THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 46, pp. 38576–38582, November 18, 2005

Specific Sequences in the N and C Termini of Apolipoprotein A-IV Modulate Its Conformation and Lipid Association*

Received for publication, June 22, 2005, and in revised form, September 12, 2005 Published, JBC Papers in Press, September 13, 2005, DOI 10.1074/jbc.M506802200

Kevin Pearson[‡1], Matthew R. Tubb[‡], Masafumi Tanaka[§], Xiu Qi Zhang[¶], Patrick Tso[‡], Richard B. Weinberg[‖], and W. Sean Davidson[‡2]

From the [‡]Department of Pathology and Laboratory Medicine, The University of Cincinnati, Cincinnati, Ohio 45237, [§]Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, [¶]Department of Chemistry, University of Illinois , Chicago, Illinois 60607, and [‖]Departments of Internal Medicine and Physiology and Pharmacology, Wake Forest University, Winston-Salem, North Carolina 27157

Apolipoprotein (apoA-IV) is a 376-residue exchangeable apolipoprotein that may play a number of important roles in lipid metabolism, including chylomicron assembly, reverse cholesterol transport, and appetite regulation. *In vivo*, apoA-IV exists in both lipid-poor and lipid-associated forms, and the balance between these states may determine its function. We examined the structural elements that modulate apoA-IV lipid binding by producing a series of deletion mutants and determining their ability to interact with phospholipid liposomes. We found that the deletion of residues 333–343 strongly increased the lipid association rate *versus* native apoA-IV. Additional mutagenesis revealed that two phenylalanine residues at positions 334 and 335 mediated this lipid binding inhibitory effect. We also observed that residues 11–20 in the N terminus were required for the enhanced lipid affinity induced by deletion of the C-terminal sequence. We propose a structural model in which these sequences can modulate the conformation and lipid affinity of apoA-IV.

Human apolipoprotein (apo)[3] A-IV is a 46-kDa glycoprotein that is the largest member of the exchangeable apolipoprotein family. It is synthesized by enterocytes of the small intestine in response to lipid absorption and is secreted into circulation on the surface of chylomicrons. As chylomicrons undergo lipolysis in the plasma compartment, apoA-IV rapidly dissociates from their surface and thereafter circulates as a lipid-free protein and in association with high density lipoprotein (1). It has been proposed that apoA-IV evolved to play a role in chylomicron assembly or catabolism (2). However, several additional functions have been proposed, including inhibition of lipid oxidation (3) and inflammatory processes (4), participation in reverse cholesterol transport (5), and regulation of food intake (6). Because plasma apoA-IV can exist both as a component of plasma lipoproteins and as a lipid-free protein, it is possible that alternate conformations perform distinct functions. However, the structural features that mediate the conversion between these two states are largely unknown.

ApoA-IV shares many features with the other exchangeable apolipoproteins, especially apoA-I and apoE. Indeed, intraexonic duplication of a primordial apoA-I gene may have led to the appearance of apoA-IV some 300 million years ago (7). A distinct feature of the primary sequences of the exchangeable apolipoproteins is a variable number of 22-residue amphipathic α-helical repeats, which likely confer the ability to bind to the surface of lipoprotein particles (8). ApoA-IV (376 residues) contains 13 such repeats (most punctuated by proline residues), located between residues 40 and 332. The first 39 amino acids of apoA-IV are encoded by a separate exon and contain potential weakly amphipathic helical domains that are similar to those found in globular proteins (9). In contrast, the C terminus (residues 333–376) is predicted to be devoid of ordered secondary structure. Residues 354–367 are composed of a repeating EQQQ sequence that is not found in any other apolipoprotein (10).

Lipid-free forms of apoA-I and apoE have been shown to exhibit a compartmentalized architecture characterized by a well organized N-terminal domain and a relatively unstable C-terminal domain (11–13) that contains lipid binding sequences. Given the similarities among these apolipoproteins, we expected that apoA-IV would exhibit a similar organization. However, we found that the α-helices in apoA-IV are organized around a single large domain (14). Furthermore, the C-terminal third of apoA-IV not only lacked a lipid-binding domain, it appeared to actually inhibit lipid interactions. Removing the C-terminal 44 amino acids of apoA-IV (Δ333–376) resulted in a mutant that reorganized liposomes significantly faster than both WT apoA-IV and apoA-I (14). The goal of the present study was to identify the sequence(s) in the C-terminal region responsible for this lipid binding inhibitory effect.

EXPERIMENTAL PROCEDURES

Materials—SDS-PAGE gels were obtained from Bio-Rad or Amersham Biosciences. Primer synthesis and DNA sequencing were performed by the University of Cincinnati DNA Core. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). IgA protease (*Igase*) was purchased from MoBiTec. BL-21 (DE3) *Escherichia coli* and the pET30 vector were from Novagen (Madison, WI). Isopropyl-β-D-thiogalactoside was purchased from Fisher. His-bind resin was purchased from Novagen (Madison, WI). Centriprep centrifugal concentrators were purchased from Millipore/Amicon Bioseparations (Bedford, MA). 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids (Birmingham, AL). All chemical reagents were of the highest quality available.

Mutagenesis of Human ApoA-IV—Human apoA-IV DNA was ligated into the pET30 expression vector using the NcoI and HindIII sites. A cleavable N-terminal poly (His) tag was appended to facilitate purification. The C-terminal mutations were created by performing

* This work was supported by National Institutes of Health Grants HL67093, HL62542 (to W. S. D.), and HL30897 (to R. B. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by a predoctoral fellowship from the Ohio Valley affiliate of the American Heart Association.
[2] To whom correspondence should be addressed: Dept. of Pathology and Laboratory Medicine, University of Cincinnati, 2120 Galbraith Rd., Cincinnati, OH 45237-0507. Tel.: 513-558-3707; Fax: 513-558-1312; E-mail: Sean.Davidson@UC.edu.
[3] The abbreviations used are: apo, apolipoprotein; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; STB, standard Tris buffer; WT, wild type.

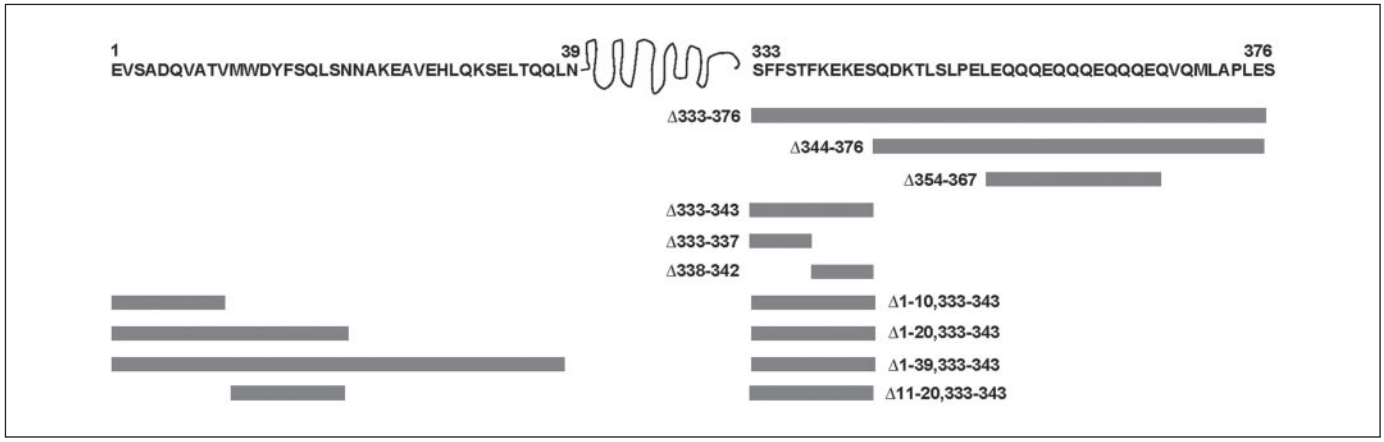

FIGURE 1. **Schematic map of human apoA-IV deletion mutants used in this study.** The *single letter amino acid code* is shown for residues 1–39 and 333–376. A *wavy line* indicates intervening residues. The *thick gray bars* identify the locations of amino acids that were removed from the C or N terminus.

PCR-based site-directed mutagenesis (Quick-Change, Novagen) (14). For the Δ333–376, Δ344–376, and Δ352–376 mutants, a stop codon was inserted in place of the first amino acid to be deleted. For internal deletion mutants, complementary forward and reverse primers were designed with clamp regions on the 5′- and 3′-ends of the sequence removed. The Δ333–343 mutant DNA contained in the pET30 vector was used as the template for the double mutants (Δ1–10, 333–343, Δ1–20, 333–343, Δ1–30, 333–343, and Δ1–39, 333–343). These mutants used a forward primer that had a 3′-clamp region in which the nucleotides encoded the amino acids at position 11, 21, 31, and 40, respectively. The primer also consisted of a 5′-flap region that encoded for the NcoI site and an *Igase* (IgA protease) cleavage site that was used to remove the N-terminal His tag (as described below). For further details, please refer to Ref. 14. Point mutagenesis was performed directly in the expression vector using the Quick-Change approach as for the introduction of the stop codons.

Protein Expression and Purification—Our protocol was similar to previous work with rat apoE (15). The expression vector was transformed into *E. coli* BL-21-competent cells and plated overnight at 37 °C. Kanamycin (30 μg/ml) was used as the selective agent. Cell colonies were picked and grown in Luria-Bertani (LB) culture media overnight in 10-ml culture tubes in a shaking incubator at 37 °C. The cells in culture media were then transferred to fresh 100-ml cultures and grown to an A_{600} of 0.6–0.7. At this point, isopropyl 1-thio-β-D-galactopyranoside was added for 2 h to induce overexpression of the protein. Cell pellets were collected by centrifugation and brought up in His-bind buffer along with protease inhibitors. Cells were disrupted by sonication and centrifuged, and the supernatant was filtered through a Millex 0.45-μm filter and added to His-bind columns according to the manufacturer's instructions. The protein was eluted, concentrated, and digested with IgA protease, a protease that cleaves before Thr in the sequence Ala-Pro-Arg-Pro-Pro-Thr-Pro. This removed the His tag and left a Thr-Pro at the N terminus. Next, the sample was passed over the His-bind column a second time to remove the cleaved tag. The His-bind column buffers in the purification contained 3 M guanidine. Finally, the samples were concentrated and dialyzed into standard Tris buffer (STB) (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, and 0.2% NaN_3) for storage.

DMPC Liposome Solubilization—DMPC in chloroform was dried in a borosilicate tube with a stream of nitrogen, solubilized in degassed STB, and bath-sonicated for 30 s to form multilamellar liposomes. Liposomes were placed in thermostated cuvettes at 24.5 °C, proteins were added to the liposomes at a final DMPC/protein mass ratio of 2.5:1, and the absorbance was continuously monitored at 325 nm in an Amersham Biosciences Ultraspec 4000 at 24.5 °C. Rate constants, k, were calculated for each mutant by fitting the first 5 min of data to a single exponential decay curve as a function of time (t): $f(t) = a^{-kt}$ using Sigma Plot 2000 (16, 17).

Interfacial Behavior at the Oil/Water Interface—The binding of apoA-IV at the oil/water interface was examined using a Tracker® automatic tensiometer (IT Concept) (28). Drops of pure triolein were injected into a sample chamber containing 25 μg/ml protein in phosphate-buffered saline buffer, and protein adsorption to the surface of the drop was monitored as the time-dependent decrease in interfacial tension.

Thermal Denaturation Studies—The thermal denaturation of the lipid-free apoA-IV mutants was monitored as the change in molar ellipticity at 222 nm over a temperature range of 20–80 °C (18). The van't Hoff enthalpy, ΔH_v, was calculated as described previously (13).

Fluorescence Spectroscopy—Protein samples were studied at 0.1 mg/ml in STB on a Photon Technology International Quantamaster spectrometer in photon counting mode at room temperature. Excitation wavelength was 295 nm, excitation and emission band passes were 3.0 nm, and emission wavelength was monitored from 302 to 375 nm. Buffer blanks were subtracted from each of the samples. Quenching studies were performed by the addition of 0–0.2 M acrylamide; Stern-Volmer quenching constants, K_{sv}, were determined as described previously (19).

RESULTS

To study the lipid association properties of human apoA-IV, we developed an efficient bacterial expression system that enables systematic deletion mutagenesis. Fig. 1 summarizes the N- and C-terminal deletion mutants that were generated for this study. The mutants were greater than 95% pure and had a size consistent with the deletions (Fig. 2). As reported previously, WT apoA-IV forms a mixture of monomers and dimers in solution with an approximate distribution of 70% dimers to 30% monomers (20). To determine whether the deletions altered the oligomerization state of the mutants, we examined the mutant proteins by non-denaturing gel PAGE. None of the deletions significantly affected the quaternary structure of apoA-IV (data not shown).

Consistent with other studies (2, 21), WT apoA-IV was only marginally able to reorganize the DMPC multilamellar liposomes into micellar particles that scatter less light (Fig. 3*A*). However, as noted in our previous study (14), apoA-IV Δ333–376 cleared DMPC vesicles at a dramatically faster rate than WT apoA-IV, surpassing apoA-I, which is well documented to bind lipid with higher affinity than apoA-IV (2, 22, 23).

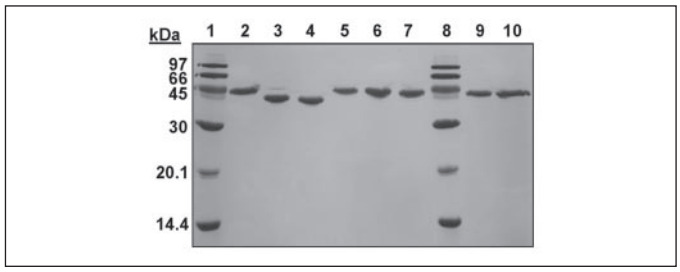

FIGURE 2. **SDS-PAGE analysis of WT apoA-IV and selected truncation mutants.** Three-microgram samples of purified apoA-IV mutants were electrophoresed on an 18% SDS-polyacrylamide gel and stained with Coomassie Blue. *Lane 1*, low molecular weight markers; *lane 2*, WT apoA-IV; *lane 3*, apoA-IV Δ344–376; *lane 4*, apoA-IV Δ333–376; *lane 5*, apoA-IV Δ333–343; *lane 6*, apoA-IV Δ333–337; *lane 7*, apoA-IV Δ338–342; *lane 8*, low molecular weight marker; *Lane 9*, apoA-IV Δ1–10,333–343; *lane 10*, apoA-IV Δ1–20,333–343.

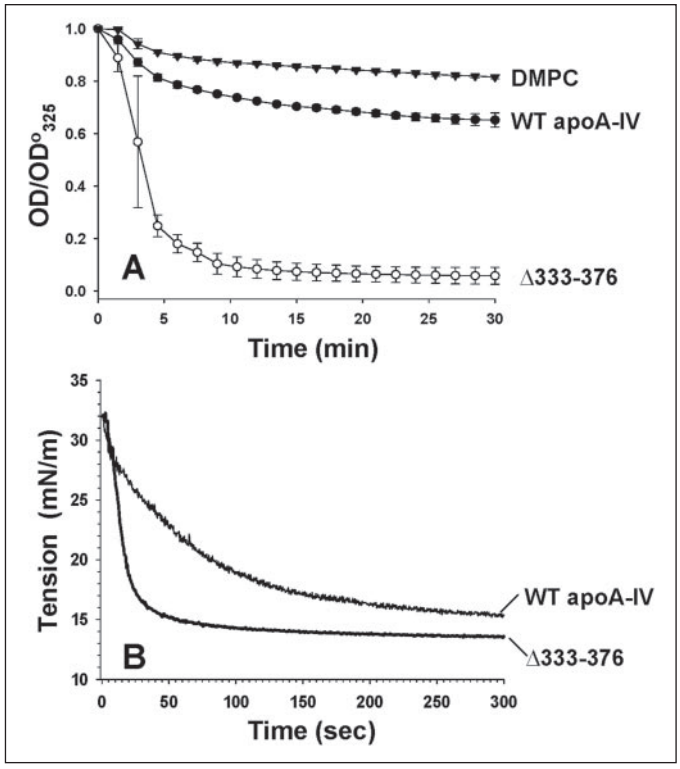

FIGURE 3. **Lipid association of WT apoA-IV and apoA-IV Δ333–376.** *A*, DMPC liposome clearance assay. DMPC multilamellar liposomes in STB were added to a cuvette maintained at 24.5 °C, and absorbance (*OD*) was continuously monitored at 325 nm following the addition of 85 μg of WT or mutant apoA-IV. Absorbance values are normalized to the initial absorbance at time zero (*OD*°). *B*, oil drop tensiometry. A Tracker® oil drop tensiometer was used to monitor the decrease in surface tension induced by the binding of WT or mutant apoA-IV to a triolein/buffer interface. Surface tension was measured at 1-s intervals following rapid injection of a 10-μl drop of pure triolein into a cuvette containing 25 μg/ml WT apoA-IV or Δ333–376.

As the DMPC assay measures the end result of a complex process that involves both an initial apolipoprotein-lipid interaction and a subsequent lipid reorganization (see reaction scheme in Ref. 24), we also examined the lipid affinity of WT and Δ333–376 apoA-IV using oil drop tensiometry (25). This method measures the rate at which apolipoproteins in solution bind to and lower the surface tension of the more hydrophobic triolein/water interface. As such, this technique provides a biophysical model for the binding of apoA-IV to the nascent triglyceride-rich chylomicron surface. As seen in Fig. 3*B*, apoA-IV Δ333–376 caused a much more rapid decrease in interfacial tension compared with WT protein, similar to its behavior in the DMPC clearance assay. In fact, for each of the mutants studied, oil drop tensiometry yielded qualitatively similar results as the DMPC clearance assay (data not shown).

We hypothesized that a specific sequence in the C-terminal 44 amino acids of apoA-IV mediated the lipid binding inhibitory effect. We therefore designed a series of truncation mutants to localize the active inhibitory sequence. Deletion of residues Δ352–376 had no effect on the rate of lipid binding compared with WT (data not shown). Likewise, internal deletion of the unique EQQQ domain from Δ354–367, which generates a "pig-like" apoA-IV (26), also had no effect on lipid binding (Fig. 4*A*). We next examined apoA-IV Δ344–376 because serine 343 is the terminal amino acid in chicken apoA-IV (2). Once again, this mutant was similar to WT in its lipid binding behavior (Fig. 4*A*).

These data suggested that the lipid binding inhibitory sequence resided between residues 333 and 343. To confirm this, we examined the behavior of an internal deletion mutant, apoA-IV Δ333–343. As shown in Fig. 4*B*, apoA-IV Δ343–343 disrupted DMPC liposomes as fast as apoA-IV Δ333–376. A consideration of the sequence and net charge conservation of residues 333–343 across seven species revealed that residues 333–337 are highly conserved, whereas residues 338–342 are much less so (Fig. 5*A*). We therefore generated two shorter internal deletion mutants, apoA-IV Δ333–337, which lacked the highly conserved sequence, and apoA-IV Δ338–342, which lacked the more variable interval. Fig. 5*B* shows that apoA-IV Δ338–342 exhibited an intermediate binding rate. However, apoA-IV Δ333–337 was extremely efficient. In fact, this mutant was one of the fastest lipid-binding apolipoproteins we have observed. These data suggest that the most potent lipid binding inhibitory sequence in apoA-IV is located between residues 333 and 337.

We previously noted that the increases in lipid binding rate displayed by the Δ333–376 and Δ271–376 deletion mutants were completely effaced by simultaneous deletion of the first 39 residues from the N terminus (14), suggesting that both domains can impact upon the lipid binding affinity of apoA-IV. To determine whether this phenomenon was maintained with the Δ333–343 mutant, we prepared a series of C- and N-terminal double deletion mutants. Deletion of the N-terminal 39 residues on the background of the apoA-IV Δ333–343 mutant (Δ1–39,333–343) converted the rapid binding Δ333–343 mutant to a slow lipid-binding protein, with kinetics similar to WT apoA-IV (Fig. 6). Similarly, a Δ1–20,333–343 deletion mutant also displayed slow binding. However, when only the first 10 residues were deleted from the N terminus, Δ1–10,333–343, the mutant maintained the rapid binding profile of the Δ333–343 single mutant. An internal deletion mutant, apoA-IV Δ11–20,333–343 that contained an intact N terminus, also displayed slow lipid binding kinetics (Fig. 6*B*).

The DMPC clearance rate constants for all apoA-IV deletion mutants in this study, calculated as described under "Experimental Procedures," are listed in TABLE ONE. The clearance rate constants for apoA-IV Δ333–376, Δ333–343, Δ333–337, and Δ1–10,333–343 were statistically different compared with WT apoA-IV. There was no difference between the clearance rate for WT apoA-IV and the Δ354–367, Δ344–376, Δ338–342, Δ1–39,333–343, and Δ1–20,333–343 mutants. Although there was no statistical difference between the clearance rate for WT apoA-IV and apoA-IV Δ338–342 over the first 5 min, it is evident from Fig. 5 that this deletion mutant was better able to reorganize lipid at longer incubation times.

The data in TABLE ONE indicate that residues 333–337 are critical for maintaining a conformation of WT apoA-IV that is relatively inefficient at interacting with lipid surfaces. To examine the impact of the deletions on protein structure we determined their thermodynamic stability using circular dichroism spectroscopy (TABLE TWO). Thermal

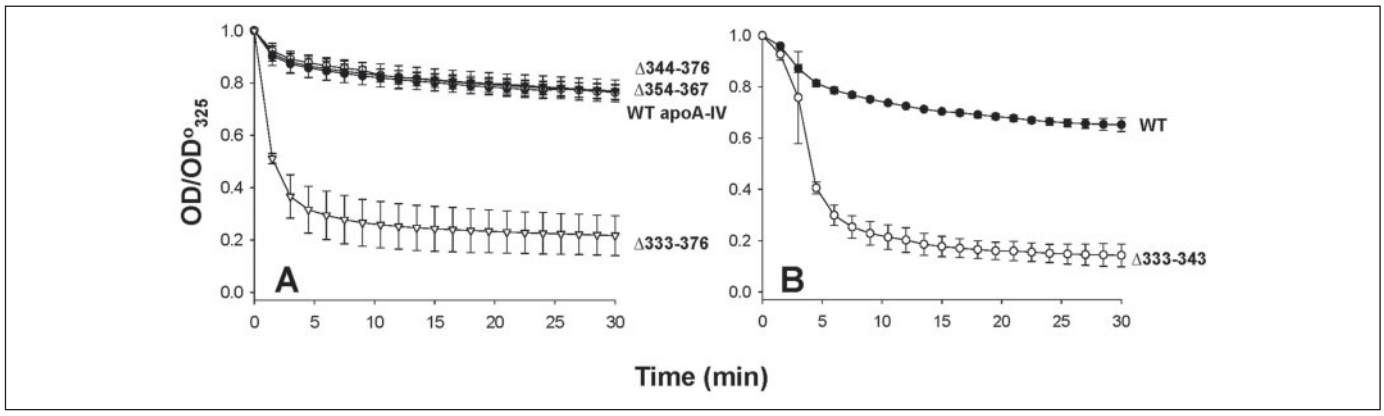

FIGURE 4. **Lipid association of WT apoA-IV and apoA-IV Δ344–376 and Δ354–367.** DMPC multilamellar liposomes in STB were maintained at 24.5 °C by a circulating water bath and monitored at 325 nm following the addition of 85 μg of protein. The time-dependent absorbance (*OD*) was normalized to the initial absorbance (*OD*°).

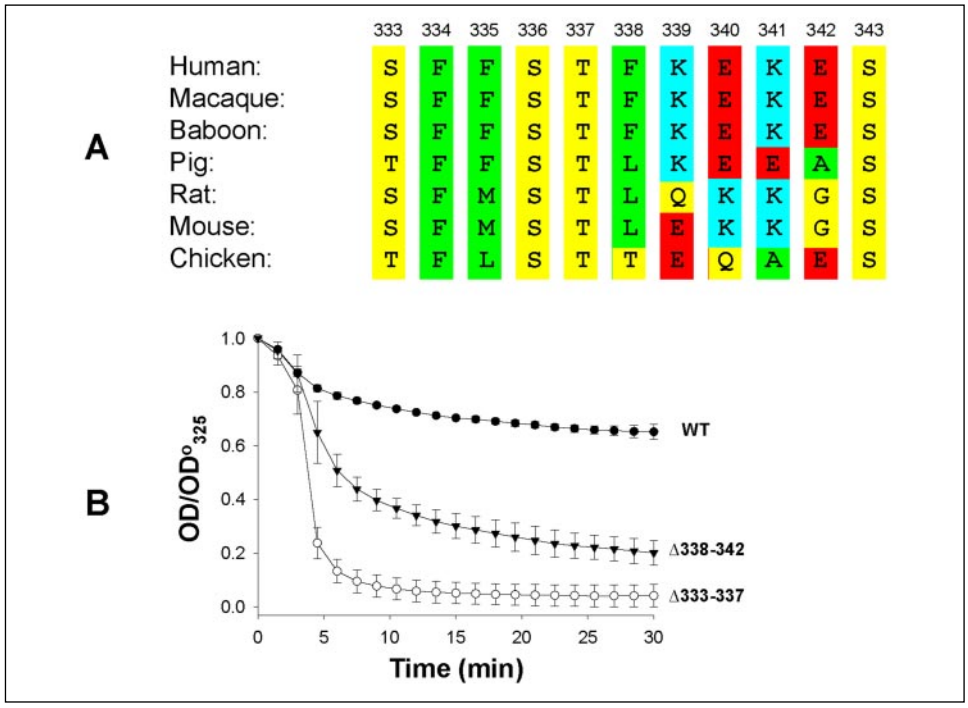

FIGURE 5. **Sequence alignment of the putative "inhibitory" region from amino acids 333 to 343 and the lipid binding performance of resulting mutants.** *A*, species sequence alignment of human apoA-IV. The amino acids are *highlighted* depending on their physical properties: *yellow*, neutrally charged polar; *green*, hydrophobic; *aqua*, positively charged; *red*, negatively charged. *B*, DMPC liposome solubilization by two mutants lacking parts of this region. This panel is organized similarly to Fig. 3*A*.

denaturation studies determined that the Δ344–376 deletion did not significantly alter global protein stability *versus* the WT, as measured by the van't Hoff enthalpy of denaturation. However, mutants that lacked the inhibitory sequence exhibited enthalpies that were slightly lower than WT apoA-IV ranging from 38 to 45 kcal/mol. We also examined the fluorescence properties of Trp-12, which has proven to be a useful measure of apoA-IV conformation (27). The wavelength of maximum fluorescence (λ_{max}) of Trp-12 in WT apoA-IV was 335 nm, indicative of a relatively hydrophobic environment (28) (TABLE THREE). ApoA-IV Δ344–376 had a similar λ_{max}; however, deletion of Δ333–376 or Δ333–343 caused a significant red shift in Trp emission, indicating that the N terminus had relocated to a more polar environment. Acrylamide fluorescence quenching studies further indicated that the change in the polarity of the N terminus was accompanied by a parallel increase in aqueous accessibility to the neutral quenching agent (Fig. 7). These observations, together with the DMPC binding data, suggest that the C-terminal inhibitory sequence may maintain the N-terminal globular domain in a more compact conformation that shields Trp-12 from the aqueous milieu.

Finally, we used point mutagenesis to determine whether specific residues could be responsible for the C-terminal inhibitory effect. When Ser-333 was replaced with an alanine, no effect on DMPC association was observed (Fig. 8). However, when either Phe-334 or Phe-335 was changed to alanine, the full lipid binding effect noted for the Δ333–337 mutant was recapitulated. Thus, dramatic changes in the lipid affinity of apoA-IV can be induced by the mutation of a single amino acid.

DISCUSSION

It is well established that apoA-IV binds to lipid with much lower affinity than other members of the exchangeable apolipoprotein family (21, 23). It has been postulated that its distinctively weak lipid binding behavior is due to: (*a*) the relative hydrophilicity and low amphipathic moment of its constitutive α-helices (29), (*b*) the fact that most of these helices are of the Y-type, which may not be capable of deeply penetrating lipid surfaces (30), and/or (*c*) the possibility that these helices are organized such that their hydrophobic regions are inaccessible for interaction with hydrophobic interfaces (2, 23, 25).

These hypotheses for the weak lipid affinity of apoA-IV notwithstanding, our data establish that human apoA-IV contains a short sequence in a region of random coil structure near the C terminus that

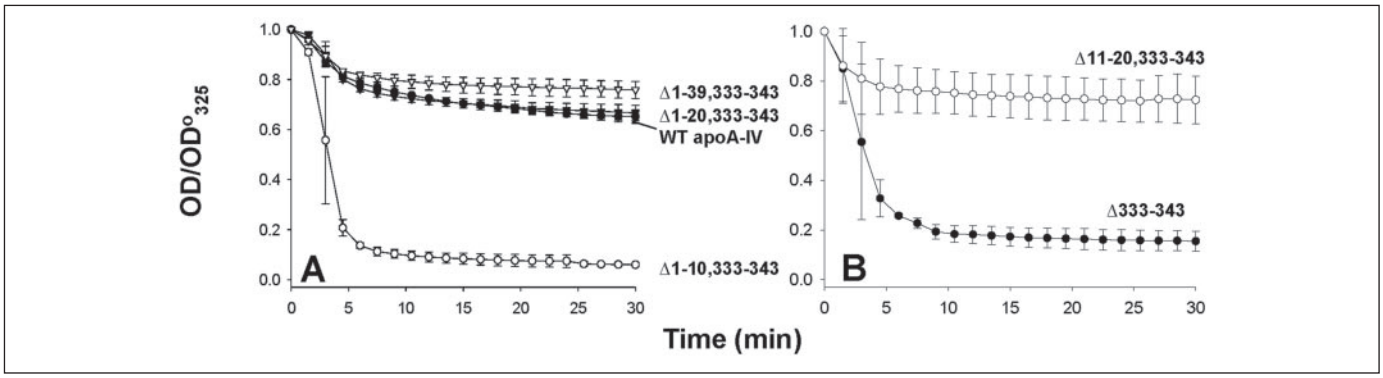

FIGURE 6. **DMPC liposome solubilization by WT apoA-IV and N-terminal mutants also lacking the inhibitory region from 333 to 343.** Both panels are organized similarly to Fig. 3*A*.

TABLE ONE

Rate constants for WT and mutant apoA-IV in the DMPC clearance assay

Mutant protein	k^a
WT apoA-IV	0.039 ± 0.012
Δ354–367	0.024 ± 0.005
Δ344–376	0.042 ± 0.017
Δ333–376	0.279 ± 0.080[b]
Δ333–343	0.171 ± 0.029[b]
Δ333–337	0.217 ± 0.039[b]
Δ338–342	0.094 ± 0.034
Δ1–39,333–343	0.040 ± 0.006
Δ1–20,333–343	0.051 ± 0.006
Δ1–10,333–343	0.260 ± 0.065[b]

[a] k is the rate constant derived from the first 5 min of the DMPC clearance assay; means ± S.D.
[b] Denotes difference of $p < 0.05$ between WT and mutant as determined by one-way analysis of variance followed by a Tukey-Kramer multiple comparisons test.

TABLE TWO

Thermal denaturation parameters of WT and mutant apoA-IV

Mutant protein	ΔH_v	DMPC association rate
	kcal/mol[a]	
WT apoA-IV	48	Slow
Δ344–376	47	Slow
Δ333–376	45	Fast
Δ333–343	40	Fast
Δ333–337	43	Fast
Δ338–342	40	Slow[b]
Δ1–10, 333–343	38	Fast
Δ1–20, 333–343	43	Slow
Δ271–376[c]	34	Fast
Δ1–39, 271–376[c]	28	Slow

[a] The estimated error on these experiments is ± 0.5 kcal/mol ($n = 2$).
[b] This mutant reaches an equilibrium that is similar to the "fast" lipid binding mutants (Fig. 5), but a statistical comparison of the rate in the first 5 min shows no significant difference from "slow" lipid binding mutants (TABLE ONE).
[c] The data for these two mutants is shown for discussion purposes and was previously published in Ref. 14.

TABLE THREE

Fluorescence quenching parameters of WT and mutant apoA-IV

Mutant protein	λ_{max}[a]	K_{sv}[b]
	nm	M^{-1}
WT apoA-IV	334.7 ± 2.1	2.84 ± 0.20
Δ344–376	335.5 ± 0.7	2.81 ± 0.23
Δ333–376	342.6 ± 1.9[c]	8.78 ± 1.09[c]
Δ333–343	338.5 ± 1.0[c]	5.53 ± 0.02[c]

[a] The λ_{max} is the wavelength of maximum fluorescence at 25 °C ($n = 3$, ± 1 S.D.).
[b] The K_{sv} is the Stern-Volmer quenching constant, indicating relative exposure of the N-terminal tryptophan residue to acrylamide.
[c] $p < 0.05$ between WT and mutant as determined by one-way analysis of variance followed by a Tukey-Kramer multiple comparisons test.

dramatically constrains its ability to associate with lipid. The mutation of a single phenylalanine in the 333–337 region can transform apoA-IV into one of the fastest lipid-binding apolipoproteins that we have measured. Clearly, apoA-IV has the innate capability to bind lipid with high efficiency, but this potential appears to be sharply attenuated by conformational factors in its lipid-free state.

Based upon a series of fluorescence studies, Weinberg *et al.* (2, 23, 25) proposed that apoA-IV exists as a loosely organized confluence/bundle of amphipathic α-helices in which the hydrophobic faces are oriented inwards toward the center of the bundle. Using a mutagenesis approach, we subsequently proposed that this bundle may exist as a single domain encompassing residues 40–332 (14). The structural organization of such helical bundles has been documented extensively for apoA-I, apoE, and lipophorin III (11, 13, 31, 32), and it has been proposed that before apolipoproteins can bind to lipid, they must first undergo a conformational transformation in which the helical bundle domain "opens," thereby reorienting the amphipathic faces of the constituent helices toward the hydrophobic lipid interface (32, 33). In apoE, intramolecular interactions between residues in distant domains stabilize the lipid-free "closed" conformation (11). In apoA-IV, we have previously observed that deletion of the N terminus increases both its stability and the cooperativity of interactions between its α-helices (14). This region is predicted to exist as a globular domain containing G-type helices that are not thought to engage in lipid binding (30). Thus, the N-terminal domain may partially destabilize the helical packing or global conformation of apoA-IV, which could promote the association of the protein with lipid under the right conditions.

As a unifying interpretation of our data, we propose that residues 334–335 can inhibit the ability of the N-terminal globular domain to destabilize the folded lipid-free form of apoA-IV. This would maintain the helical bundle in a more tightly packed, closed conformation, which, in turn, would result in slow binding to lipid interfaces. Deletion or mutation of residues 334 and/or 335 would release the N-terminal globular domain and thus destabilize or "loosen" the packing of the multihelical bundle promoting lipid binding. It is clear from our fluorescence quenching studies that deletion of residues 333–343 can dramatically affect the environment of the Trp residue in the N terminus. Whether

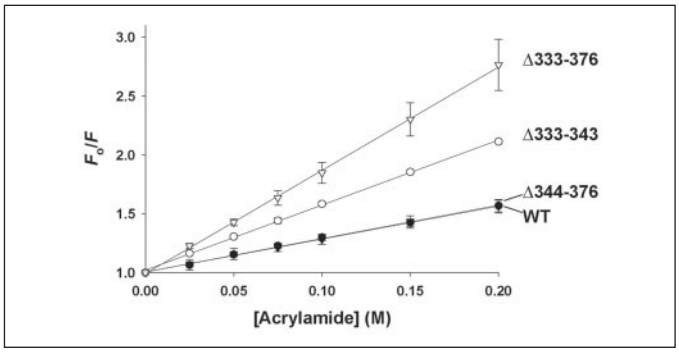

FIGURE 7. **Steady-state Stern-Volmer plot of WT apoA-IV and deletion mutants.** Fluorescence emission spectra of WT and mutant apoA-IV at concentrations of 0.1 mg/ml were collected in STB at room temperature and excitation wavelength of 295 nm. Fluorescence emission was scanned from 302 to 375 nm to determine F_o/F, the ratio of the maximum intensity in the absence of acrylamide to the intensity in the presence of the indicated concentration of acrylamide. Each data point is the average of three separate observations (± S.D.). The *lines* are linear regressions of the data; the slopes of the lines give the Stern-Volmer quenching constants.

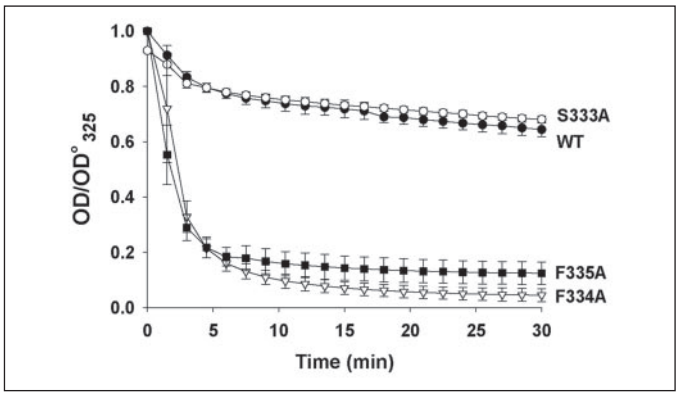

FIGURE 8. **DMPC liposome solubilization by WT apoA-IV and individual point mutants within the putative inhibitory region.** This figure is organized similarly to Fig. 3*A*.

this occurs by direct interaction of the two sites in the tertiary structure or via a long range conformational change will require further study. Nevertheless, this model is consistent with the observations that deletion of the inhibitory sequence: (*a*) reduced the van't Hoff enthalpy (albeit by only 7–15% compared with the 40–45% reduction seen with deletions that disrupt the helical bundle domain (14)) and (*b*) dramatically increased the rate of DMPC vesicle clearance. This model also predicts that if the N-terminal globular domain were disrupted by mutagenesis, it would no longer function as a destabilizing factor, and apoA-IV would revert to its native slow binding behavior as we observed.

It has been documented that disruption of apolipoprotein structure can result in enhanced binding to lipids (34). Thus, it could be argued that the disruptions caused by mutating the C-terminal inhibitory domain may simply increase the thermodynamic drive to move from a relatively unstable solution state to a more stable lipid-bound form. However, several of our observations argue against this possibility. Although all mutants exhibited a lower van't Hoff enthalpy of denaturation than the WT protein, there was no consistent relationship between lipid binding rate and thermodynamic stability. For example, as seen in TABLE TWO, the Δ333–337 and Δ1–20,333–343 mutants have identical ΔH_v values; yet one is a fast binder, whereas the other is slow. Moreover, in our previous study (14), deletion of the C terminus into the putative helical bundle domain (Δ271–376, TABLE TWO) yielded a mutant with an extremely low stability (ΔH_v = 34 kcal/mol) that was a fast lipid binder, yet simultaneous deletion of the N terminus resulted in an even less stable mutant (ΔH_v = 28 kcal/mol) that was a slow lipid binder. These observations indicate that the lipid affinity of a given apoA-IV mutant does not strictly correlate with its thermodynamic stability, suggesting that the N- and C-terminal domains induce changes in conformation that modulate lipid affinity.

What could be the biological function of this inhibition of lipid binding in native apoA-IV? One possibility is that the varied metabolic functions of apoA-IV may require differential but adaptable lipid affinity. For example, it has been postulated that apoA-IV plays a role in chylomicron assembly (1, 35). However, once lipolysis of chylomicrons begins, apoA-IV is rapidly shed from their surface and thereafter circulates predominantly as a lipid-free protein. Perhaps the N and C termini evolved to "lock" the protein in a closed conformation with relatively low lipid affinity to maintain it in a free form required for other metabolic functions once its role in chylomicron assembly was completed. In this regard, although apoA-IV is present at a lower concentration than apoA-I in plasma, a much higher fraction exists in a lipid-free form. Consequently its partitioning into the interstitial space may be much greater than that of apoA-I (36). Because lipid-free apolipoproteins are required for interaction with the ATP binding cassette transporter A1, it is possible that this low lipid affinity of apoA-IV may allow it to participate in apolipoprotein-mediated cholesterol efflux in the periphery. Perhaps the lipid-free form of apoA-IV is required for its antioxidant and anorexic roles as well (3, 37). Finally, it is possible that localized intracellular or extracellular environmental factors, such as pH or ionic concentration, could induce the conformational changes in apoA-IV that modulate its lipid affinity (23).

The powerful impact of the C-terminal residues on the structure and function of apoA-IV is evidenced by the fact that the most common polymorphisms of apoA-IV are two single nucleotide polymorphisms that encode a T347S (apoA-IV-Ser) and a Q360H (apoA-IV-2) substitution in the apoA-IV molecule (38). These substitutions have significant biophysical consequences: the His isoprotein displays increased lipid affinity (39), whereas the Ser isoprotein binds lipid with lower affinity (25) compared with the WT protein. Most likely as a direct result of these changes in lipid binding, individuals carrying a His allele display more rapid post-prandial triglyceride-rich lipoprotein clearance than Gln allele homozygotes, whereas individuals carrying a Ser allele display delayed clearance (40). Thus, it appears that even subtle modifications of the C terminus in apoA-IV can have important physiological consequences.

In conclusion, we have established the exact location of a C-terminal sequence in human apoA-IV that dramatically constrains its ability to bind to lipid interfaces. Given the potential importance of lipid affinity in the biological function of apoA-IV, this finding provides a guide to the design of informative apoA-IV mutants. When introduced into cell and transgenic mouse models such mutants will be powerful tools to delineate the role of apoA-IV lipid binding on its function in chylomicron assembly and in lipoprotein metabolism in general.

REFERENCES

1. Green, P. H., Glickman, R. M., Riley, J. W., and Quinet, E. (1980) *J. Clin. Investig.* **65,** 911–919
2. Weinberg, R. B., Anderson, R. A., Cook, V. R., Emmanuel, F., Denefle, P., Hermann, M., and Steinmetz, A. (2000) *J. Lipid Res.* **41,** 1410–1418
3. Qin, X., Swertfeger, D. K., Zheng, S., Hui, D. Y., and Tso, P. (1998) *Am. J. Physiol.* **274,** H1836–H1840
4. Vowinkel, T., Mori, M., Krieglstein, C. F., Russell, J., Saijo, F., Bharwani, S., Turnage, R. H., Davidson, W. S., Tso, P., Granger, D. N., and Kalogeris, T. J. (2004) *J. Clin. Investig.* **114,** 260–269
5. Remaley, A. T., Stonik, J. A., Demosky, S. J., Neufeld, E. B., Bocharov, A. V., Vishnyakova, T. G., Eggerman, T. L., Patterson, A. P., Duverger, N. J., Santamarina-Fojo, S.,

and Brewer, H. B., Jr. (2001) *Biochem. Biophys. Res. Commun.* **280,** 818–823
6. Fujimoto, K., Cardelli, J. A., and Tso, P. (1992) *Am. J. Physiol.* **262,** G1002–G1006
7. Karathanasis, S. K., Oettgen, P., Haddad, I. A., and Antonarakis, S. E. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8457–8461
8. Boguski, M. S., Elshourbagy, N., Taylor, J. M., and Gordon, J. I. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 992–996
9. Elshourbagy, N. A., Walker, D. W., Paik, Y. K., Boguski, M. S., Freeman, M., Gordon, J. I., and Taylor, J. M. (1987) *J. Biol. Chem.* **262,** 7973–7981
10. Weinberg, R. B. (1994) *J. Lipid Res.* **35,** 2212–2222
11. Wilson, C., Wardell, M. R., Weisgraber, K. H., Mahley, R. W., and Agard, D. A. (1991) *Science* **252,** 1817–1822
12. Davidson, W. S., Hazlett, T., Mantulin, W. W., and Jonas, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13605–13610
13. Saito, H., Dhanasekaran, P., Nguyen, D., Holvoet, P., Lund-Katz, S., and Phillips, M. C. (2003) *J. Biol. Chem.* **278,** 23227–23232
14. Pearson, K., Saito, H., Woods, S. C., Lund-Katz, S., Tso, P., Phillips, M. C., and Davidson, W. S. (2004) *Biochemistry* **43,** 10719–10729
15. Pearson, K., Liu, M., Shen, L., Tso, P., and Davidson, W. S. (2005) *Protein Expression Purif.* **41,** 447–453
16. McLean, L. R., and Hagaman, K. A. (1993) *Biochim. Biophys. Acta* **1167,** 289–295
17. Panagotopulos, S. E., Witting, S. R., Horace, E. M., Hui, D. Y., Maiorano, J. N., and Davidson, W. S. (2002) *J. Biol. Chem.* **277,** 39477–39484
18. Acharya, P., Segall, M. L., Zaiou, M., Morrow, J., Weisgraber, K. H., Phillips, M. C., Lund-Katz, S., and Snow, J. (2002) *Biochim. Biophys. Acta* **1584,** 9–19
19. Lehrer, S. S. (1971) *Biochemistry* **10,** 3254–3263
20. Weinberg, R. B., and Spector, M. S. (1985) *J. Biol. Chem.* **260,** 14279–14286
21. Weinberg, R. B., Ibdah, J. A., and Phillips, M. C. (1992) *J. Biol. Chem.* **267,** 8977–8983
22. Weinberg, R. B., and Spector, M. S. (1985) *J. Lipid Res.* **26,** 26–37
23. Weinberg, R. B., and Spector, M. S. (1985) *J. Biol. Chem.* **260,** 4914–4921
24. Segall, M. L., Dhanasekaran, P., Baldwin, F., Anantharamaiah, G. M., Weisgraber, K. H., Phillips, M. C., and Lund-Katz, S. (2002) *J. Lipid Res.* **43,** 1688–1700
25. Weinberg, R. B., Cook, V. R., Beckstead, J. A., Martin, D. D., Gallagher, J. W., Shelness, G. S., and Ryan, R. O. (2003) *J. Biol. Chem.* **278,** 34438–34444
26. Navarro, M. A., Acin, S., Iturralde, M., Calleja, L., Carnicer, R., Guzman-Garcia, M. A., Gonzalez-Ramon, N., Mata, P., Isabel, B., Lopez-Bote, C. J., Lampreave, F., Piniero, A., and Osada, J. (2004) *Gene* (*Amst.*) **325,** 157–164
27. Weinberg, R. B. (1988) *Biochemistry* **27,** 1515–1521
28. Davidson, W. S., Arnvig-McGuire, K., Kennedy, A., Kosman, J., Hazlett, T. L., and Jonas, A. (1999) *Biochemistry* **38,** 14387–14395
29. Weinberg, R. B. (1987) *Biochim. Biophys. Acta* **918,** 299–303
30. Segrest, J. P., Garber, D. W., Brouillette, C. G., Harvey, S. C., and Anantharamaiah, G. M. (1994) *Adv. Protein Chem.* **45,** 303–369
31. Silva, R. A., Hilliard, G. M., Fang, J., Macha, S., and Davidson, W. S. (2005) *Biochemistry* **44,** 2759–2769
32. Wang, J., Sykes, B. D., and Ryan, R. O. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 1188–1193
33. Weisgraber, K. H. (1994) *Adv. Protein Chem.* **45,** 249–302
34. Weers, P. M., Narayanaswami, V., Choy, N., Luty, R., Hicks, L., Kay, C. M., and Ryan, R. O. (2003) *Biophys. Chem.* **100,** 481–492
35. Kalogeris, T. J., Rodriguez, M. D., and Tso, P. (1997) *J. Nutr.* **127,** (suppl.) 537–543
36. Roheim, P. S., Edelstein, D., and Pinter, G. G. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 1757–1760
37. Ostos, M. A., Conconi, M., Vergnes, L., Baroukh, N., Ribalta, J., Girona, J., Caillaud, J. M., Ochoa, A., and Zakin, M. M. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21,** 1023–1028
38. Weinberg, R. B. (2002) *Curr. Opin. Lipidol.* **13,** 125–134
39. Weinberg, R. B., Jordan, M. K., and Steinmetz, A. (1990) *J. Biol. Chem.* **265,** 18372–18378
40. Hockey, K. J., Anderson, R. A., Cook, V. R., Hantgan, R. R., and Weinberg, R. B. (2001) *J. Lipid Res.* **42,** 211–217