Disruption of the C-terminal helix by single amino acid deletion is directly responsible for impaired cholesterol efflux ability of apolipoprotein A-I Nichinan

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Abstract Apolipoprotein A-I (apoA-I) Nichinan, a naturally occurring variant with ΔE235 in the C terminus, is associated with low plasma HDL levels. Here, we investigated the tertiary structure, lipid-binding properties, and ability to induce cellular cholesterol efflux of apoA-I Nichinan and its C-terminal peptide. Thermal and chemical denaturation experiments demonstrated that the ΔE235 mutation decreased the protein stability compared with wild type (WT). ApoA-I Nichinan exhibited capabilities to bind to or solubilize lipid vesicles that are intermediate to that of WT and a L230P/L233P/Y236P variant in which the C-terminal α-helix folding is completely disrupted and forms relatively larger and unstable discoidal complexes, indicating that perturbation of the C-terminal α-helical structure by the ΔE235 mutation leads to reduced lipid binding. Supporting this, apoA-I 209-241/ΔE235 peptide showed significantly decreased ability to form α-helix both in the lipid-free and lipid-bound states, and reduced efficiency to solubilize vesicles. In addition, both apoA-I Nichinan and its C-terminal peptide exhibited reduced activity in ABCA1-mediated cellular cholesterol efflux. Thus, the disruption of the ability of the C-terminal region to form α-helix caused by the E235 deletion appears to be the important determinant of impaired lipid binding and cholesterol efflux ability and, consequently, the low plasma HDL levels of apoA-I Nichinan probands.—Kono, M., T. Tanaka, M. Tanaka, C. Vedhachalam, P. S. Chetty, D. Nguyen, P. Dhanasekaran, S. Lund-Katz, M. C. Phillips, and H. Saito. **Disruption of the C-terminal helix by single amino acid deletion is directly responsible for impaired cholesterol efflux ability of apolipoprotein A-I Nichinan.** ***J. Lipid Res.*** **2010.** 51: **809–818.**

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Apolipoprotein A-I (apoA-I) is the major protein of plasma HDL and functions as a critical mediator in reverse cholesterol transport, a process by which excess cholesterol in peripheral cells is transferred via HDL to the liver for catabolism (1–3). It is generally thought that the first step in the reverse cholesterol transport pathway involves the efflux of cellular lipids to lipid-poor apoA-I, which is mediated by its interactions with ABCA1 (1–3). Thus, mutations in the genes of apoA-I (4, 5) or ABCA1 (6, 7) lead to inadequate lipid transport from cells to extracellular spaces, resulting in the failure of HDL biogenesis and, consequently, low plasma HDL levels.

Human apoA-I is a 243-residue polypeptide that contains 11- and 22-residue repeats of amphipathic α-helices (8). It has been demonstrated that the apoA-I molecule folds into two tertiary structure domains, comprising an N-terminal α-helix bundle spanning residues 1–187 and a separate less organized C-terminal region spanning the remainder of the molecule (9–11). The helical segments in the N-terminal domain are involved in the activation of LCAT (12, 13), whereas the C-terminal helix is involved in the strong lipid-binding properties of this protein (14–16). The interaction between the N- and C-terminal helical regions appears to contribute to the overall conformational stability of the apoA-I molecule in solution (17–20).

In humans, over 40 natural mutations in apoA-I have been reported to date, of which roughly half can reduce

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; apoA-I, apolipoprotein A-I; BHK, baby hamster kidney; CD, circular dichroism; DMPC, dimyristoyl phosphatidylcholine; GdnHCl, guanidine hydrochloride; ITC, isothermal titration calorimetry; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; SUV, small unilamellar vesicle; UV, ultraviolet; WMF, wavelength of maximum fluorescence; WT, wild type.

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plasma HDL levels and increase the incidence of cardiovascular disease (4, 5). The large majority of mutations associated with low plasma HDL levels are clustered in the N-terminal helix bundle domain, whereas very few mutations are in the C-terminal domain (5). ApoA-I Nichinan, which contains the deletion of residue E235 in the C-terminal helical region, is known to be associated with low plasma HDL cholesterol and apoA-I levels (21, 22). Interestingly, this ΔE235 mutant is associated with normal plasma LCAT activity but a decrease in the ratio of HDL_2 to HDL_3 particles. Because a number of studies have demonstrated the importance of the C-terminal region of apoA-I in the ABCA1-mediated lipid efflux from cells and HDL biogenesis (23–29), it is possible that impaired lipid-binding ability caused by the deletion of E235 in apoA-I Nichinan could underlie the decreased plasma HDL concentrations seen in carriers of the mutation. However, the molecular basis for these effects, especially the structure-function relationship of apoA-I Nichinan, has not been established yet.

In the present study, we investigated the effects of the E235 deletion in apoA-I and its C-terminal peptide on the structure, lipid-binding properties, and ability to induce ABCA1-mediated cellular cholesterol efflux. The results suggest that the disruption of the helix-forming ability of the C-terminal region caused by the E235 deletion is the critical determinant of impaired cholesterol efflux ability and, consequently, low plasma HDL levels in apoA-I Nichinan probands.

MATERIALS AND METHODS

Preparation of apoA-I proteins and peptides

The mutation in human apoA-I to delete residue E235 was made using the QuikChange site-directed mutagenesis kit (Stratagene). Human wild type (WT) apoA-I and engineered mutants were expressed and purified as described (15, 16). The apoA-I preparations were at least 95% pure as assessed by SDS-PAGE. The C-terminal apoA-I 209-241 and 209-241/ΔE235 peptides were synthesized using Fmoc chemistry as described (30, 31). The N and C termini were capped with an acetyl group and an amide group, respectively. Peptide purity was verified by analytical HPLC (>97%) and mass spectrometry. In all experiments, apoA-I variants and peptides were freshly dialyzed from 6 M guanidine hydrochloride (GdnHCl) solution into the appropriate buffer before use.

CD spectroscopy

Near- and far-ultraviolet (UV) circular dichroism (CD) spectra were recorded with a Jasco J-810 or an Aviv 62DS spectropolarimeter. After dialysis from 6 M GdnHCl solution, the apoA-I protein or peptide solutions of 25–50 μg/ml in 10 mM sodium phosphate buffer (pH 7.4) were subjected to near-UV measurements by scanning from 185 to 260 nm in a 1–2 mm cuvette, and solutions of 0.3 mg/ml apoA-I concentration were used for near-UV measurements (270–320 nm) in a 1 cm cuvette. For the mixture with small unilamellar vesicles (SUVs), the apoA-I protein or peptide was incubated for 1 h prior to the measurement with egg phosphatidylcholine (PC) SUVs prepared by sonication as described before (32). The results were corrected by subtracting the baseline for an appropriate blank sample. The α-helix content was calculated from the molar ellipticity at 222 nm ($[\theta]_{222}$) using the equation: % α-helix = $[(-[\theta]_{222} + 3000)/(36000 + 3000)] \times 100$ (33). Thermal unfolding was monitored from the change in $[\theta]_{222}$ over the temperature range of 20–90°C, as described (34). Although there was some variability in the initial and final α-helix contents, the apoA-I variants exhibited reversible thermal unfolding. The van't Hoff enthalpy, ΔH_v, was calculated from the slope of the line fitted by linear regression to the equation, $\ln K_D = -(\Delta H_v/R)\ 1/T + \text{constant}$, where K_D is the equilibrium constant describing the unfolding of apoA-I at each temperature, R is the gas constant, and T is temperature. The kinetics of thermal unfolding of apoA-I in discoidal complexes with 1-palmitoyl-2-oleoyl PC (POPC) prepared using the cholate dispersion method (35) was monitored by temperature-jump analysis from 25 to 80–90°C (36).

Chemical denaturation studies

For monitoring chemical denaturation, lipid-free proteins at a concentration of 25–50 μg/ml were incubated overnight at 4°C with GdnHCl or urea at various concentrations. When complexed with POPC, apoA-I was completely denatured by incubating for 72 h (37). K_D at a given denaturant concentration was calculated from the change in either $[\theta]_{222}$ or wavelength of maximum fluorescence (WMF) of intrinsic Trp residues (15). The Trp emission fluorescence spectrum was recorded from 300 to 420 nm using an excitation wavelength of 295 nm with a Hitachi F-7000 fluorescence spectrophotometer in Tris buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4). The Gibbs free energy of denaturation in the absence of denaturant, ΔG_D°, the midpoint of denaturation, $D_{1/2}$, and m value, which reflects the cooperativity of denaturation in the transition region, were determined by the linear equation, $\Delta G_D = \Delta G_D^\circ - m[\text{denaturant}]$, where $\Delta G_D = -RT \ln K_D$ (19, 33).

ANS fluorescence measurements

8-Anilino-1-naphthalenesulfonic acid (ANS) fluorescence measurements were carried out with a Hitachi F-4500 fluorescence spectrophotometer. The extent of ANS binding to hydrophobic sites on the apoA-I variants was determined by measuring ANS fluorescence spectra recorded from 400 to 600 nm at an excitation wavelength of 395 nm, in the absence or presence of 50 μg/ml protein and an excess of ANS (250 μM) (15).

Isothermal titration calorimetry (ITC) measurements

Heats of apoA-I binding to SUV were measured with a MicroCal MCS isothermal titration calorimeter at 25°C (32). To ensure that the injected protein bound completely to the SUV surface, the PC to protein molar ratio was kept over 10,000. Heat of dilution determined by injecting apoA-I solution into buffer was subtracted from the heat for the corresponding apoA-I-SUV binding experiments. The decay rate constants for the heats of binding were obtained from fitting the titration curves to a one-phase exponential decay model (15).

DMPC clearance assay

The kinetics of solubilization of dimyristoyl PC (DMPC) vesicles by the apoA-I variants or peptides were measured by monitoring the time-dependent decrease in turbidity at 24.6°C. For the apoA-I proteins, DMPC large unilamellar vesicles (LUVs) extruded through a 200-nm filter at a concentration of 0.25 mg/ml were mixed with apoA-I samples (0.02–0.2 mg/ml), and incubated for 15 min to monitor the light scattering intensity at 325 nm with a Shimadzu UV-2450 spectrophotometer (20, 38). For the apoA-I peptides, much lower concentrations of DMPC multi-

lamellar vesicles (MLVs) and peptides (typically 34 μg/ml DMPC and 2–20 μg/ml peptides) were used to avoid the aggregation of DMPC/peptide complexes during experiments. The decrease in turbidity was monitored by the right-angle light scattering intensity using a Hitachi F-4500 spectrophotometer with both excitation and emission wavelengths set at 600 nm (31, 39).

Monolayer exclusion pressure measurements

The relative affinities of apoA-I peptides for the lipid-water interface were determined with a surface balance technique, as described previously (40, 41). Linear extrapolation of the initial surface pressure of egg PC monolayer in the absence of peptides (π_i) versus the change in surface pressure in the presence of peptides ($\Delta\pi_i$) curve to the point at which $\Delta\pi_i = 0$ gave the monolayer exclusion pressure (π_e). The π_e value indicates the surface pressure at which the peptides are no longer able to penetrate into the egg PC monolayer.

Cell culture and cholesterol efflux

J774 murine macrophages were grown and maintained in RPMI 1640 supplemented with 10% FBS and 0.5% gentamycin. For efflux experiments, these cells were seeded in 12-well plates, grown to 80–90% confluence, and then labeled by incubating the cells for 24 h in RPMI medium supplemented with 1% FBS, 2 μg/ml CP-113,818 ACAT inhibitor, and 3 μCi/ml [^{3}H]cholesterol (25). After labeling, the cells were washed with MEM-Hepes and incubated with RPMI medium containing 0.2% BSA, 2 μg/mlCP-113,818ACATinhibitor,and0.3mM8-(4-chlorophenylthio)-cAMP for 12 h, to upregulate the expression of ABCA1. Cells were then washed with MEM-Hepes and incubated with or without apoA-I proteins or peptides at the indicated concentrations for 4 h. To determine the free unesterified cholesterol efflux, aliquots were removed from the incubation medium at specific time points, filtered, and radioactivity was determined by liquid scintillation counting. The percent cholesterol efflux was calculated after subtracting the background cholesterol efflux (without apoA-I) as follows: (counts/min in medium at 4 h/cpm in cells at $t = 0$) × 100.

For efflux experiments with baby hamster kidney (BHK) cells, BHK cells transfected with human ABCA1 (a generous gift from Dr. John Oram) were grown and maintained in DMEM containing 10% FBS and 0.5% gentamycin (42). These cells were seeded in 12-well plates and then labeled by incubating the cells for 24 h in DMEM medium supplemented with 2.5% FBS, 2 μg/ml CP-113,818 ACAT inhibitor, and 3 μCi/ml [^{3}H]cholesterol. ABCA1 was induced by incubating the labeled cells for 18 h in DMEM containing 0.2% BSA and 10 nM mifepristone. Cells were then washed with MEM-Hepes and incubated with or without apoA-I proteins or peptides under the indicated conditions of concentration for 4 h and the percentage cholesterol efflux was calculated as mentioned above.

RESULTS

Secondary structure, thermal unfolding, and near-UV CD spectra of apoA-I C-terminal mutants

The secondary structure and thermal unfolding of apoA-I Nichinan were analyzed by far-UV CD spectroscopy, in comparison with the apoA-I L230P/L233P/Y236P mutant in which the C-terminal α-helix is completely disrupted (19, 25). The α-helix contents of the lipid-free apoA-I Nichinan and L230P/L233P/Y236P variants were no different from WT (**Table 1**), indicating that the deletion of E235 and the proline insertions either *1*) do not perturb the local α-helical structure significantly or *2*) the C-terminal region is not helical in the lipid-free state. **Figure 1A** shows the thermal unfolding curves for WT and the Nichinan variant. As summarized in Table 1, both apoA-I Nichinan and the L230P/L233P/Y236P variant displayed a lower midpoint (T_m) and higher van't Hoff enthalpy (ΔH_v) compared with WT, indicating that the E235 deletion and the proline insertions decrease the protein stability but increase the unfolding cooperativity of apoA-I (19). In contrast, near-UV CD spectra of WT and the Nichinan variant were very similar, exhibiting the negative trough with a minimum at 292 nm (Fig. 1B). This suggests that the orientational ordering of the Trp residues located in the N-terminal domain is not affected by the Nichinan mutation (16, 43).

Chemical denaturation of apoA-I C-terminal mutants

Next, we monitored chemical denaturation profiles of apoA-I variants by the molar ellipticity at 222 nm as well as by the change in WMF of Trp fluorescence spectra. Molar ellipticity measures the unfolding of all α-helices in the protein molecule whereas Trp fluorescence measures the stability of the N-terminal helix bundle because human apoA-I contains four Trp residues, all of which are located in the N-terminal domain. In **Fig. 2A**, the denaturation curve of apoA-I Nichinan monitored by the molar ellipticity was shifted to lower concentrations of urea compared with WT. As a result, the Nichinan variant exhibited significant decreases in the Gibbs free energy and midpoint of denaturation compared with WT apoA-I, whereas the L230P/L233P/Y236P mutation had little effect on these parameters (Table 1). Figure 2B compares the free energies of denaturation with GdnHCl and urea for apoA-I variants as monitored by Trp fluorescence. It is thought

TABLE 1. α-Helix content and parameters for thermal and urea denaturation of apoA-I variants monitored by molar ellipticity

ApoA-I variant	α-Helix (%)	Thermal Denaturation		Urea Denaturation		
		T_m[a]	ΔH_v[b]	$\Delta G_D°$	m	$D_{1/2}$
		°C	*kcal/mol*	*kcal/mol*		*M*
WT	46 ± 3	60	33	3.9 ± 0.3	1.5 ± 0.2	2.7 ± 0.1
Nichinan (ΔE235)	46 ± 2	57	41	3.3 ± 0.3*	1.4 ± 0.1	2.5 ± 0.1*
L230P/L233P/Y236P	46 ± 2	57	43	4.0 ± 0.3	1.6 ± 0.2	2.6 ± 0.1

[a] The reproducibility in T_m is ±1.5°C.
[b] Estimated error is ±0.5 kcal/mol.
*P<0.05 compared with WT apoA-I.

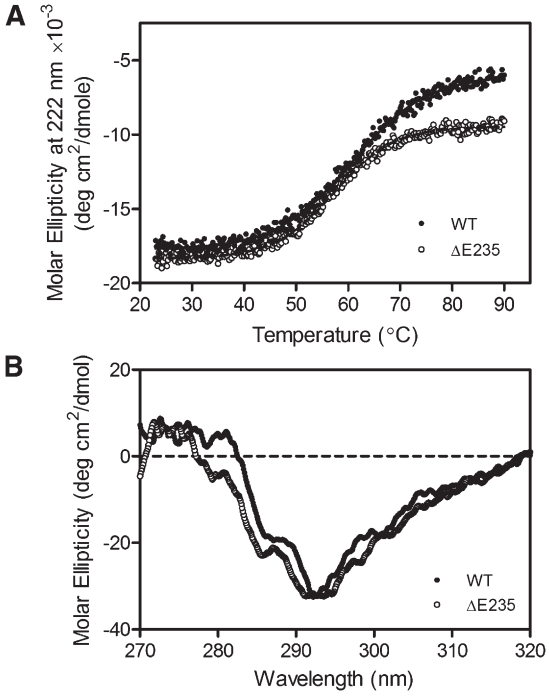

Fig. 1. Thermal denaturation curves monitored by the molar ellipticity at 222 nm (A) and near-UV CD spectra (B) of human apoA-I WT (●) and ΔE235 variant (○). Protein concentrations were 50 μg/ml (A) and 0.3 mg/ml (B).

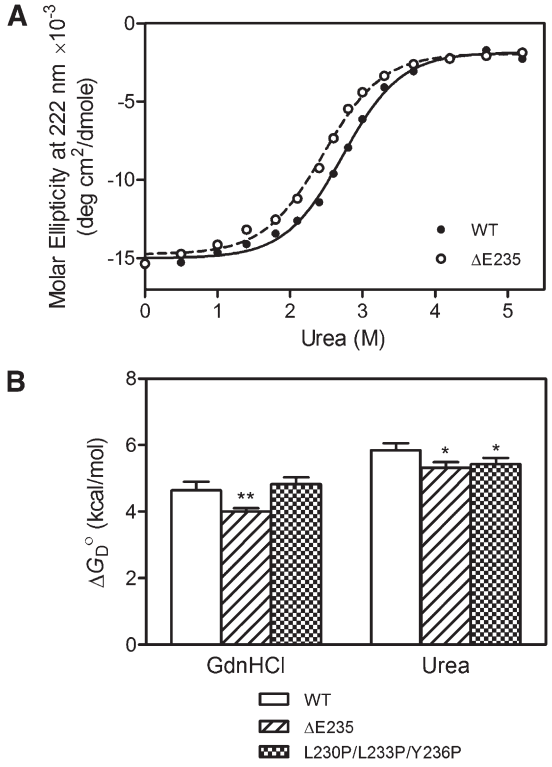

Fig. 2. Chemical denaturation of the C-terminal mutants of apoA-I monitored by molar ellipticity (A) or Trp fluorescence (B). A: Urea-induced denaturation curves of apoA-I WT (●) and ΔE235 variant (○). B: Comparison of the free energies of denaturation induced by GdnHCl and urea. $*P < 0.05$, $**P < 0.01$ compared with WT apoA-I.

that the electrostatic interactions between amino acids in proteins are shielded by GdnHCl whereas uncharged urea has no effect on the electrostatic interactions (44). With both denaturants, significant decreases in the free energy of denaturation for the Nichinan variant were observed compared with WT, indicating that the E235 deletion destabilizes the N-terminal helix bundle of apoA-I via both electrostatic and nonelectrostatic interactions. In addition, large differences in ΔG_D° value monitored by molar ellipticity and Trp fluorescence such as 3.3 versus 5.3 kcal/mol for the Nichinan variant (Fig. 2B and Table 1) appear to come from the fact that molar ellipticity measures only unfolding of α-helical structure whereas Trp fluorescence measures unfolding of tertiary structure as well so that disruption of helix-helix contacts is included in the free energy change (20).

ANS binding

To compare the exposure of hydrophobic regions in the apoA-I C-terminal variants, ANS binding experiments were performed. Compared with free ANS, a great increase in ANS fluorescence spectrum was observed for WT apoA-I (**Fig. 3**), indicating the presence of ANS accessible hydrophobic surface (15, 45, 46). Because the deletion or disruption of the C-terminal helical region such as the L230P/L233P/Y236P mutation greatly decrease the hydrophobic sites created by the C-terminal domain (15, 19), the finding that the ΔE235 mutation displayed the intermediate effect on ANS fluorescence between WT and L230P/L233P/Y236P apoA-I (Fig. 3) suggests that the deletion of E235 alters the conformation of the C-terminal domain and partially disrupts the hydrophobic sites.

Lipid interactions of apoA-I C-terminal mutants

To assess the lipid-binding properties of the apoA-I variants, we measured the heats of binding of apoA-I to egg PC SUVs using ITC (16, 32). As listed in **Table 2**, WT apoA-I exhibited a large exothermic heat of about −93 kcal/mol upon binding to SUVs; this exothermic enthalpy of binding is the major component of the favorable free energy of apoA-I binding to SUVs, and the increase in α-helix content associated with such binding is largely responsible for this enthalpy (32, 47). Thus, the large reduction in the binding enthalpy and small increase in α-helical amino acids upon binding to SUVs for the L230P/L233P/Y236P variant compared with WT appear to result from impaired ability to form α-helix caused by the proline insertions (19). The intermediate effects of the Nichinan variant on the binding enthalpy and increase in α-helical residues upon binding to SUVs seen in Table 2 suggest that the E235 deletion partially disrupts the ability of the C-terminal region to form α-helical structure, resulting in impaired lipid-binding capability for apoA-I Nichinan.

To further characterize the interactions of the apoA-I variants with lipids, we used a DMPC clearance assay for comparing their abilities to solubilize DMPC vesicles (16, 20, 38). The typical timecourses of clearance in **Fig. 4A**

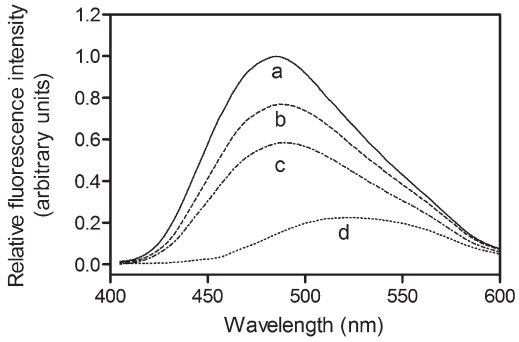

Fig. 3. ANS fluorescence emission spectra bound to apoA-I WT (a), ΔE235 (b), and L230P/L233P/Y236P (c). Spectrum of free ANS (d) is also shown for comparison. Protein and ANS concentrations were 50 μg/ml and 250 μM, respectively.

and the dependence of clearance efficiency on protein concentrations in Fig. 4B demonstrate that the solubilizing efficiency of the apoA-I variants decreases in the order, WT > Nichinan > L230P/L233P/Y236P, which is similar to the results for the SUV binding (Table 2). Because solubilization of DMPC vesicles by apolipoprotein to form discoidal complexes involves a structural reorganization of the protein molecule (48), the self-association state of protein can affect solubilization kinetics (38). Cross-linking experiments demonstrated that apoA-I Nichinan at relatively high protein concentration (1 mg/ml) tends to form more oligomers compared with WT apoA-I (data not shown). However, the decreased DMPC clearance efficiency of the Nichinan mutant was seen even at a much lower concentration range of 25–200 μg/ml (Fig. 4B), indicating that the reduced solubilizing efficiency of apoA-I Nichinan is not mainly due to the self-association state of this protein.

We also compared the stabilities of WT and the Nichinan mutant in apoA-I-POPC discoidal complexes. Electron microscopy and nondenaturating polyacrylamide gradient gel analyses demonstrated that WT apoA-I-POPC discs have a major diameter of 11 nm whereas, under the same conditions, the Nichinan mutant forms much larger discs with a major diameter of 15 nm (data not shown). In **Fig. 5A**, the denaturation curve of apoA-I Nichinan in a POPC discoidal complex monitored by the molar ellipticity was shifted to lower concentrations of GdnHCl compared with WT, and the resultant Gibbs free energy and midpoint of denaturation for the Nichinan variant were significantly decreased compared with WT apoA-I (2.2 ± 0.2 vs. 3.0 ± 0.1 kcal/mol

TABLE 2. Parameters of binding of apoA-I variants to egg PC SUV

ApoA-I Variant	ΔH	α-Helix Content on SUV	Increase in α-Helical Residues[a]
	–kcal/mol	*%*	*amino acids*
WT	92.6 ± 5.3	72 ± 3	65
Nichinan (ΔE235)	72.0 ± 2.3	69 ± 4	54
L230P/L233P/Y236P	56.1 ± 4.8	64 ± 4	43

[a] Increase in α-helical residues was estimated from the difference in α-helix contents of apoA-I in the lipid-free state (Table 1) and bound to SUV.

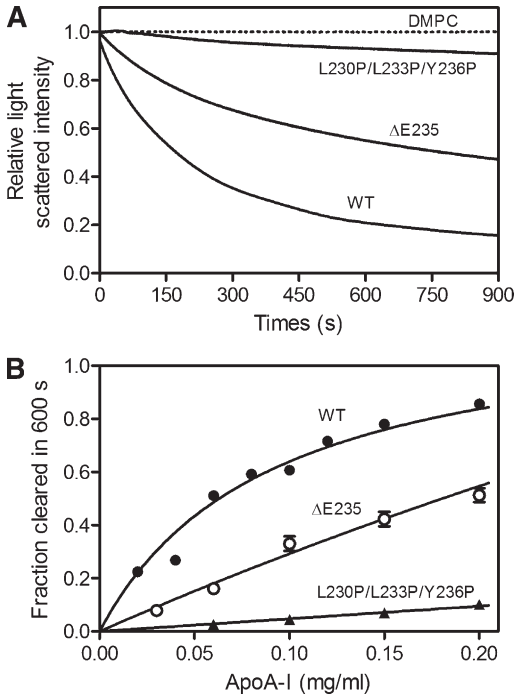

Fig. 4. Solubilization of DMPC LUVs by the C-terminal mutants of apoA-I. A: Timecourses of the decrease in turbidity at 325 nm after incubation of DMPC LUVs (0.25 mg/ml) with 0.15 mg/ml protein at 24.6°C. B: Increase in fraction of turbidity cleared in 600 s with increasing concentration of protein. ApoA-I WT (●), ΔE235 (○), L230P/L233P/Y236P (▲).

in ΔG_D° and 2.7 ± 0.1 vs. 3.2 ± 0.1 M in $D_{1/2}$, respectively). In addition, comparison of the timecourse of the protein unfolding induced by a rapid increase in temperature (Fig. 5B) showed that the unfolding of apoA-I Nichinan is faster than that of WT apoA-I in POPC discs. These results indicate that apoA-I Nichinan forms less stable and larger discoidal complexes with lipids compared with WT.

Effects of E235 deletion on structure and lipid interaction of apoA-I C-terminal peptides

The effects of the ΔE235 mutation on the C-terminal α-helix properties of apoA-I were also examined using synthetic apoA-I C-terminal peptides. Like WT apoA-I, the 33-mer peptide, apoA-I 209-241, can interact with lipids (39, 41) and promote ABCA1-mediated cellular cholesterol efflux (25, 49). **Table 3** summarizes the monolayer exclusion pressure, π_e, and α-helix contents of apoA-I 209-241 and 209-241/ΔE235 peptides. Although both peptides displayed similar lipid affinity as assessed by π_e, the deletion of E235 greatly decreased the α-helix content of apoA-I 209-241 peptide both in the absence and presence of SUVs, consistent with the previous result for a shorter C-terminal peptide, apoA-I 220-241 (31). This directly demonstrates that the E235 deletion reduces the ability of the C-terminal region of apoA-I to form α-helix upon lipid binding. As shown in **Fig. 6A** and 6B, such a weak propensity of apoA-I 209-241/ΔE235 to form α-helix resulted in a reduced ability to solubilize DMPC vesicles compared with apoA-I 209-241 (50); this effect is similar to that seen for the apoA-I C-terminal variants (Fig. 4).

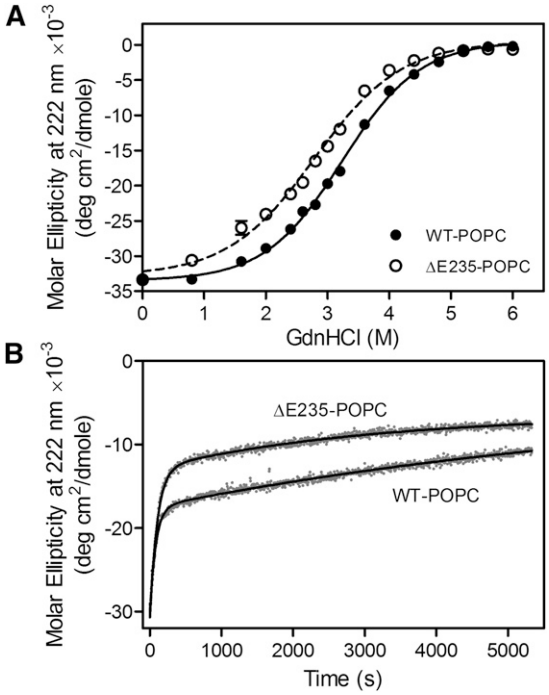

Fig. 5. Denaturation of apoA-I variants on POPC discs monitored by the molar ellipticity at 222 nm. A: Denaturation curves of apoA-I WT (●) and ΔE235 variant (○) on POPC discoidal complexes after incubation with GdnHCl for 72 h. B: Kinetics of apoA-I unfolding in temperature-jumps from 25 to 85°C.

Cholesterol efflux ability of apoA-I Nichinan variant and peptide

To assess the ability of the apoA-I Nichinan variant and its C-terminal peptide to promote ABCA1-mediated cellular cholesterol efflux, the concentration-dependence of cholesterol efflux from ABCA1-expressing J774 and BHK cells to apoA-I was determined. **Figure 7** shows hyperbolic relationships between cholesterol efflux and the apoA-I concentrations for WT apoA-I and the Nichinan variant in both types of cells. The greater difference between the cholesterol efflux to WT apoA-I and apoA-I Nichinan observed with BHK cells (Fig. 7B) compared with J774 cells (Fig. 7A) may be due to the fact that J774 macrophages express mouse ABCA1 whereas BHK cells are transfected with human ABCA1. Apparent V_{max} and K_m values derived by fitting these hyperbolic curves to the Michaelis-Menten equation are listed in **Table 4**. In both types of cells, the apparent K_m value for apoA-I Nichinan was much higher than that for WT, and the catalytic efficiency expressed as the ratio V_{max}/K_m for the Nichinan variant was about half of that of WT apoA-I (2.5 vs. 4.7 for J774 cells and 1.6 vs. 3.6 for BHK cells, respectively). This indicates that the deletion of E235 greatly reduces the efficiency of cholesterol efflux to apoA-I in both types of cells (21, 22).

Similar to previous results (25, 49), apoA-I 209-241 peptide retained cholesterol efflux ability, but a linear relationship between cholesterol efflux and peptide concentration in J774 cells (**Fig. 8**) indicates that ABCA1-mediated cholesterol efflux to this peptide is a low affinity process. Strikingly, apoA-I 209-241/ΔE235 peptide at up to 20 μg/ml failed to induce a cholesterol efflux, indicating that the deletion of E235 abolishes the ability of the C-terminal helical region of apoA-I to induce ABCA1-mediated cholesterol efflux. This finding is consistent with previous studies with amphipathic α-helical peptides demonstrating that lipid efflux via ABCA1 is sensitive to the lipid-binding affinity of the helix (41) as well as the distribution of acidic residues (49). In addition, neither the apoA-I 209-241 nor the 209-241/ΔE235 peptide gave any cholesterol efflux from BHK cells in the concentration range studied (data not shown).

TABLE 3. Parameters for lipid binding affinity of apoA-I C-terminal peptides

ApoA-I Peptide	Exclusion Pressure π_e[a]	α-Helix Content	
		Lipid-Free	SUV
	dyn/cm	%	
209-241	33	59 ± 2	70 ± 2
209-241/ΔE235	34	23 ± 3	50 ± 2

[a] Standard error is ±1 dyn/cm.

DISCUSSION

It is known that human apoA-I mutations can reduce plasma HDL levels and increase the incidence of cardiovascular disease (4, 5). ApoA-I Nichinan that contains the

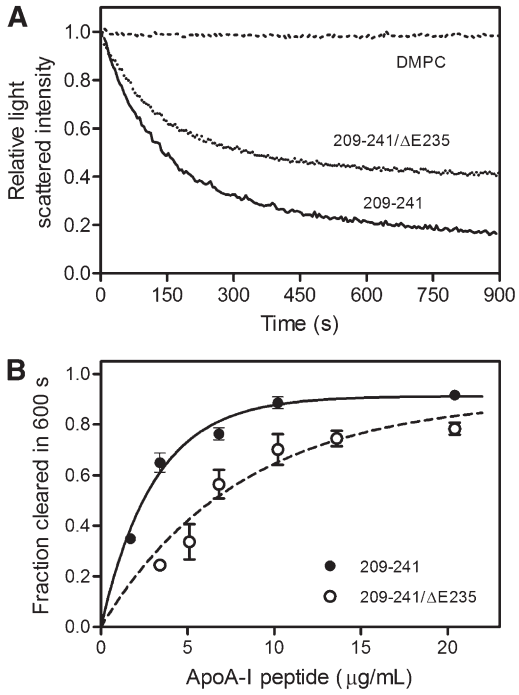

Fig. 6. Solubilization of DMPC MLVs by the apoA-I C-terminal peptides. A: Timecourses of the decrease in right-angle light scattering intensity at both excitation and emission wavelengths of 600 nm after incubation of DMPC MLVs (34 μg/ml) with 6.8 μg/ml peptide at 24.6°C. B: Increase in fraction of scattering intensity cleared in 600 s with increasing concentration of peptide. ApoA-I 209-241 (●), 209-241/ΔE235 (○).

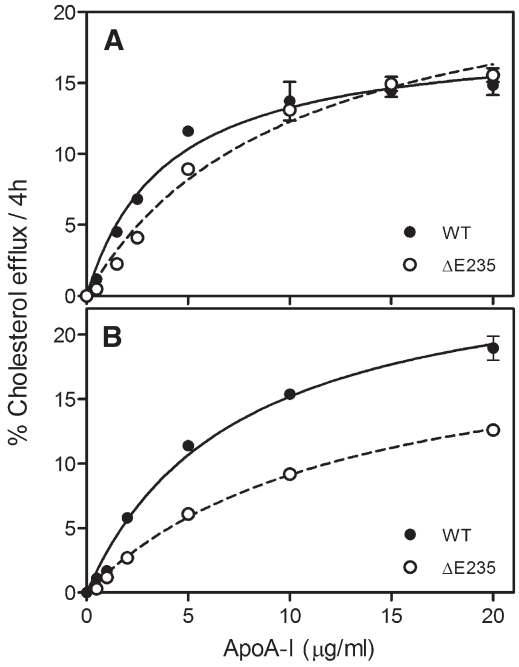

Fig. 7. Effect of apoA-I concentration on cholesterol efflux via ABCA1 from J774 macrophages (A) and BHK cells (B) to apoA-I WT (●) and ΔE235 variant (○). J774 macrophages or BHK cells transfected with ABCA1 were labeled with [^{3}H]cholesterol, and then J774 macrophages were treated with 8-(4-chlorophenythio)-cAMP or BHK cells were treated with mifepristone to upregulate the expression of ABCA1 as described in Materials and Methods. The cells were incubated with various concentrations of apoA-I for 4 h, and % of cholesterol efflux was determined.

deletion of residue E235 in the C-terminal helical region impairs lipid binding and is associated with low HDL cholesterol and plasma apoA-I levels (21, 22). To understand the structural basis for such dysfunctions of the Nichinan mutation in terms of alterations in apoA-I tertiary structure, we examined the structure, lipid-binding properties, and ability to induce cellular cholesterol efflux of apoA-I Nichinan and its C-terminal peptide.

Effects of deletion of E235 on apoA-I structure

Lipid-free human apoA-I is organized into two structural domains comprising an N-terminal helix bundle domain and a separate C-terminal domain (9, 11, 15). Because the mutation in apoA-I Nichinan occurs in the C-terminal helical region, the effects of the E235 deletion on apoA-I tertiary structure are expected to be primarily in the C-terminal domain. Indeed, there was no or little difference in the α-helix content, thermal denaturation parameters between WT and the Nichinan variant (Table 1), and similar tertiary packing of Trp residues in the N-terminal helix bundle (Fig. 1B), in contrast to the situation with a mutation in the N-terminal domain such as apoA-I Milano (R173C), which markedly destabilizes the protein structure (51). However, when the changes in molar ellipiticity and Trp fluorescence caused by Gdn-HCl or urea denaturation were monitored, significant reductions in the conformational stability, ΔG_D°, and midpoint of denaturation for apoA-I Nichinan were observed compared with WT apoA-I (Table 1 and Fig. 2). This indicates that the deletion of E235 not only affects the conformational stability of the C-terminal domain but also destabilizes the N-terminal helix bundle domain.

We have demonstrated recently that intramolecular interaction between the N- and C-terminal tertiary structure domains in apoA-I modulates the conformational stability of the N-terminal helix bundle, and the C-terminal helical region is responsible for this stabilizing interaction (20). Because the deletion of E235 significantly destabilizes the apoA-I structure, it is possible that the absence of E235 that removes a negative charge at this position and alters the charge distribution of the C-terminal region may result in alterations in stabilizing electrostatic interactions between the N- and C-terminal domains in apoA-I. The finding that the E235A mutation did not destabilize the apoA-I structure (unpublished observations) indicates that the E235 residue itself is not involved in the interaction with the N-terminal domain. Rather, the alteration in the charge distribution of the C-terminal region caused by the E235 deletion may disrupt optimal electrostatic interaction between the N- and C-terminal domains, resulting in the destabilization of the protein structure, as seen in human-mouse hybrid apoA-I molecules where the human C-terminal sequence is replaced with that of mouse apoA-I (16, 20).

Alternatively, it may be possible that the disruption of the ability of the C-terminal region to form α-helix caused by the deletion of E235 inhibits the hydrophobic helix-helix interaction between the N- and C-terminal domains in apoA-I Nichinan. The fact that the α-helix content of ~80% found in the crystal structure (11) is much higher than that for monomeric apoA-I in solution (~50%) (9, 32) indicates that the true conformation in solution is more flexible and less organized than the compactly folded, highly ordered structure in the crystal state. Indeed, whereas the C-terminal residues 219-242 are shown to be helical in the crystal structure (11), our recent hydrogen-deuterium exchange analysis of lipid-free apoA-I in dilute solution demonstrates that the C-terminal domain is entirely nonhelical (unpublished results). Thus, it is unlikely that the deletion of E235 affects the stability of

TABLE 4. Kinetic parameters for cholesterol efflux to apoA-I variants

ApoA-I Variant	J774 Cells		BHK Cells	
	V_{max}	K_m	V_{max}	K_m
	% cholesterol efflux/4 h	*μg of apoA-I/ml*	*% cholesterol efflux/4 h*	*μg of apoA-I/ml*
WT	18.5 ± 0.9	3.9 ± 0.6	26.4 ± 1.4	7.3 ± 0.9
Nichinan (ΔE235)	24.4 ± 1.5	9.9 ± 1.3	20.8 ± 1.0	12.8 ± 1.2

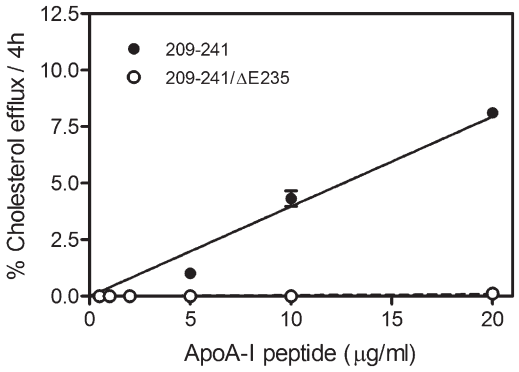

Fig. 8. Effect of apoA-I peptide concentration on cholesterol efflux via ABCA1 from J774 macrophages to apoA-I peptides, apoA-I 209-241 (●) and apoA-I 209-241/ΔE235 (○). J774 macrophages were labeled with [^{3}H]cholesterol and treated with 8-(4-chlorophenythio)-cAMP as described in the legend of Fig. 6.

the N-terminal helix bundle through disrupting the C-terminal helical structure in the lipid-free apoA-I. Rather, it is likely that the helix-disrupting effect of the Nichinan mutation in the apoA-I molecule is relevant only to the lipid-bound state. In addition, the higher α-helix content seen for apoA-I 209-241 peptide than that for apoA-I 209-241/ΔE235 in the lipid-free state is likely due to the self-association of apoA-I 209-241 peptide at the concentration studied (52).

Effects of deletion of E235 on apoA-I lipid binding

On the basis of the two-domain tertiary structure of apoA-I, we have proposed a two-step mechanism for binding of apoA-I to a phospholipid surface; the initial binding step occurs through hydrophobic α-helices in the C-terminal domain, followed by a conformational opening of the N-terminal helix bundle to interact with lipids (10, 15, 53). Thus, removal (15, 32) or modification (19, 23) of the C-terminal helical region drastically reduces the lipid-binding ability of apoA-I. The moderate effects of the E235 deletion in apoA-I Nichinan on the binding affinity to SUV (Table 2) and DMPC clearance efficiency (Fig. 4) are consistent with the notion that the deletion of E235 partially disrupts local α-helix formation of the C-terminal region. Indeed, comparison of the α-helix contents between synthetic C-terminal peptides, apoA-I 209-241 and 209-241/ΔE235 in the lipid-free or SUV-bound states (Table 3) clearly demonstrates that the E235 deletion markedly reduces the ability of the C-terminal region of apoA-I to form α-helix both in the absence and presence of lipids. Because the helical propensity of apolipoproteins is critical for their lipid-binding affinities (19, 31, 50, 54), such a weak propensity to form α-helix caused by the E235 deletion results in the less effective ability of the Nichinan protein and its C-terminal peptide to solubilize DMPC vesicles (Figs. 4 and 6). In agreement with this, apoA-I Nichinan forms relatively larger and unstable discoidal complexes with lipids compared with WT (Fig. 5). Interestingly, it was reported that the E235K mutation in apoA-I does not affect the lipid-solubilizing efficiency (23), indicating that the absence of negative charge of E235 itself does not appear to be involved in the impaired lipid-binding ability of apoA-I Nichinan. Rather, it is possible that the loss of E235 residue cause a "frameshift" that could disrupt the optimal charge distribution on the polar and nonpolar faces of the C-terminal helix, leading to the impaired α-helix formation and lipid binding. Supporting this, the E235A mutation in apoA-I protein (unpublished observations) and in the C-terminal peptide (31) has little effect on the DMPC solubilizing ability.

Effects of deletion of E235 on the cholesterol efflux ability of apoA-I

It is well established that the C-terminal α-helical region of apoA-I is important for effective cellular lipid efflux (23–25, 55) and formation of nascent HDL particles (26, 56) via the ABCA1 pathway. In this apoA-I/ABCA1 reaction, the microsolubilization of exovesiculated domains of cell plasma membrane by apoA-I is a rate-limiting step to create discoidal HDL particles (28). Consequently, the ability of apoA-I variants to solubilize phospholipid bilayer such as DMPC vesicles is correlated with the efficiency to efflux cellular lipids via the ABCA1 pathway (28). As the solubilization process requires penetration of apoA-I helices into the phospholipid bilayer with the C-terminal α-helix playing a key role (15, 16, 41), it follows that the decreased lipid-binding ability of the C-terminal helical region in apoA-I Nichinan should lead to the reduced rate of cholesterol efflux, as actually seen in the previous (22) and this study (Fig. 7). In addition, it is likely that the decrease in the hydrophobic binding site in the C-terminal domain of apoA-I Nichinan (Fig. 3) prevents the formation of preβ-HDL particles because this site is important for the acquisition of a few phospholipid molecules for the formation of preβ-HDL (56). It should be noted that the reduced ability of apoA-I Nichinan to mediate cholesterol efflux is unlikely to result from impairment of direct interactions between apoA-I and ABCA1 because the C-terminal deletion mutants, Δ190-243 and Δ223-243 apoA-I, which have reduced lipid affinity, cross-link to ABCA1 as effectively as WT apoA-I (27).

Such a reduction in the abilities to induce cellular cholesterol efflux and formation of HDL particles in the apoA-I Nichinan probands could result in a large pool of lipid-free apoA-I that is susceptible to rapid catabolism (57), consistent with a previous report that used in vivo turnover studies in rabbits to demonstrate that the Nichinan mutant is rapidly cleared (22% faster) compared with WT apoA-I (21). In addition, the fact that the size distribution of HDL particles is altered in the apoA-I Nichinan probands despite normal plasma LCAT activity (21) appears to be consistent with the notion that the stability of the N-terminal helix bundle and the lipid-binding affinity of the C-terminal domain are key factors that modulate the distribution of apoA-I among HDL subclasses (16, 53). In addition, the finding that the Nichinan mutant forms less stable and larger discoidal complexes with lipids compared with WT (Fig. 5) may also underlie the higher HDL_2 levels seen in the Nichinan probands.

In summary, the present study demonstrated that the disruption of the helix-forming ability of the C-terminal region by the mutation of E235 deletion contributes not

only to the decreased lipid-binding affinity but also to the destabilized protein structure of apoA-I Nichinan. Such alterations in physical properties of the apoA-I Nichinan protein appear to impair the abilities to induce cellular cholesterol efflux and biogenesis of HDL particles, resulting in lower plasma HDL cholesterol and apoA-I levels, as well as an altered HDL particle size distribution.

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