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The roles of C-terminal helices of human apolipoprotein A-I in formation of high-density lipoprotein particles

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Abstract

Apolipoprotein A-I (apoA-I) accepts cholesterol and phospholipids from ATP-binding cassette transporter AI (ABCA1)-expressing cells to form high-density lipoprotein (HDL). Human apoA-I has two tertiary structural domains and the C-terminal domain (approximately amino acids 190–243) plays a key role in lipid binding. Although the high lipid affinity region of the C-terminal domain of apoA-I (residues 223–243) is essential for the HDL formation, the function of low lipid affinity region (residues 191–220) remains unclear. To evaluate the role of residues 191–220, we analyzed the structure, lipid binding properties, and HDL formation activity of Δ 191–220 apoA-I, in comparison to wild-type and Δ 223–243 apoA-I. Although deletion of residues 191–220 has a slight effect on the tertiary structure of apoA-I, the Δ 191–220 variant showed intermediate behavior between wild-type and Δ 223–243 regarding the formation of hydrophobic sites and lipid interaction through the C-terminal domain. Physicochemical analysis demonstrated that defective lipid binding of Δ 191–220 apoA-I is due to the decreased ability to form α -helix structure which provides the energetic source for lipid binding. In addition, the ability to form HDL particles *in vitro* and induce cholesterol efflux from ABCA1-expressing cells of Δ 191–220 apoA-I was also intermediate between wild-type and Δ 223–243 apoA-I. These results suggest that despite possessing low lipid affinity, residues 191–220 play a role in enhancing the ability of apoA-I to bind to and solubilize lipids by forming α -helix upon lipid interaction. Our results demonstrate that the combination of low lipid affinity region and high lipid affinity region of apoA-I is required for efficient ABCA1-dependent HDL formation.

Keywords

ABCA1; apoA-I; HDL; Cholesterol

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1. Introduction

Since the excess accumulation of cholesterol, an essential component of cellular membranes, is harmful to cells, several mechanisms are utilized to regulate cellular and whole body cholesterol levels [1–3]. High-density lipoprotein (HDL) formation is an important means of elimination of excess cholesterol from peripheral tissues. Apolipoprotein A-I (apoA-I), a major protein component of HDL, accepts cellular cholesterol and phospholipids from ATP-binding cassette protein A1 (ABCA1)-expressing cells to form HDL [4–7]. Mutations in *ABCA1* and *APOA1* genes cause low HDL levels, prominent cholesterol-ester accumulation in tissue macrophages, and premature atherosclerotic vascular disease [8–12]. Recently, it was reported that the capacity of serum to mediate the cholesterol efflux from macrophages is strongly and inversely associated with both carotid intima-media thickness and the likelihood of angiographic coronary artery disease, independent of HDL cholesterol levels [13], emphasizing the importance of HDL formation by ABCA1. Despite the physiological importance of this pathway, however, the details of HDL formation remain unclear [4].

Human apoA-I (243 amino acid residues) contains 11- and 22-amino acid repeats that form amphipathic α -helices [14]. It has been shown that apoA-I is folded into two tertiary structure domains; the N-terminal domain (residues 1–186) forms an α -helix bundle and the C-terminal domain has less organized structure [15, 16]. It has been reported that the C-terminal domain has higher affinity for lipid than the N-terminal domain [17], and apoA-I initially binds to a lipid surface through amphipathic α -helices in the C-terminal domain, followed by opening of the helix bundle in the N-terminal domain [18, 19]. The C-terminal domain changes conformation from random coil to α -helix upon incorporation into lipoprotein particles [20], and this α -helix formation is required for high affinity binding of apoA-I to lipids [21, 22]. Thus, the C-terminal domain of apoA-I plays important roles in lipid binding and HDL formation.

Because deletion of the entire C-terminal domain (residues 190–243) or C-terminal helix (residues 223–243) of apoA-I drastically decreases the lipid binding property and HDL formation activity, it is apparent that residues 223–243 are critical for the functionality of apoA-I [5, 23–27]. Although a peptide consisting of residues 220–241 can solubilize dimyristoyl phosphatidylcholine (DMPC) vesicles, the peptide does not mediate cholesterol and phospholipid efflux from ABCA1-expressing cells [25, 26, 28]. In contrast, a peptide consisting of residues 209–241 possesses more than 60% of cholesterol efflux activity compared to full length apoA-I [25, 26, 28], and has higher lipid affinity than peptide 220–241 in monolayer exclusion pressure measurements [25, 29]. Furthermore, Mitsche et al. showed the contribution of residues 198–219 to adsorption and desorption of apoA-I at surface of lipoprotein [30]. It was also reported that difference between human and mouse in residues around 165 to 209 are involved in the determination of lipoprotein subclass distribution [31]. These results suggested that residues 223–243 are essential, but not sufficient for the interaction with lipids and formation of HDL particles by apoA-I, and that the remaining part of the C-terminal domain of apoA-I also has an important role in HDL formation.

To evaluate the function of residues 191–220 in the context of human apoA-I, we analyzed the effects of deletion of residues 191–220 on the structure, lipid binding property, and cholesterol efflux activity by ABCA1-expressing cells, in comparison to the deletion of residues 223–243. Our results demonstrate the importance of residues 191–220 as well as of residues 223–243 for lipid interaction and HDL formation by the apoA-I molecule.

2. Materials and Methods

2.1. Materials

Human apoA-I and engineered deletion mutants were expressed as thioredoxin fusion proteins in *E. coli* strain BL21-DE3 host and then cleaved and purified as described previously [18]. The apoA-I preparations were at least 95% pure as assessed by SDS-PAGE. In all experiments, apoA-I was freshly dialyzed from 6 M guanidine hydrochloride (GdnHCl) solution into the appropriate buffer before use. The remaining chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

2.2. Circular Dichroism (CD) Spectroscopy

Far-UV CD spectra were recorded from 185 to 260 nm at 25°C using a Jasco J-600 spectropolarimeter. The apoA-I solutions of 50 µg/ml in 10 mM Tris buffer (pH 7.4) were subjected to CD measurements in a 2-mm quartz cuvette, and the results were corrected by subtracting the buffer base line. The α -helix content was derived from the molar ellipticity at 222 nm ($[\theta]_{222}$) using the equation: % of α -helix = $(-[\theta]_{222} + 3,000)/(36,000 + 3,000) \times 100$ [32].

2.3. Fluorescence Measurements

Fluorescence measurements were carried out with a Hitachi F-4500 fluorescence spectrophotometer at 25°C. To monitor the exposure of hydrophobic sites on the apoA-I variants, 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence spectra were collected from 400 to 600 nm at an excitation wavelength of 395 nm in the presence of 50 µg/ml protein and an excess of ANS (250 µM) [18]. To access the local environment of apoA-I, Trp emission fluorescence was recorded from 300 to 420 nm using a 295-nm excitation wavelength to avoid tyrosine fluorescence. For monitoring chemical denaturation, lipid-free proteins at a concentration of 25 µg/ml were incubated overnight at 4°C with GdnHCl at various concentrations. K_D was calculated from the change in the ratio of fluorescence intensity at 335 nm and 350 nm of intrinsic Trp residues. 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 $\Delta G_D = -RT \ln K_D$ [29, 33].

2.4. ApoA-I Binding to Small Unilamellar Vesicles (SUVs)

The binding of apoA-I to SUV was assayed by gel filtration chromatography as described [21]. The apoA-I variants were radiolabeled to a specific activity of ~1 µCi/mg of protein by reductive methylation of lysine residues with [^{14}C]formaldehyde. This trace labeling of apoA-I leads to modification of less than 1 lysine residue in the molecule, and there is no detectable change in the physical properties of the protein. Typically, fresh SUV (1 mg/ml egg phosphatidylcholine (PC)) containing a trace amount of [^3H]cholesterol was incubated with shaking for 1 h at room temperature with increasing concentrations (10–100 µg/ml) of ^{14}C -labeled apoA-I. The mixtures were then applied to a Sepharose CL-6B column (1 × 28 cm), and 0.5-ml fractions were collected. Aliquots of each fraction were counted using liquid scintillation procedures to determine the levels of [^3H]cholesterol (SUV) and [^{14}C]apoA-I. The elution chromatograms were fitted with Gaussian distribution functions using Origin software (MicroCal Inc., Northampton, MA). Binding isotherms were obtained by non-linear regression analysis (GraphPad Prism) using a one-binding-site model.

2.5. Isothermal Titration Calorimetry (ITC) Measurements

Heats of apoA-I binding to egg PC SUV were measured with a MicroCal MCS isothermal titration calorimeter at 25°C [21]. 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.

2.6. DMPC Clearance Assay

The kinetics of solubilization of DMPC vesicles by the apoA-I variants were measured by monitoring the time-dependent decrease in turbidity at 24.6°C. DMPC vesicles extruded through a 200-nm filter at a concentration of 0.25 mg/ml were mixed with apoA-I samples (0.05–0.2 mg/ml), and incubated for 15 min to monitor the light scattering intensity at 325 nm with a Shimadzu U-3900H spectrophotometer [34, 35].

2.7. Characterization of HDL Particles by Gel Filtration

DMPC multilamellar vesicles (MLVs) (0.6 mg/ml) containing 0.05 mol% NBD-PE were incubated with apoA-I (0.2 mg/ml) at 24.6°C for 4 or 24 h. The resultant HDL particles were fractionated by gel filtration chromatography on a Superdex 200 column calibrated by the proteins of known diameter (particle diameter range, 6.1–17.0 nm). ApoA-I and lipid were detected by absorbance at 280 nm and fluorescence of NBD-PE (excitation at 463 nm, emission at 536 nm), respectively.

2.8. Cell Culture

BHK/ABCA1 cells [36] were grown in a humidified incubator (5% CO₂) at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C in 5% CO₂.

2.9. Cellular Lipid Release Assay

BHK/ABCA1 cells were subcultured in 24-well plates at a density of 4×10^4 cells in DMEM containing 10% FBS. After a 24-h incubation, the cells were washed twice with PBS and incubated with or without 10 nM mifepristone in DMEM containing 0.02% BSA for 16 h. The cells were incubated with wild-type or mutant forms of apoA-I (0.625–10 µg/ml) in DMEM containing 0.02% BSA for 4 h. The cholesterol content in the medium was determined by a fluorescence enzyme assay [37]. Prior to lipid release assay THP-1 cells were treated with 200 ng/ml phorbol 12-myristate 13-acetate for 2 days to facilitate differentiation into macrophages. The adherent macrophages were incubated with 10 µM TO901317 to induce ABCA1 expression for 24 h. Cells were washed twice with PBS, and incubated with RPMI containing 0.02% BSA and wild-type or mutant forms of apoA-I (2.5 or 5 µg/ml) for 4 h [38]. The cholesterol content in the medium was determined as described above.

3. Results

3.1. Effect of deletion of residues 191–220 on the structure and stability of apoA-I

To analyze the effect of deletion of this segment on the secondary structure of apoA-I, lipid-free structures of apoA-I variants were analyzed by far-UV CD spectroscopy. Although Δ 191–220 apoA-I exhibited a CD spectrum typical for α -helical structure (Fig 1A) similarly to wild-type apoA-I, it has lower α -helix content (36%) and the number of α -helical residues (77 residues) compared to wild-type (α -helical content of 43% and 104 α -helical residues). Since residues 191–220 in the lipid-free apoA-I molecule appear to be predominantly in

random coil structure [39], such discrepancy suggests that deletion of residues 191–220 affects the N-terminal helix bundle structure [34].

The effects of deletion of the C-terminal domain on the structure and stability of the N-terminal domain were evaluated by Trp fluorescence. Because all of the Trp residues are located in the N-terminal domain of the apoA-I molecule and fluorescence of Trp residues is sensitive to the hydrophobic environment, monitoring Trp fluorescence is useful for probing conformational changes in the N-terminal domain [18]. Fig. 1B shows GdnHCl-induced denaturation curves of the apoA-I variants monitored by Trp fluorescence. The conformational stability (ΔG_D°), midpoint of denaturation ($D_{1/2}$) and cooperativity of denaturation in the transition region (m value) calculated from data in Fig. 1B are listed in Table 1. These denaturation parameters for two deletion variants are close to those of wild-type apoA-I despite an about 10% reduction in ΔG_D° value. Based on the finding that the N-terminal deletion variants show marked reduction in ΔG_D° by 40 to 80% [18], it is likely that deletion of residues 191–220 has a slight effect on the structure and stability of the N-terminal helix bundle.

3.2. Contribution of residues 191–220 to the tertiary structure and lipid interactions of the apoA-I C-terminal domain

ANS binding experiments were performed to compare the exposure of hydrophobic regions in the C-terminal deletion variants (Table 2). Compared to wild-type apoA-I which shows a great increase in ANS fluorescence due to the presence of ANS accessible hydrophobic surface [18], a drastic reduction in ANS binding for the deletion of residues 223–243 (Table 2) indicates that residues 223–243 contribute to the formation of hydrophobic surface by the C-terminal domain [18]. The $\Delta 191$ –220 variant showed an intermediate value of ANS fluorescence indicating that these residues are also involved in the tertiary conformation of the C-terminal domain.

To evaluate the role of residues 191–220 in lipid interaction, binding of apoA-I variants to egg PC SUV was analyzed by gel filtration. In our experimental conditions, there was no change in elution profiles of SUV after incubation with apoA-I, indicating that bound apoA-I did not disrupt the structural integrity of the vesicles [21]. From elution profiles with various concentrations of protein, the binding isotherms of wild-type and $\Delta 191$ –220 to SUV were obtained (Fig. 2A). The binding parameters derived from curve-fitting of these isotherms are listed in Table 2. Similar to the $\Delta 223$ –243 variant, deletion of residues 191–220 drastically decreased the binding affinity of apoA-I to SUV, suggesting that not only residues 223–243 but also residues 191–220 are required for the high affinity binding of apoA-I to lipids.

Because α -helix formation provides the energetic source for high affinity binding of apoA-I to lipid [21], α -helical contents of apoA-I variants upon SUV binding were estimated by far-UV CD analysis (Fig. 2B). The increase in the number of α -helical residues of $\Delta 191$ –220 upon SUV binding was about one-third of that of wild-type apoA-I (66 residues in wild-type; 23 residues in $\Delta 191$ –220), indicating that residues 191–220 significantly contribute to the formation of α -helices upon lipid binding.

To further obtain thermodynamic parameters for the binding of apoA-I variants to SUV, the heats of the binding of apoA-I variants were determined by ITC measurements. The injection of $\Delta 191$ –220 apoA-I to SUV gave large exothermic heats (data not shown), and the complete thermodynamic parameters for binding of $\Delta 191$ –220 to SUV were calculated using the binding constants from binding isotherms (Table 2). As listed in Table 2, deletion of residues 191–220 led to an intermediate reduction of the favorable enthalpy of binding among the C-terminal deletion variants, consistent with residues 191–220 playing an

important role in the binding of apoA-I to lipid. Previously, we have reported that the enthalpy and the free energy of binding are linearly correlated with the number of residues in the apoA-I variants forming α -helix upon lipid binding [21]. The values for the $\Delta 191$ –220 variant fit the linear correlations well (Fig. 2C and 2D), indicating that defective lipid binding of $\Delta 191$ –220 apoA-I is due to the decreased ability to form α -helix structure which provides the energetic source for high affinity binding of apoA-I to lipids.

3.3. Role of residues 191–220 in formation of HDL particles

To analyze the role of residues 191–220 in solubilization of lipids, a DMPC clearance assay was performed [40, 41]. As previously reported, deletion of residues 223–243 caused a drastic decrease in the ability of apoA-I to clear DMPC vesicles, indicating that residues 223–243 play an essential role in the solubilization of lipids [5] (Fig. 3). The time courses of clearance (Fig. 3A) and the dependence of clearance efficiency on protein concentration (Fig. 3B) demonstrate that $\Delta 191$ –220 apoA-I has a markedly decreased efficiency of DMPC vesicle solubilization compared to wild-type apoA-I. The relative catalytic efficiency ($V_{\max}/K_m$) decreased to 0.17 for $\Delta 191$ –220 apoA-I compared to wild-type apoA-I (set to 1.0) (Table 3). These results suggest that residues 191–220 are important for the solubilization of lipids *in vitro*.

To further examine the ability to form HDL particles by apoA-I variants *in vitro*, HDL-like discoidal particles (a mimetic of nascent HDL particle) formed by incubation of apoA-I and DMPC were analyzed by gel filtration chromatography (Fig. 4). After incubation of DMPC and wild-type apoA-I at 3 : 1 (w/w) ratio for 4 h, more than 80% of apoA-I was converted to discoidal HDL particles of 11 nm hydrodynamic diameter (Fig. 4A), indicating efficient formation of HDL particles by wild-type apoA-I. By contrast, less than 30% of $\Delta 191$ –220 apoA-I was converted to HDL particles after 4 h, although after 24 h incubation, 80% of the protein was found in HDL fraction (Fig. 4B). Reflecting the decreased lipid solubilization activity, about 50% of $\Delta 223$ –243 apoA-I was recovered in the lipid-free fraction even after 24 h incubation (Fig. 4C). Furthermore, the C-terminal deletion variants produced a more heterogeneous HDL particle distribution after 24 h incubation, consistent with the low lipid solubilizing capabilities of these variants [40] reducing formation of smaller HDL particles [42]. These results demonstrate that not only residues 223–243 but also residues 191–220 are involved in efficient lipid solubilization and HDL formation *in vitro*.

The effect of deletion of apoA-I residues 191–220 on HDL formation by ABCA1-expressing cells was examined (Fig. 5). As previously reported, cholesterol efflux from BHK/ABCA1 cells in which human ABCA1 is expressed under the control of a mifepristone-inducible promoter is dependent on the concentration of apoA-I and can be described by the Michaelis-Menten equation [42] (Fig. 5A). Cholesterol efflux from BHK/ABCA1 cells to $\Delta 191$ –220 apoA-I was intermediate to that of wild-type and $\Delta 223$ –243 apoA-I, and the catalytic efficiency for the $\Delta 191$ –220 variant was 40% of that for wild-type apoA-I (Table 3). We also analyzed the effect of deletion of apoA-I residues 191–220 on cholesterol efflux from THP-1 macrophage. Consistent with the result obtained in BHK/ABCA1 cells, $\Delta 191$ –220 apoA-I showed lower cholesterol efflux than wild-type apoA-I when incubated with THP-1 macrophages (Fig. 5B). These results suggest that residues 191–220, as well as residues 223–243, are required for efficient HDL formation when apoA-I is incubated with ABCA1-expressing cells.

4. Discussion

The apoA-I molecule adopts a two-domain tertiary structure and the properties of these domain modulate the ability to form HDL particles. The C-terminal helix (residues 223–243) is the most non-polar segment of the human apoA-I molecule and this hydrophobicity

plays a crucial role in apoA-I to solubilize lipids and promote cholesterol efflux [43]. Studies of fragment peptides demonstrated that residues 191–220 have much lower lipid affinity compared to residues 223–243 [44, 45]. However, the addition of these residues with low lipid affinity to the strong lipid-binding residues is necessary for effective lipid solubilization and ABCA1-mediated cholesterol efflux [25, 26, 28], indicating the importance of combination of multiple helices to the functionality of apolipoprotein [46]. In this study, we evaluated the role of residues 191–220 of human apoA-I in the lipid interaction and HDL formation by comparing the C-terminal deletion mutants, Δ 191–220 and Δ 223–243. To our knowledge, this is the first study that directly compares the role of C-terminal helices in the HDL formation processes.

Although the C-terminal domain of apoA-I doesn't form α -helix in the lipid-free state [39], CD analysis demonstrated that deletion of residues 191–220 decreases the number of amino acids forming α -helix (Fig. 1A). Because intra-molecular interaction between the N- and C-terminal domains of apoA-I is shown to be involved in the stabilization of apoA-I in the lipid-free state [33], it is possible that residues 191–220 are involved in this interaction. However, we observed only slight differences in the protein stability among apoA-I variants monitored by Trp fluorescence which represents the structure of N-terminal helix bundle of apoA-I (Fig. 1B and Table 1), indicating that deletion of residues 191–220 has a slight effect on the tertiary structure of apoA-I in the lipid-free state.

In ANS binding (Table 2) and SUV binding (Fig. 2A) experiments, the Δ 191–220 variant demonstrated properties intermediate between wild-type and Δ 223–243 apoA-I. This finding indicates that residues 191–220 are involved in the formation of a hydrophobic domain and lipid interaction by the C-terminal domain, although the contribution of residues 191–220 is much less than that of residues 223–243. The formation of HDL particles *in vitro* (Figs. 3 and 4) and cholesterol efflux ability from ABCA1-expressing cells (Fig. 5) by the Δ 191–220 variant are also intermediate between the values for wild-type and Δ 223–243 apoA-I. Furthermore, Δ 190–243 and Δ 223–243 apoA-I showed similar activity in DMPC vesicle clearance and cholesterol efflux from ABCA1-expressing cells [18, 26]. Together with the fact that a synthetic peptide corresponding to residues 187–219 of apoA-I does not mediate DMPC vesicle solubilization and ABCA1-dependent lipid efflux [25], these results indicate that residues 191–220 enhance the activity of residues 223–243 to bind to and solubilize lipids, although residues 191–220 themselves have weak capability of lipid binding and solubilization. Consistent with this, deletion of residues 190–220 reduces the level of apoA-I binding to HDL and lipid emulsion particles, compared to the effects of removal of residues 223–243 [47].

Despite the inability of the peptide consisting of residues 220–241 to mediate cholesterol and phospholipid efflux from ABCA1-expressing cells, combination of residues 220–241 with the remaining part of C-terminal domain confers the ABCA1-dependent cholesterol efflux activity [25, 26]. It is known that combination of high and low lipid affinity helices is required for the ABCA1-dependent cholesterol efflux in bihelical peptides and small apolipoproteins. 37pA is a synthetic peptide which consists of two high lipid affinity helices and it mediates both ABCA1-dependent and -independent lipid efflux from cells [48]. Sethi *et al.* [49] demonstrated that peptide 5A, in which five hydrophobic amino acids in the second helix of 37pA are substituted for alanine to decrease the lipid affinity of the second helix, stimulated cholesterol and phospholipid efflux by ABCA1 with higher specificity than 37pA. In addition, bihelical peptide comprising helices 2 and 3 of apoA-II mediates cholesterol efflux from ABCA1-expressing cells although each helix of apoA-II alone cannot mediate ABCA1-dependent cholesterol efflux. Combination of high and low lipid affinity helices in apoC-I is required for effective cholesterol efflux [46], suggesting that the requirement of combination of helices with different lipid affinities generally applies to

HDL formation by apolipoproteins. Therefore, it is likely that the combination of low lipid affinity region (residues 191–220) and high lipid affinity region (residues 223–243) of apoA-I is required for efficient ABCA1-dependent HDL formation.

In the apoA-I molecule, the C-terminal domain in the lipid-free state is in a predominantly non-helical conformation [39] and lipid binding induces a random coil to α -helix transition in this domain [16, 22]. From a thermodynamic point of view, this helix formation in the C-terminal domain contributes to the free energy of binding, providing the energetic source for high affinity binding of apoA-I to lipids [20, 21, 50]. Our finding that the $\Delta 191$ –220 variant also fits into a linear correlation between *the* thermodynamic parameters (the enthalpy and free energy of binding) and the number of amino acids forming α -helix among a series of deletion mutants (Figs. 2C and 2D) clearly demonstrates that residues 191–220 play a crucial role in the initial lipid interaction of apoA-I through the C-terminal domain despite its low lipid binding affinity. Thus, the formation of α -helix in residues 191–220 provides much of the energetic source required for lipid binding and solubilization, supporting our conclusion that residues 191–220 enhance the lipid binding and lipid solubilization activity of residues 223–243. Since the lipid binding and the lipid-solubilizing capabilities of the C-terminal domain are important contributors to the ABCA1-mediated cholesterol efflux to apoA-I [5, 42], it is plausible that residues 191–220 play an important role in this phenomenon (Fig. 5).

In this study, we have demonstrated the contribution of the C-terminal helices of human apoA-I to the HDL formation process. Apolipoproteins including apoA-I cannot accept lipids from cells that are not expressing ABCA1. This strict ABCA1-dependence of lipid efflux is important to avoid excess lipid efflux can disrupt cellular membrane and induce cellular toxicity. By combining low and high lipid affinity helices, the lipid binding and solubilization properties of apolipoproteins are modulated to allow controlled and efficient lipid secretion. This study enhances understanding of how apoA-I structure influences the process of HDL formation by ABCA1.

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Abbreviations

ABC	ATP-binding cassette
ANS	8-anilino-1-naphthalenesulfonic acid
apo	apolipoprotein
BHK	baby hamster kidney
BSA	bovine serum albumin
CD	circular dichroism
DMEM	Dulbecco's modified Eagle's medium
DMPC	dimyristoyl phosphatidylcholine
FBS	fetal bovine serum
GdnHCl	guanidine hydrochloride

HDL	high-density lipoprotein
ITC	isothermal titration calorimetry
MLV	multilamellar vesicle
PBS	phosphate-buffered saline
PC	phosphatidylcholine
SUV	small unilamellar vesicle
UV	ultraviolet

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Highlights

High lipid affinity region in C-half of apoA-I is essential for the HDL formation

The function of low lipid affinity region in C-half of apoA-I remains unclear

Lipid binding induced α -helix formation in low lipid affinity region of apoA-I

Low lipid affinity region of apoA-I was required for lipid binding and HDL formation

Combination of low and high lipid affinity region is required for HDL formation

Figure 1. Secondary structure and stability of the C-terminal deletion apoA-I variants
 (A) Far-UV CD spectra of wild-type (solid line) and $\Delta 191-220$ (dotted line) apoA-I in the lipid-free state. (B) GdnHCl-induced denaturation of wild-type (●), $\Delta 191-220$ (○) and $\Delta 223-243$ (▲) apoA-I were monitored by Trp fluorescence.

Figure 2. Lipid binding property of the $\Delta 191-220$ apoA-I variant

(A) Binding isotherms of wild-type (●) and $\Delta 191-220$ (○) apoA-I to egg PC SUV. (B) Far-UV CD spectra of wild-type (solid line) and $\Delta 191-220$ (dotted line) apoA-I in the presence of SUV. Correlation of the enthalpy (C) and the free energy (D) of binding of apoA-I variants with the increase in α -helix content upon binding to egg PC SUV. Data of deletion variants and human apoA-I (plasma) are added from [21].

Figure 3. Lipid solubilizing properties of the apoA-I C-terminal deletion variants
 (A) Time courses of the decrease in turbidity at 325 nm after incubation of DMPC vesicles (0.25 mg/ml) with 0.15 mg/ml protein at 24.6°C. (B) Increase in fraction of turbidity cleared in 10 min with increasing concentration of protein. Wild-type (●), $\Delta 191-220$ (○), and $\Delta 223-243$ (▲) apoA-I. The average values are plotted with SD.

Figure 4. HDL formation by the C-terminal apoA-I deletion variants *in vitro*

Gel filtration profiles of HDL particles formed by incubation of DMPC vesicles with wild-type (A), $\Delta 191-220$ (B), and $\Delta 223-243$ (C) apoA-I for 4h (dotted line) and 24 h (solid line). The fractional distributions of apoA-I are plotted.

Figure 5. Cholesterol efflux from ABCA1-expressing cells to the C-terminal apoA-I deletion variants

(A) Cholesterol efflux from BHK/ABCA1 cells to wild-type (●), $\Delta 191-220$ (○), and $\Delta 223-243$ (▲) (0.625–10 $\mu\text{g}/\text{ml}$) apoA-I. (B) Cholesterol efflux from THP-1 macrophages to indicated concentrations of wild-type and $\Delta 191-220$ apoA-I. Experiments were performed in triplicate, and the average values are plotted with SE.

Table 1

Thermodynamic parameters of apoA-I variants in GdnHCl-induced denaturation.

	<u>Thermodynamic parameters of denaturation</u>		
	ΔG_D°	m	$D_{1/2}$
	<i>kcal/mol</i>		<i>M</i>
Wild-type	5.1 ± 0.1	4.5 ± 0.1	1.13 ± 0.05
$\Delta 191-220$	4.7 ± 0.1	4.2 ± 0.1	1.12 ± 0.07
$\Delta 223-243$	4.5 ± 0.1	4.1 ± 0.1	1.10 ± 0.06

Table 2

ANS binding and lipid binding parameters of apoA-I variants.

ANS		Parameters of binding of apoA-I variants to PC SUV at 25°C				
F.I. ^a	K _d	μg/ml	B _{max}	ΔH	ΔG ^b	ΔS ^c
		amino acids/mol PC		kcal/mol	kcal/mol	cal/mol/K
Wild-type	1.0	2.6 ± 1.3	0.42 ± 0.05	-92.6 ± 5.3 ^d	-12.0 ± 0.4	-270
Δ191-220	0.67	59 ± 23	0.24 ± 0.05	-60.4 ± 2.4	-10.1 ± 0.3	-169
Δ223-243	0.57	> 100 ^d	N.D. ^e	-40.6 ± 1.9 ^d	-9.9 ^d	-104 ^d

^aF.I. ; Fluorescence intensity.

^bThe Gibbs free energy was calculated according to $\Delta G = -RT \ln 55.5 (1/K_d)$.

^cThe entropy of binding was calculated from $\Delta G = \Delta H - T\Delta S$.

^dData are added from [21].

^eN.D. ; Not determined.

Table 3

Parameters for DMPC clearance and cholesterol efflux by apoA-I variants.

DMPC clearance				Cholesterol efflux (BHK/ABCA1)			
Relative	K_m	Relative		Relative	K_m	Relative	
V_{max}	($\mu\text{g/ml}$)	catalytic	efficiency	V_{max}	($\mu\text{g/ml}$)	catalytic	efficiency
Wild-type	1.0 ± 0.1	62 ± 14	1.0	1.0 ± 0.1	1.5 ± 0.5	1.0	
$\Delta 191-220$	1.1 ± 0.1	384 ± 167	0.17	1.1 ± 0.2	4.3 ± 1.6	0.40	

The data for $\Delta 223-243$ apoA-I could not be fitted with the Michaelis-Menten equation.