptor binding activity. From the examination of point mutations in apoE3, we found that a single amino acid, Arg172, was responsible for this effect. Finally, sequence comparison of apoE3Δ167–185 with intact apoE3 implicated the importance of the presence of Arg172 and its possible positioning on a lipid surface for LDL receptor binding activity.
R at the end of an internal primer name indicates it is the reverse primer of the one listed above it. In the primary PCRs, the 5’-flanking primer is used with the reverse internal primer, and the 3’-flanking primer is used with the forward internal primer. The two resulting PCR products have overlapping ends and are thus primer and template in the secondary PCR. (The 3’ and 5’ flanking primers are also used in the secondary PCR.)

### MATERIALS AND METHODS

**Construction of ApoE Variants**—The cDNAs of human apoE3 and apoE4 were inserted into a modified thioredoxin (Trx) fusion expression vector (pET32a; Novagen) as described previously (33). Internal deletions and point mutations of apoE were made by overlapping polymerase chain reaction (PCR) (23) with *Pfu* polymerase (Stratagene) and appropriate oligonucleotides (Oligos Etc.) (Table I). The DNA was then digested with the appropriate restriction enzymes, subjected to agarose gel electrophoresis, and purified with a gel extraction kit. The purified inserts were ligated into the expression plasmid using T4 Ready-To-Go ligase (Amersham Pharmacia Biotech). The sequence of the DNA insert was verified by double-stranded DNA sequencing.

**Expression and Purification of ApoE and Variants**—Plasmids containing cDNA of human apoE or variants were transformed into *Escherichia coli* (strain BL21 (DE3); Novagen). Several transformants were grown in LB medium at 37 °C to an optical density of 0.5–0.6 (550 nm). Before cleavage, the fusion protein was solubilized in 6 M guanidine HCl (0.1 M Tris, pH 7.4, 0.1 M DTT, and 0.1% bovine serum albumin) complexed with dimyristoylphosphatidylcholine (DMPC) to protect the protein to remove Trx from apoE. Before cleavage, the fusion protein was then cleaved with thrombin affinity column. The fusion protein was then cleaved with thrombin. The protein was solubilized in 100 mM NH₄HCO₃ and stored at −20 °C. All apoE variants were structurally similar to apoE3 as determined by circular dichroism measurements (data not shown).

**Preparation of ApoE-DMPC Complexes and Determination of Receptor Binding Activity**—Purified recombinant human apoE and variants were complexed with DMPC and isolated by KB gel centrifugation as described (34). The apoE-DMPC discs were negatively stained on the surface of carbon fluid grids. Electron micrographs were made at a magnification of × 200,000 and imported with a video camera into an Image I/AT image-analysis system. Particle size was analyzed by automated sizing and counting programs available on system software (mVscion version 4.03a; Universal Imaging Corp.). Multiple areas on a single grid were sampled. The complexes formed by the apoE variants and DMPC were the same size as normal apoE3 (disc diameter ranged from 15 to 17 nm). The receptor binding activities of the apoE-DMPC complexes were determined in a receptor competition assay with 125I-labeled human LDL and human primary fibroblasts in culture (34).

### RESULTS AND DISCUSSION

When complexed with DMPC, the 22-kDa fragment of apoE3 binds to the LDL receptor with high affinity (19). LDL receptor-binding studies of carboxyl-terminal truncations of the apoE3 22-kDa fragment showed that a mutant consisting of residues 1–183 of apoE3, apoE3(1–183), had essentially normal binding activity, but further truncation to residue 170, apoE3(1–170), reduced binding activity to less than 1% of that of full-length apoE3 (32). Since arginine residues are important in binding activity (2, 26), it was hypothesized that one or more arginines in this region interact directly with the receptor or act to stabilize the receptor-binding region (32). To confirm that the region within residues 171–183 was indeed significant for apoE receptor binding activity and that the previous findings were not influenced by the absence of the major lipid-binding region in apoE (the 10-kDa carboxyl-terminal fragment), we constructed internal deletions of apoE, rather than truncations. The first internal deletion (apoE3 Δ167–185) was designed to eliminate four arginine residues (167, 172, 178, and 180) in this region. There are no lysines in the deleted region. The second variant (apoE4Δ186–223), in which residues adjacent to 167–185 were deleted, was constructed previously for studies of lipoprotein binding (25).

The binding of apoE3Δ186–223 to the LDL receptor was essentially identical to that of apoE3 and apoE4. (In this assay, apoE3 and apoE4 have identical binding to the LDL receptor (28).) However, apoE3Δ167–185 bound to the LDL receptor with only 15% of the binding activity of apoE3 (Fig. 1, Table II). These results are consistent with the data from the carboxyl-terminal truncation experiments, which suggested that part of the hinge region affects receptor binding activity.

To determine if the arginines in this region are important for receptor binding activity, we created four mutants in which an arginine was replaced with an alanine (apoE3 R167A, apoE3 R172A, apoE3 R178A, and apoE3 R180A). Of these four mu-

### Table I

| Primer | Sequence (5’ → 3’) |
|--------|--------------------|
| 5’-Flanking | ATGGACGAGACCATGAGGAGATTGAAAG |
| Internal primers | TCCGAATTCAGTGATTGTCTGGCAC |
| 167ala | TACCGAGCGGGGCAGAAAGGCGGGCGGT |
| 167alaR | AGCCGGCCGCGCGCGCCGCGGA |
| 172ala | AGGGGGCCAGGGCCGCGCCAT |
| 172alaR | ATGGCGGCTGAGGCCCTCGGCGCCAT |
| 178ala | AGGGCCAGGGCCGCGCCAT |
| 178alaR | ATGGCGGCTGAGGCCCTCGGCGCCAT |
| 180ala | AGGGGGCCAGGGCCGCGCCAT |
| 180alaR | ATGGCGGCTGAGGCCCTCGGCGCCAT |
| 172lys | AGGGCCAGGGCCGCGCCAT |
| 172lysR | ATGGCGGCTGAGGCCCTCGGCGCCAT |
| 167–185′170′ala | AGGGGGCCAGGGCCGCGCCAT |
| 167–185′170′alaR | ATGGCGGCTGAGGCCCTCGGCGCCAT |
| 167–185′172′ala | AGGGGGCCAGGGCCGCGCCAT |
| 167–185′172′alaR | ATGGCGGCTGAGGCCCTCGGCGCCAT |
| 167–185′170′172′ala | AGGGGGCCAGGGCCGCGCCAT |
| 167–185′170′172′alaR | ATGGCGGCTGAGGCCCTCGGCGCCAT |
ApoE Arg172 Modulates Binding to the LDL Receptor

FIG. 1. Ability of apoE3 DMPC (●), apoE4Δ186–223 DMPC (■), and apoE3Δ167–185 DMPC (◆) to compete with human 125I-LDL for binding to LDL receptors on normal human fibroblasts. Cells incubated in medium containing 10% human lipoprotein-deficient serum received the same medium with 2 µg/ml of 125I-LDL and the indicated concentrations of apoE-DMPC complexes. After a 2-h incubation on ice, the cells were extensively washed, and the amount of 125I-LDL bound to cells was determined. Data presented are from one of at least three experiments.

FIG. 2. Ability of variants of apoE-DMPC particles to compete with human 125I-LDL for binding to normal human fibroblasts. ●, apoE3; ■, apoE3 R167A; , apoE3 R172A; ○, apoE3 R178A; and □, apoE3 R180A. Cells incubated in medium containing 10% human lipoprotein-deficient serum received the same medium with 2 µg/ml 125I-LDL and the indicated concentrations of apoE-DMPC complexes. After a 2-h incubation on ice, the cells were extensively washed, and the amount of 125I-LDL bound to cells was determined. Data presented are from one of at least four experiments.

FIG. 3. Ability of apoE3 DMPC (●), apoE3 R172A-DMPC (■), and apoE3 R172K-DMPC (◆) to compete with human 125I-LDL for the LDL receptor on normal human fibroblasts. Cells incubated in medium containing 10% human lipoprotein-deficient serum received the same medium with 2 µg/ml 125I-LDL and the indicated concentrations of apoE-DMPC complexes. After a 2-h incubation on ice, the cells were extensively washed, and the amount of 125I-LDL bound to cells was determined. Data presented are from one of at least four experiments.

FIG. 4. Comparison of residues 167–185 with 186–204 of apoE3. In apoE3Δ167–185, amino acid residues 186–204 are essentially shifted down so that residue 186 is now in position 167. Thus, in apoE3Δ167–185, residue 186 can be renamed as residue 167, and residue 204 is 185. The arginines and proline present in this region of apoE3 and apoE3Δ167–185 are highlighted from 186–204, at positions 189 (170′) and 191 (172′). Furthermore, at position 183, a proline, which disrupts a-helical structure, was replaced with another proline, residue 202 (183′). Instead of clarifying the difference in receptor binding activity between the two mutants, this analysis produces more questions. If Arg172 is important, why was the receptor binding activity of apoE3Δ167–185 defective?

To explain this contradiction, we examined the predicted secondary structure of residues 167–204. Segrest et al. (35) predicted that residues 167–182 would form a class A am-

Table II
Summary of the ability of apoE and variants to compete with LDL for the LDL receptor

| Concentration of apoE-receptor-DMPC complex that displaced 50% of the 125I-LDL from the LDL receptor on cultured human fibroblasts ± S.D. | Relative competitive ability of apoE versus apoE3 | n |
|---|---|---|
| ApoE | µg protein/ml | % apoE3 activity |
| ApoE3 | 0.042 ± 0.015 | 100 ± 11 |
| ApoE4 | 0.038 ± 0.013 | 105 ± 7 |
| ApoE3Δ167–185b | 0.407 ± 0.232 | 15 ± 9 |
| ApoE3Δ186–223b | 0.020 ± 0.005 | 194 ± 65 |
| ApoE3 R167A | 0.043 ± 0.022 | 92 ± 11 |
| ApoE3 R172Ab | 1.850 ± 0.580 | 2 ± 1 |
| ApoE3 R178A | 0.062 ± 0.038 | 69 ± 24 |
| ApoE3 R180A | 0.034 ± 0.012 | 129 ± 40 |
| ApoE3 R172Kb | 0.967 ± 1.664 | 6 ± 5 |
| ApoE3Δ167–185 R189Ab | 0.077 ± 0.050 | 54 ± 11 |
| ApoE3Δ167–185 R170Ab | 0.280 ± 0.050 | 14 ± 12 |
| ApoE3Δ186–185 R191Ab | 3.209 ± 1.976 | 1 ± 1 |

* Determined within a single experiment and then averaged between experiments.

b p < 0.05 versus apoE3.

* Arg172

However, there were some paradoxes in the results. Specifically, apoE3Δ167–185 had consistently higher binding activity than apoE3 R172A (15 versus 2%, p = 0.018). As a result, we decided to compare this section of the hinge region, residues 167–185, in apoE3 and apoE3Δ167–185. In apoE3Δ167–185, residues 186–204 are essentially shifted down to replace the deleted residues 167–185; therefore, residue 186 can be referred to as 167′ in the apoE3Δ167–185 variant and residue 204 as 186′. The other residues between 186 and 204 may be similarly renamed. When residues 186–204 (167′–185′) were aligned with 167–185 (Fig. 4), it was revealed that an arginine at position 172 in apoE3 was replaced with an arginine in apoE3Δ167–185. (There are two arginines within the sequence
hydrophilic faces are indicated. The helices were generated using Insight oriented to align Arg191 (172 left charged residues, in this case arginine, are located at the polar-nonphilic face. As is typical for class A amphipathic helices, positively charged residues at the polar-nonpolar face of the helix. The model of interaction of a class A amphipathic helix with phospholipid suggests that the hydrophobic face inserts into the lipid layer while the hydrophilic face is exposed to the aqueous environment, and the positively charged residues at the polar-nonpolar interface interact with the phospholipid head groups.

**FIG. 5.** Residues 167–182 and 186–201 in apoE3 modeled as α-helices. A residues 167–182 fit a model of a class A amphipathic helix, with leucine, isoleucine, and valine residues on the hydrophobic face of the helix and negatively charged glutamic acids on the hydrophilic face. As is typical for class A amphipathic helices, positively charged residues, in this case arginine, are located at the polar-nonphilic face. B, the helix on the left is oriented to align Arg191 (172) with Arg172 in A. Arg172 (170) is on the opposite side of the helix from Arg191 (172). Thus, if the face of the helix that inserts into the phospholipid layer contains only hydrophobic residues, then the position of Arg191 (172) in apoE3A167–185 would rotate by approximately 90° (helix on the right), altering its potential interactions with Arg172 in apoE3. The hydrophobic and hydrophilic faces are indicated. The helices were generated using Insight II (Molecular Simulations, San Diego). Residues are as follows: yellow, arginine; red, glutamic acid; brown, valine, leucine, and isoleucine; blue, glutamine; gray, glycine, serine, alanine, and threonine.

With the possibility that segments within residues 167–204 form α-helices, we modeled residues 167–182 and 186–201 (167–182') as α-helices using the computer program Insight II (Fig. 5). Comparison of the helices suggested an explanation for the paradoxical receptor binding of apoE3A167–185, specifically that positioning of Arg172 on a lipid surface, with respect to the LDL receptor, could be critical. The α-helix modeled from residues 167–182 is amphipathic, with its opposing polar and nonpolar faces oriented along the long axis of the helix (35). Arginines 167, 178, and 180 are clustered at the polar-nonpolar interface, typical of a class A amphipathic α-helix (35). It has been hypothesized that the arginine residues at the polar-nonpolar interface interact with the phospholipid head groups at the lipoprotein surface, while the hydrophobic face inserts into the phospholipid layer (35). This model of association is consistent with recent studies examining the interaction of class A amphipathic peptides with phospholipid bilayers (39) and thus suggests that arginines 167, 178, and 180 would interact with the phospholipid head groups on the phospholipid disc. However, Arg172 is positioned in the center of the polar face of the helix, where it may be available to interact with other residues in apoE or other proteins (e.g., the LDL receptor) rather than phospholipid head groups. When residues 186–201 (167–182') are modeled as an α-helix, Arg172 (170') is on the opposite side of the helix from Arg191 (172'), and one could predict that the position of Arg191 (172') would shift by approximately 90° relative to Arg172 in apoE3, so that Arg189 (170') and Arg191 (172') would lie at the phospholipid interface (Fig. 5). Thus, the potential interactions of Arg191 (172') with other amino acid residues would be altered compared with Arg172 in apoE3. Additionally, this stretch of residues is not very amphipathic. While one side of the putative helix contains polar and charged residues, and Arg189 (170') and Arg191 (172') may define the polar-nonpolar interface, the nonpolar face is not very hydrophobic, containing only one valine and a few alanine residues; therefore, its potential orientation or association with phospholipid is questionable. Alternatively, it is possible that residues 186–201 are not helical and that the receptor-binding results may be explained by a disruption of secondary structure in this region.

If residues in the vicinity of Arg191 (172') are helical, we speculated that we could further modulate receptor binding activity of apoE3A167–185 by mutating arginine to alanine at positions 189 (170') and 191 (172'). To assess this possibility, we created three additional point mutations (apoE3D167–185 R189A, apoE3A167–185 R191A, and apoE3A167–185 R189A,R191A) and tested them for receptor binding activity (Fig. 6, Table I). ApoE3A167–185 R189A had increased binding activity (54% of apoE3). Loss of Arg191 (172') to shift close to the position Arg172 may occupy in intact apoE3. This result implies that residues 186–201 or a short segment within this region is probably helical. ApoE3A167–185 R191A (R172A) had approximately 15% of the binding activity of apoE3. Without Arg191 (172') at the opposite side of the helix, Arg189 (170') may partially compensate for the loss of the Arg at 191 (172'), although poorly. The double mutant (apoE3A167–185 R189A,R191A) displayed only 1% of receptor binding activity, similar to the R172A mutation in intact apoE3.

The results of these mutagenesis studies show that Arg172 is important for LDL receptor binding activity; they also imply that its proper orientation, relative to the receptor-binding region, is critical. The structure of the receptor-active conformation of apoE is not known, but it does require association...
with phospholipids (40). One hypothesis is that the four-helix bundle of the apoE 22-kDa fragment opens up when associating with phospholipid, promoting proper conformation of the receptor-binding region in apoE (4). These current studies also suggest the possibility that phospholipids promote the correct positioning of Arg172 so that it can interact directly with the ligand-binding region of the LDL receptor or with other residues of apoE to maintain an active conformation of the apoE receptor-binding region. However, precisely how Arg172 affects receptor binding activity cannot be established until the structure of apoE3 on phospholipid has been determined.

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