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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 A1 (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 Δ191–220 apoA-I, in comparison to wild-type and Δ223–243 apoA-I. Although deletion of residues 191–220 has a slight effect on the tertiary structure of apoA-I, the Δ191–220 variant showed intermediate behavior between wild-type and Δ223–243 regarding the formation of hydrophobic sites and lipid interaction through the C-terminal domain. Physicochemical analysis demonstrated that defective lipid binding of Δ191–220 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 Δ191–220 apoA-I was also intermediate between wild-type and Δ223–243 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.

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

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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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 is 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 *Escherichia 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 $= ((-[\theta]_{222} + 3000) / (36{,}000 + 3000)) \times 100$ [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, $\Delta G_D = \Delta G_D^\circ - m$[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 [^{3}H]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 [^{3}H]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% CO_2) at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented