N-terminal mutation of apoA-I and interaction with ABCA1 reveal mechanisms of nascent HDL biogenesis

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Abbreviations: ApoA-I, apolipoprotein A-I; BODIPY, boron-dipyrromethene; CAD: coronary artery disease; CD, circular dichroism; CE, cholesteryl ester; CTB, cholera toxin subunit B; DDM: n-Dodecyl-β-D-Maltoside; DMEM, Dulbecco's modified Eagle's medium; DMPC, dimyristoyl phosphatidylcholine; DMSO, dimethyl sulfoxide; FC, free cholesterol; GM1, monosialotetrahexosylganglioside; HEK293, human embryonic kidney cells 293; nHDL, nascent HDL; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEI, Polyethylenimine; PL, phospholipid; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin layer chromatography; TMB, 3,3′,5,5′-Tetramethylbenzidine.
Abstract

ApoA-I and the ATP-binding cassette transporter A1 (ABCA1) play important roles in nascent HDL (nHDL) biogenesis—the first step in the pathway of reverse cholesterol transport that protects against cardiovascular disease. On the basis of the crystal structure of a C-terminally truncated form of apoA-I(Δ[185-243]) determined in our laboratory, we hypothesized that opening the N-terminal helix bundle would facilitate lipid binding. To that end, we structurally designed a mutant (L38G/K40G) to destabilize the N-terminal helical bundle at the first hinge region. Conformational characterization of this mutant in solution revealed minimally reduced α-helical content, a less-compact overall structure, and increased lipid-binding ability. In solution-binding studies, apoA-I and purified ABCA1 also showed direct binding between them. In ABCA1-transfected HEK293 cells, L38G/K40G had a significantly enhanced ability to form nHDL, which suggests that a destabilized N-terminal bundle facilitates nHDL formation. The total cholesterol efflux from ABCA1-transfected HEK293 cells was unchanged in mutant versus wild-type apoA-I, though, which suggests that cholesterol efflux and nHDL particle formation might be uncoupled events. Analysis of the particles in the efflux media revealed a population of apoA-I–free lipid particles along with nHDL. This model improves knowledge of nHDL formation for future research.
Introduction

Cardiovascular diseases remain the leading cause of death in the US and other developed countries today (1). High density lipoprotein (HDL) has long been known to have a cardio-protective effect due to its role in the reverse cholesterol transport (RCT) pathway (2, 3). During RCT, HDL delivers excess peripheral cholesterol to the liver, thus preventing the formation of foam cells that may ultimately lead to atherosclerosis (4). The first step in HDL formation involves the interaction between apolipoprotein A-I (apoA-I) and ATP Binding Cassette A1 (ABCA1) (5). Mutations in ABCA1 lead to Tangier Disease, which results in an enlarged spleen and liver, yellow tonsils, < 5% normal plasma HDL level and premature coronary artery disease (CAD) (6, 7). Mutations in apoA-I are also associated with premature CAD (8). In this first step, apoA-I recruits effluxed lipid promoted by ABCA1 and makes discoidal nascent HDL particles (nHDL) (5, 9-14). However, the detailed molecular mechanism underlying this process has remained unclear. In previous work, we determined the structure of a C-terminally truncated apoA-I, apoA-I(Δ(185-243)), in a dimeric form (15). In this structure, the N-terminal region forms a helix-bundle, stabilized by a cluster of hydrophobic residues. These aromatic clusters (W8, F71, W72 from one apoA-I molecule and F33, F104, W108 from the symmetry related molecule) together with π-cation interactions (K23-W50 and W8-R61) are major forces that hold the helical bundle together (15, 16). However, on discoidal nHDL particles, the helices are thought to be extended with two apoA-I molecules forming a double belt structure (17). We suggested that one step in the opening of the helical bundle occurs at the hinge region A37-Q41(16), which shows high flexibility (temperature factor) as revealed by the crystal structure. In this study, we designed an N-terminus destabilized mutant of apoA-I by perturbing the first hinge region at A37-Q41. Mutation of each residue flanking G39 to a glycine residue (L38G/K40G) (Figure 1) resulted in tandem triple Gly (3X Gly), which, due to highly unconstrained ψ, φ angles, can act as a helix breaker (18). We hypothesized that the unconstrained hinge would open more freely and the buried hydrophobic core would be readily accessible to lipid molecules.
During nHDL biogenesis, it is controversial whether apoA-I interacts directly with ABCA1. Vedhachalam \textit{et al.} suggested that apoA-I binds to ABCA1 first, and activates ABCA1 to move lipids from the inner leaflet to the outer leaflet of the plasma membrane forming a bulge-like structure to release surface tension. This structure was considered essential for apoA-I to bind and form nHDL (13). Vaughan \textit{et al.} suggested that there is no direct interaction between apoA-I and ABCA1, but rather lipids only get loaded to apoA-I through passive diffusion (19). The only evidence for a direct interaction between apoA-I and ABCA1 has been crosslinking-based studies (20). Thus, to investigate further whether there is directed interaction between the two proteins, we extracted and solubilized ABCA1 from HEK293 cells overexpressing ABCA1 and tested the interaction between purified ABCA1 and purified apoA-I in solution. We demonstrated, for the first time, that there is a direct interaction between apoA-I and ABCA1.

Material and Methods

\textbf{Materials:} Dimyristoylphosphatidylcholine (DMPC) and ANS were purchased from Sigma. DH5α competent cells were purchased from NEB. Complete EDTA-free protease-inhibitor cocktail was purchased from Roche. Dulbecco\textquotesingle s modified Eagle\textquotesingle s medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen. Bovine serum albumin, methyl-β-cyclodextrin, HRP-conjugated antibodies to goat and mouse were purchased from Sigma. BODIPY-cholesterol (Topfluor) was purchased from Avanti. Genjet was purchased from SinaGen laboratories. Polyethylenimine (PEI, MW 25,000 liner) and amphipol 8-35 were purchased from Polysciences. Rhodopsin-1d4-tag antibody beads and eluting peptide were purchased from Cubebiotech. TMB substrate (3,3’,5,5’-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA) was purchased from Sigma. ApoA-I polyclonal goat antibody was from Abcam. ABCA1 mouse monoclonal antibody was from Gateway. Anti-LDH and anti-flotillin-2 mouse monoclonal antibody was from Santa Cruz Biotechnology. Denville Blue protein stain was
purchased from Denville Scientific. ATPase activity kit was purchased from Innova Biosciences. AmplexRed Cholesterol Kit was purchased from ThermoFisher.

Construct generation: pcDNA1 containing the human ABCA1 gene was a generous gift from Dr. Freeman’s lab at Harvard. A rho1d4 tag (T-E-T-S-Q-V-A-P-A) was added to the C-terminus of ABCA1 using the Q5® Site-Directed Mutagenesis Kit from the New England Biolab. Primers used for making this construct were:

Forward: GTGGCGCCGCGTGAATCCTGTTCATACGGGG
Reverse: CTGGCTGGTTTCGGTTAATACATAGCTTTCTTTCACTTTC

Circular Dichroism: Refolded apoA-I and mutant proteins were placed in 1 or 2 mm quartz cuvettes for the experiments. Data were recorded on Aviv 62DS or Aviv 215 spectropolarimeters (Aviv Associates, Lakewood, NJ). Freshly-refolded proteins at a concentration ~ 0.02 to 0.2 mg/ml (below the self-aggregation concentration of apoA-I as characterized in previous studies (21) in 10 mM phosphate buffer pH 7.4 at 25 °C were used in these experiments.

For Far-UV spectra, wavelengths 250 nm-185 nm were scanned with 1 nm bandwidth, 1 nm step size and 5 s accumulation time at 25 °C. Each sample was scanned 3 times. The PO₄ buffer baseline was subtracted for later calculations. α-helical content was calculated as follows: α-helix% = (-[θ]₂₂₂ + 3000)/39000 (22).

For thermal unfolding experiments, the ellipticity of protein samples at 222 nm was monitored from 5 to 95 °C with 1 °C step size and 90 s accumulation time for each data point. Melting temperature was derived from the peak of the first derivative of the thermal unfolding curve calculated using the Origin Software (Microcal).

DMPC clearance assay: DMPC (10 mg) was dissolved in a glass tube in chloroform/methanol (2:1) and then dried under nitrogen and left in a desiccator at 4 °C 20-24 h to remove the residual solvent. An aliquot of 2 ml of phosphate-buffered saline (PBS) was added to the tube and the solution was vigorously vortexed.
An aliquot of 50 µl stock solution (5 mg/ml) was mixed with 1 ml 0.1 mg/ml WT or mutant forms of apoA-I pre-equilibrated at 24 °C to give a 2.5 : 1 (w : w) lipid to protein ratio, respectively. The rate of DMPC clearance by WT and mutant forms of apoA-I was monitored by the change of the turbidity of the solution (absorbance at 325 nm) at 24 °C for 1 hour. The plot of Absorbance at 325 nm versus time (min) was recorded.

**ANS binding assay:** 8-anilino-1-naphthalenesulfonate (ANS) fluorescence binding assays were performed on a fluroMax-2 fluorescence spectrometer (Instruments S.A. Inc) at 25°C. Excitation slit width was 5 nm and emission slit width was 2.5 nm. ANS fluorescence emission was recorded at concentration 0.25 mM in the presence of mutant L38G/K40G or WT apoA-I at 0.05mg/ml in 5 mM phosphate buffer pH=7.4. ANS fluorescence was excited at 395 nm and the emission spectra were recorded from 400 nm to 580 nm.

**Cholesterol efflux nHDL formation and expressed ABCA1 level:** Human Embryonic Kidney cells 293 (HEK293 cells) were plated at 60% - 70% confluence in 24-well collagen-coated plates in Growth media (Dulbecco's modified Eagle's medium (DMEM) (low glucose) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin). After 20-24 h incubation, the cells were transiently-transfected with pcDNA1 containing human ABCA1 cDNA or pcDNA1 alone (empty vector, as a negative control). For each well, 0.8 µg of DNA was transfected. Twenty-four hours post-transfection, BODIPY-cholesterol (1 µM) solubilized in dimethyl sulfoxide (DMSO) or mixed with cholesterol (24 µg/ml)/methyl-β-cyclodextrin complex was added to the cells and incubated for 1 hour (23-27). BODIPY-cholesterol solubilized in DMSO was used to label cells with basal level cholesterol while cholesterol/methyl-β-cyclodextrin/BODIPY-cholesterol complexes were used to label cells with elevated cholesterol. This treatment elevated cholesterol level in the cells by 13.6% ± 1.3%. Measurement of cholesterol level was achieved using AmplexRed Cholesterol Kit according to manufacturer’s instruction. Cells were then washed with DMEM and incubated with 4 to 10 µg/ml apoA-I or L38G/K40G mutant. Albumin at a concentration of 0.01% (a molar ratio of 8:1 to apoA-I (5 µg/ml) added was used as a negative control).
Media were collected after incubation for 20-24 h. For kinetic studies, 20 µl of media were collected at time points 0h, 2.5 h, 5 h, 7.5 h, 10 h, and 24 h. The conditioned media were filtered through a 0.22 µm membrane to remove cell debris and the cells were lysed with 1% sodium cholate at room temperature (25 °C) for 4 hours. Fluorescence in both media and cells was measured with a Tecan Infinite M1000 plate reader at excitation wavelength 490 nm (bandwidth 10 nm) and emission wavelength 520 nm (bandwidth 20 nm). Fluorescence derived from empty-vector transfected cells was subtracted to obtain the baseline. The percentage efflux was calculated by taking the fraction of fluorescence in the media over total fluorescence in both media and the cells. Nascent HDL formation was detected by immunoblotting following a 4%-15% gradient native PAGE where membranes were probed with anti-apoA-I polyclonal antibody. ABCA1 level was determined by SDS-PAGE followed by immunoblotting using antibodies to ABCA1. The same membrane was subsequently probed with antibodies to actin which served as loading control.

Lipid analysis: Efflux media were collected and filtered as described above. For density gradient fractionation, solid KBr was added to the media and mixed thoroughly to reach a density of 1.25 g/ml. The media were then overlaid with 1.05 g/ml KBr. The samples were spun in a SW41 rotor, at 39k rpm at 11 °C for 24 hours. One-ml fractions were collected from the top and density determined using an Abbe Refractometer (American Optical Corp.). Fluorescence was measured in a Tecan Infinite M1000 plate reader for cholesterol content. Protein and the ganglioside GM1 were analyzed by dot blot or by 4%-15% gradient native PAGE followed by immuno- and ligand blotting, respectively. For non-gradient separation, efflux media were adjusted to 1.21 g/ml with KBr and ultracentrifuged in SW41 rotor at 39k rpm at 11°C for 24 hours. After ultracentrifugation, all lipid-containing particles were in the top 2-ml fraction. The lipid composition of this fraction was analyzed by Thin Layer Chromatography (TLC).

After ultracentrifugation, lipids were extracted as described (28). The chloroform phase was dried under nitrogen. Lipids were dissolved in 40-50 µl chloroform and loaded on a TLC plate. The plate was first
developed to 25% in a polar system with solvent composition CHCl₃: MeOH: H₂O: Acetic acid (65:25:4:1) and then a neutral system with solvent composition Hexane: Diethylether: Acetic acid (70:30:1). The plate was exposed to iodine vapors to visualize the lipids.

*Electron microscopy of apoA-I-free particles:* Carbon-coated grids were glow-discharged before use. Four µl aliquots from ultracentrifugation fractions were loaded onto the grid and incubated for 5 mins and then blotted with filter paper. The grid was rinsed with Tris buffered saline 10 times. Freshly-prepared uranyl acetate (1%) stain was applied and incubated for 10s, twice. Excess stain was blotted and the grid was air-dried for 3 mins. The grids were observed in the CM12 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands).

*Binding of apoA-I to ABCA1 in the cells:* HEK293 cells were plated in 24-well collagen-coated plates and transfected as described. Forty-eight hours post transfection, cells were incubated with 5 µg/ml apoA-I for 30 min at 37 °C followed by 30 min at 4 °C. For cholesterol-elevated cells, cholesterol (24 µg/ml )/methyl-β-cyclodextrin complex was added to the cells for 1 h at 37 °C before apoA-I addition. Cells were washed twice with ice-cold PBS. Paraformaldehyde (2.5%) was added and cells incubated at room temperature for 30 min. The cells were then washed with PBS 3 times and incubated at 37 °C for 30 min with 5% non-fat milk in PBS to block non-specific binding of antibodies. Goat anti-apoA-I polyclonal antibodies were added and incubated at room temperature for 1 h. After washing 3 times with PBS, HRP-conjugated antibody to goat IgGs was added and cells incubated at room temperature for 1 h. After three washes with PBS, 3,3′,5,5′-Tetramethylbenzidine (TMB) was added and incubated for 30 min. After TMB incubation, the solution was supplemented with 3 M H₂SO₄ (final concentration 1.5 M) to stop the reaction. The solution then turned yellow. Absorbance at 450 nm was determined in a Tecan Infinite M1000 plate reader.

*ABCA1 and apoA-I purification:* ApoA-I proteins were expressed and purified as described previously (15, 29).
ABCA1 purification: HEK293F cells were grown in Freestyle Expression Media (Invitrogen) in 1 L rotating flasks at 130 rpm, 8% CO2, 37 °C. Once cell density reached 2.5 x 10^6 /ml, the cells were transfected with 3 µg ABCA1-rho DNA and 9 µg polyethylenimine (PEI) per 1 ml of cell culture. Twenty-four hours post transfection, the culture was supplemented with 2 mM valproic acid to help stabilize protein expression (30). Forty-eight hours post transfection, cells were harvested and lysed with ice-cold 10 mM HEPES pH 7.5 hypotonic buffer supplemented with protease inhibitor cocktail (Roche) using a Dounce homogenizer. Cell lysate was first centrifuged at 1,000 x g to remove cell debris and nuclei, and then centrifuged at 100,000 x g for 40 minutes to pellet membranes. The membrane pellet was resuspended in 50 mM HEPES, 150 mM NaCl, 2 mM TCEP pH 7.5 and 1 % (w/v) n-Dodecyl-β-D-Maltoside (DDM) and gently agitated for 1 hour. This suspension was centrifuged at 100,000 x g for 40 minutes and the supernatant was collected. The supernatant was incubated with rho1d4 antibody-conjugated agarose beads for 20-24 h. The beads were then washed twice with 10x bed volume of wash buffer (50 mM HEPES, 150 mM NaCl, 2 mM TCEP pH 7.5, 0.02% DDM). For DDM solubilized-ABCA1, the protein was eluted with 3 fractions of elution buffer (50 mM HEPES, 150 mM NaCl pH 7.5, and 0.02% DDM, with 500 µM rho1d4 peptide) for 1 h each at 4 °C. For amphipol solubilized-ABCA1, DDM was substituted with amphipol 8-35 (ABCA1: amphipol = 1:3 w:w) for 1 h at room temperature followed by 1 h at 4 °C. The beads were washed three times with 10x bed volume wash buffer without DDM. ABCA1-rho was eluted by 50 mM HEPES and 150 mM NaCl pH 7.5, with 500 µM rho1d4 peptide for 1 h per fraction. Three fractions were collected.

ABCA1 and apoA-I oligomerization states probed by crosslinking: Bis(sulfo)succinimidyl)suberate (BS3) (5 mM) in 25 mM phosphate buffer, pH 7.4 was incubated with 100 µg/ml DDM solubilized-ABCA1 or apoA-I at room temperature for 1 h. The crosslinking reaction was stopped by the addition of 25 mM Tris buffer for 15 min at room temperature. Proteins were then resolved on SDS-PAGE at 200 V for 45 min followed by immunoblotting and membrane probed by anti-ABCA1 and anti-apoA-I antibody.
ATPase activity: The ATPase activity was measured by Innova Biosciences’ ATPase activity kit. The content of Pi released from ATP molecules was measured using a colorimetric method according to the manufacturer’s protocol. Activity was determined by absorbance between 590 nm to 660 nm using Tecan Infinite M1000 plate reader.

In-solution apoA-I-ABCA1 interaction: After exchange for amphipol on the beads, 100 to 150 µg/ml eluted ABCA1 was incubated with 10 to 15 µg/ml of apoA-I (1:1 molar ratio of apoA-I:ABCA1) at room temperature for 1 hour. The concentration of apoA-I was the same as in the efflux assay and cross-linking experiments described. After 1 h incubation, samples were resolved on native-PAGE and analyzed by immuno blotting and membranes probed with anti-apoA-I antibody. The same blot was then stripped with stripping buffer (0.1 M glycine pH 2) and probed again with anti-ABCA1 antibody.

Results

In-solution characterization of apoA-I mutant L38G/K40G -- secondary structure, stability, and lipid binding properties

Figure 2A shows the Far-UV CD spectra of mutant L38G/K40G and WT apoA-I. Both forms exhibited negative peaks at 222 nm and 208 nm, characteristic of α-helical structure. Based on the [θ]_222 signal, the calculated α-helical content of WT was 54 ± 2%, consistent with previously-reported helical content of WT apoA-I (21). Helical content of L38G/K40G was similar to that of WT, 51 ± 2%. This result indicates that this mutation had minimally affected the helical content of the lipid-free apoA-I form, rather than perturbing the overall helical structure.

Interestingly, for the L38G/K40G mutant, although the melting temperature remained the same as WT, at 62 ± 2 °C, the thermal unfolding cooperativity decreased (more flattened curve, rather than a sigmoidal
shape in WT shown in Figure 2B), suggesting that although the protein’s stability was not affected, it had become less compact.

On binding to hydrophobic surfaces, intrinsic ANS fluorescence is enhanced and blue-shifted. Therefore, we used the ANS fluorescence assay to access the exposed hydrophobic surface of apoA-I and its mutant as described in previous work (15, 21). As shown in Figure 2C, ANS alone in phosphate buffer had a very low-intensity emission at 518 nm. When WT apoA-I was added, the emission peak of ANS fluorescence blue shifted to 478 nm, and the intensity increased 6-fold. With mutant L38G/K40G, the emission peak was at 474 nm, and the intensity increased 10-fold. This finding suggested that mutant L38G/K40G has a larger exposed hydrophobic surface, indicative of a loosely-packed structure, exposing the hydrophobic pocket in apoA-I buried within the helical bundle.

Clearance of DMPC multilamellar vesicles (MLV) provides a measure of the kinetics of mutant L38G/K40G and WT apoA-I to form DMPC-apoA-I complexes. This assay reflects the apoA-I lipid binding ability. As shown in Figure 2D, the turbidity of DMPC MLV was reduced faster by L38G/K40G than WT apoA-I, suggesting that L38G/K40G had better lipid binding ability. Because mutating the leucine residue at position 38 and lysine residue at position 40 to glycine does not change the calculated hydrophobicity of apoA-I significantly, we attribute this increased lipid binding ability to the more-easily-opened N-terminal helical bundle, where the hydrophobic core is buried.

Collectively, the in-solution characterization studies validated our mutation design and supported our hypothesis that opening the N-terminal helix bundle facilitates lipid binding.
Cholesterol Efflux and nHDL formation from ABCA1-transfected HEK293 cells

The ABCA1 construct was a generous gift by Dr. Freeman at Massachusetts General Hospital. Dr Freeman’s group characterized this construct extensively including the cellular distribution of ABCA1 in HEK293 (31). Their studies have shown that ABCA1 was efficiently expressed by this construct and was localized to the plasma membrane. Figure 3A illustrates the nHDL particles formed by mutant L38G/K40G and WT apoA-I. ABCA1-transfected cells were examined in triplicates. Both L38G/K40G (lane 1-3) and WT (lane 5-8) apoA-I formed nHDL particles from ABCA1-transfected HEK293 cells, but not from EV-transfected cells (lane 4, 8) or when no acceptor was added (lane 9), substantiating that the process of particle formation was specifically mediated by ABCA1 and apoA-I. Based on size, nHDL particles could be classified into the following species; pre-β1 (7.1 nm), pre-β2 (8.2 nm), pre-β3 (10.4 nm), pre-β4 (12.2 nm) and pre-β5 (17 nm) (32, 33). As shown in lanes 1-3 and lanes 5-8, all five species were formed by both mutant L38G/K40G and WT apoA-I and the migration positions of particles were the same in the two groups. These results suggested that mutant L38G/K40G and WT apoA-I probably adopted the same conformation on each nHDL species. However, quantification of the bands shown in Figure 3C, revealed a 50% increase in nHDL particles following incubation of cells with mutant L38G/K40G, suggesting that this mutation enhanced nHDL formation. This difference was not caused by different expression levels of ABCA1 due to transient transfection, or different internalization and degradation levels of ABCA1 (34) (35), because ABCA1 levels following incubation with L38G/K40G or WT apoA-I were the same (Figure 3B). Surprisingly, as shown in Figure 3D, measurement of total cholesterol efflux from L38G/K40G and WT showed no difference. Albumin (0.01%, at a molar ratio of 8:1 to apoA-I (5 µg/ml) added did not promote significantly higher cholesterol efflux compared to the no acceptor added group, excluding the possibility that BODIPY-cholesterol was loosely binding to the membrane and able to bind to any hydrophobic surface in the media, independent of apoA-I and ABCA1 activity.
To investigate further how mutant L38G/K40G is different from WT in forming nHDL particles, a time-course experiment was carried out. Following addition of L38G/K40G or WT apoA-I, media were collected at time points 2.5 h, 5 h, 7.5 h, 12 h and 24 h. As shown in Figure 3E, some particles started to emerge at 2.5 h incubation with mutant L38G/K40G, while no particles were visible following incubation with WT. At 12 h, the majority of L38G/K40G had interacted with lipid to form nHDL particles, while WT was largely unlipidated. After incubation for 24 h, almost all L38G/K40G was incorporated into nHDL particles. In contrast, WT still showed a significant amount of free protein. Overall and consistent with previous results, L38G/K40G formed more nHDL particles than WT after 24 h. These kinetic experiments showed that L38G/K40G could form nHDL faster, further supporting our hypothesis that destabilization of the N-terminal hinge facilitates opening of the N-terminal helical bundle, thereby facilitating nHDL particle formation.

Figure 3F shows the quantification of the blot in Figure 3E. The plot of lipidation level versus time fitted a straight line, suggesting that lipidation was occurring at a steady rate during the 24 h incubation time. Surprisingly, the kinetics of cholesterol efflux (Figure 3G) suggested that efflux almost plateaued as early as 8 h. In other words, after incubation of cells for 8 h, cholesterol efflux almost reached maximum and remained constant, but nHDL particles continued to form until 24 h. Thus, there was a lag time between nHDL formation and cholesterol efflux. Maximum cholesterol efflux preceded nHDL particle formation. This difference in cholesterol efflux kinetics and nHDL formation kinetics is consistent with the seemingly paradoxical finding that different levels of apoA-I lipidation could occur with the same cholesterol efflux in the media. Combined, these results suggested that cholesterol efflux and nHDL formation might be uncoupled processes.

Analysis of apoA-I-promoted lipids effluxed from ABCA1-transfected HEK293 cells

To understand further how cholesterol efflux promotion was uncoupled from nHDL formation, we analyzed the lipid composition from the efflux media after 24 h incubation with L38G/K40G and WT apoA-I. Lipids
from effluxed media collected after 24 h were extracted and analyzed by thin layer chromatography (Figure 4A). As shown in lane 2 and lane 3, cholesterol efflux promoted by L38G/K40G and WT was the same, consistent with our fluorescence measurements. However, although cholesterol levels were the same for L38G/K40G and WT, a small decrease in PC and sphingomyelin (SM) levels in media derived from L38G/K40G compared to WT was observed, suggesting that the lipid composition may be different in apoA-I- and mutant-promoted efflux media. We also observed that even without apoA-I as the lipid acceptor (lane 1), ABCA1 was still able to transport some cholesterol from the cells into the media, which is consistent with previous reports documenting “apoA-I-independent, ABCA1-dependent cholesterol efflux” (36). This observation was not a result of cell death, since probing for lactate dehydrogenase (LDH), a marker for cell death, showed no obvious cell death as very little LDH was detected (Figure 4B). These results are also consistent with our efflux data showing that ABCA1-transfected cells with no apoA-I as lipid acceptor always yielded higher efflux than empty vector (mock) -transfected cells (data not shown).

We suspected that this apoA-I-free, cholesterol-containing lipid population, were the microparticles proposed by Duong et al (37), and that these microparticles might explain the discrepancy between cholesterol efflux level and nHDL biogenesis. Earlier characterization of these microparticles by Duong et al reported that the microparticles contained the ganglioside GM1. Therefore, we separated nHDL particles from apoA-I-free lipids in the efflux media by density gradient fractionation, and probed for GM1, as well as cholesterol and apoA-I levels. As shown in Figure 4C, D, and E, cholesterol, apoA-I, and GM1 levels, respectively, in each of the fractions were quantified (expressed as a fraction of total cholesterol, apoA-I, or GM1 after baseline subtraction of 38/40 EV and WT EV signal). Fractions 3 to 7 contained cholesterol, apoA-I, and GM1, in agreement with the data shown by the western blot (Figure 4C) demonstrating that these fractions contained nHDL particles. Fractions 8 to 13 contained mainly lipid-free apoA-I, and therefore, were low in cholesterol and GM1 content. However, fraction 1 contained no apoA-I, but was cholesterol- and GM1- abundant, indicating that this low-density fraction was apoA-I-free lipid mixture.

We analyzed the density fractions further by negative-stain electron microscopy (EM). In the ABCA1-transfected, apoA-I added group (Figure 5A) the particles in fraction 1 appeared different from the nHDL
in the higher-density fractions (fractions 3-6, Figure 5F). These particles ranged in size from 20 to 60 nm, with the majority at ~30 nm, much larger than nHDL particles, which are typically in the range of 7-12 nm, as shown in Figure 5F. The particles in fraction 1 were specific to ABCA1 transfected groups (Figure 5A and B), and are more abundant when apoA-I was added. Importantly, these particles were not observed in the empty-vector transfected group (Figure 5C). In the control experiments, we examined fraction 1 derived from cells killed with 0.5% azide (Figure 5D), and media containing BODIPY-cholesterol/cholesterol/cyclodextrin complexes (Figure 5E). The particles generated from ABCA1-transfected cells clearly showed different size and morphologies compared to these control groups, suggesting that the particles were not membrane fragments released from dead cells or from artifacts of the BODIPY-cholesterol/cholesterol/cyclodextrin complex.

These data suggest a possible mechanism in which cholesterol efflux and nHDL formation might be two different steps and that there is an apoA-I-free lipid population in the media. These findings raise the question as to whether apoA-I is binding only to the apoA-I-free lipids, presumably microparticles shed by ABCA1 activity (37, 38), but not to ABCA1 located in the cell membrane. Therefore, we tested the binding of apoA-I to ABCA1 on the cell surface as well as to purified ABCA1 in solution.

Crosslinking of apoA-I to ABCA1-expressing cells

Figure 6A shows apoA-I crosslinked to ABCA1-expressing HEK293 cells. In both basal level cholesterol and cholesterol-elevated cells, more apoA-I was crosslinked to ABCA1-transfected cells than to empty vector-transfected cells, suggesting that crosslinking of apoA-I to cells was dependent on ABCA1 expression as previously suggested by Chroni et al (20). In ABCA1-expressing cells with elevated cholesterol, the amount of apoA-I crosslinked to the cells was approximately 2-fold higher than the amount of apoA-I crosslinked to ABCA1-expressing cells with basal level cholesterol. In cells mock-transfected with empty vector, the level of apoA-I crosslinked to ABCA1-expressing cells was similar in basal level cholesterol and in the cholesterol-elevated group, confirming a direct apoA-I-ABCA1 interaction. In a
parallel experiment, instead of being crosslinked to apoA-I by paraformaldehyde, cells with different cholesterol levels were lysed and probed for ABCA1. As shown in Figure 6B, the ABCA1 level was the same in basal level cholesterol and cholesterol elevated groups. Therefore, the difference in crosslinking was not caused by different ABCA1 levels, but caused by different binding efficiencies for apoA-I as a consequence of increased activation of ABCA1 that presumably leads to a more favorable conformation for binding apoA-I. Collectively, these results demonstrated that apoA-I was binding to ABCA1 and/or ABCA1-rearranged cell membranes (13), and exclude the possibility that apoA-I was only binding to the shed apoA-I-free particles in the media.

Interaction between apoA-I and ABCA1 in solution

To investigate whether apoA-I was binding to ABCA1 directly or binding to the ABCA1-rearranged cell membrane, we probed the interaction between apoA-I and purified ABCA1 in solution, without the interference of on the cell membrane lipids. A large-scale mammalian expression system was established and ABCA1 with a rhodopsin 1d4 (rho1d4) tag was purified. Addition of the rho1d4 tag to the C terminus of ABCA1 did not affect nHDL formation (Figure 7A). SDS-PAGE of purified ABCA1 (Figure 7B) showed a sharp single band with apparent MW of slightly more than 250 kDa, consistent with the size of ABCA1 monomers based on the reported calculated molecular weight (39). This band was confirmed to be ABCA1 by Western blotting (Figure 7C) and mass spectrometry. As shown by SDS-PAGE, the elution fractions were pure after this single-step affinity purification.

To exclude the interference of detergent micelles, DDM was exchanged with amphipol, which has been shown to bind to membrane proteins with such a high affinity that no amphipol micelles remain in the solution (40). This property allowed us to minimize the interaction of apoA-I with detergent micelles in solution and facilitate interaction with ABCA1. Native-PAGE of amphipol-solubilized ABCA1 (Figure 7D) showed a single band at MW over 440 kDa, possibly due to glycosylation, amphipol binding, and shape
difference compared to the globular size marker. This amphipol-solubilized ABCA1 showed four-fold higher ATPase activity compared to the control group (ATP self-decay in buffer only) (Figure 7E), affirming that the protein was correctly folded during purification.

We then incubated this amphipol-solubilized ABCA1 with apoA-I. Figure 7F shows an immunoblot probed with antibodies to apoA-I followed by antibodies to ABCA1. When probed with anti-apoA-I antibody (left panel), apoA-I alone (lane 1) appeared only at the lower molecular weight region, showing the same migration pattern as apoA-I in phosphate buffer, suggesting that residual rho1d4 peptide in the solution did not affect apoA-I conformation. After incubation with ABCA1, a fraction of apoA-I was seen at the position of ABCA1 (lane 3), suggesting that apoA-I was in complex with ABCA1. This binding was abolished by adding a detergent-like phospholipid (PIP2) to the solution (lane 4). This result is the first direct evidence in solution that apoA-I binds to ABCA1. However, only a small fraction of apoA-I bound to ABCA1 under these experimental conditions, possibly due to a lack of lipid environment that facilitates the binding in cells or intrinsic low binding affinity.

Discussion

In this study, we probed the effect of an N-terminal mutation at the first hinge region (L38G/K40G) of apoA-I both from a structural and a functional standpoint. As expected, this mutation made the first hinge region more flexible, potentially facilitating opening of the helical bundle. Helical structure and melting temperature remained similar to that of WT apoA-I, suggesting that the secondary structure was not disrupted by the mutation. However, a decreased unfolding cooperativity revealed a slightly less compact tertiary structure. This observation was confirmed by the ANS binding and DMPC clearance assays. Mutant L38G/K40G showed an increased ANS binding and DMPC clearing ability, suggesting a more exposed hydrophobic surface and consequent lipid binding ability. Therefore, overall structure was maintained in the mutant and lipid interaction was facilitated by the increased flexibility.
The facilitated opening of the N-terminal bundle led to an enhanced ability to form nHDL by mutant L38G/K40G. However, mutant L38G/K40G promoted the same cholesterol efflux from cells compared to WT apoA-I. Further analysis of the efflux media revealed an apoA-I-free lipid population in the media, which may be membrane-derived microparticles shed by ABCA1-expressing cells, thus leading to the concept that apoA-I may not bind directly to ABCA1, but rather, binds only to the shed microparticles and solubilizes them to form nHDL. Our in-solution binding studies have demonstrated a direct interaction between apoA-I and ABCA1, thus excluding the possibility that apoA-I binds solely to microparticles. Under our experimental conditions, only a small fraction of apoA-I binds to ABCA1 in solution. This result is in agreement with reports by Phillips (41) and Hassan et al (42) suggesting that only 10% of apoA-I is directly bound to ABCA1.

Since apoA-I was observed at the position of the ABCA1 monomer (slightly above 440 kDa on Native-PAGE), it is likely that apoA-I binds directly to the ABCA1 monomer, rather than, as proposed, that ABCA1 dimerization drives apoA-I binding (43). However, the low resolution of the gel precluded evaluation of whether apoA-I monomer or dimer binds to the ABCA1 monomer based on shift in migration. The recent cryo-EM structure of ABCA1 has revealed structural details of ABCA1 (44). However, it did not provide suggestions on how ABCA1 interacts with apoA-I, and did not elucidate whether the functional form of ABCA1 is a monomer or dimer.

In addition to Duong et al’s characterization of microparticles in J774 macrophages, in which they reported microparticles of 24 ± 2 nm in diameter containing CD14 (37), microparticles have also been characterized by Hafiane et al (45) using other cell types, including baby hamster kidney (BHK) cells, THP-1 monocytes, and HepG2 cells. These investigators reported the existence of flotillin-2 and CD36 on the particles and the particles’ density was similar to that of exosomes, between 1.10 to 1.19 g/ml (46, 47); Their size ranged from 50 to 250 nm diameter (the size of exosomes ranges from 40 to 100 nm (48)). However, in our study, the density of the particles was clearly out of the range of exosomes, at < 1.06 g/ml; and the size ranges from 20 to 60 nm, with the majority at 30 to 40 nm. Also, we could not identify flotillin-2 or CD14 in the
apoA-I-free particles using anti-flotillin and anti-CD14 antibodies (data not shown), suggesting that these apoA-I free-particles might be different from the microparticles described by previous groups, or that these particles vary in composition depending on cell types. Furthermore, we did not concentrate the media before gradient ultracentrifugation as was done in the studies from the other two groups, which may also contribute to the difference in size and density of these apoA-I-free particles. We also observed that these particles were very unstable since after incubation at 4 °C 20-24 h they were no longer visible by EM, possibly due to fusion of smaller particles into bigger aggregates that were difficult to attach to the grid.

Our lipid analysis has revealed the presence of GM1 in both apoA-I-free particles and for the first time, in nHDL. However, it is unlikely that ABCA1 is transporting the major lipids, including phospholipid, cholesterol, and the bulky and highly glycosylated GM1. GM1 may be transferred to nHDL through lipid exchange from the apoA-I-free microparticles. GM1 is a lipid raft marker (49). Previous work by Sorci-Thomas et al documented that the lipid composition of nHDL resembles lipid rafts (50). However, another lipid raft marker, flotillin, was not found in our apoA-I-free particles. Therefore, it remains unclear whether these particles originated from lipid rafts.

Based on the findings in this and previous studies, especially the model proposed by Vedhachalam et al, (16, 51-55), we propose a model for the formation of nHDL (Figure 8). In Step 1, when apoA-I is absent, ABCA1 works at basal activity and translocates cholesterol and other lipids between membrane leaflets. When the outer leaflet accumulates more lipids, a bulge-like structure is formed to relieve the surface tension. This bulge can then detach from the membrane in the form of apoA-I-free particles, as suggested by Duong et al (37). These apoA-I-free particles represent the apoA-I-independent lipid efflux which has been described by us and other groups (20).

When apoA-I is present, the apoA-I-independent efflux pathway still operates, but the apoA-I-dependent pathway becomes important. Under physiological conditions, it is most likely that apoA-I is in its monomeric form due to low concentration. When apoA-I reaches the cell membrane where ABCA1 resides,
two events occur (Step 2): the hydrophobic C-terminus of apoA-I docks to the cell membrane that was rearranged by ABCA1, and the N-terminus of apoA-I interacts with ABCA1, unfolds, allowing for apoA-I to dimerize. Subsequently in Step 3, phospholipid and cholesterol transported by ABCA1 are recruited to apoA-I. When sufficient lipids are recruited, the nHDL particle is formed and released from the membrane, which then gains more lipids, including GM1, from apoA-I-free particles produced in the apoA-I-independent pathway or from membrane bulges via passive diffusion to form larger pre-β nHDL species (Step 4).

Our model differs from the model proposed by Vedhachalam et al in that we propose that after ABCA1 rearranges the membrane lipid distribution and creates a high-curvature structure, the apoA-I - ABCA1 interaction is required for the formation of nHDL. ApoA-I binding to the high-curvature membrane surface followed by spontaneous solubilization of the exovesiculated domain does not produce nHDL because if high-curvature membrane is sufficient for apoA-I to generate nHDL particles, the shed particles should also be solubilized by apoA-I spontaneously and we would have observed apoA-I on these shed particles. However, these particles were apoA-I free. Based on the prior literature and our studies on apoA-I (56), we suggest that ABCA1 binding unfolds the N-terminal bundle of apoA-I, making it accessible for phospholipid and cholesterol binding. We also suggest that there is another step in which newly-generated nHDL acquire lipids from apoA-I-free particles that contain GM1.

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Figures

A. ApoA-I consists of ten tandem 11/22mer helices separated by proline residues in the exon-4 encoded region (in cyan and purple) and an exon-3 encoded N-terminal region (in green) (57). Each helix is composed of either sequence unit A (consensus sequence PLAEELRARLR, cyan) or sequence unit B (consensus sequence AQLEELRERLG, purple) with proline kinks (58).

B. Crystal structure of a C-terminal truncated apoA-I. This structure is one copy of monomer from the crystal dimer. We introduced a 3-tandem-glycine mutation (L38G/K40G) in the first hinge region to disrupt the local helical structure, in order to make the hinge more flexible, hence easier to open. Mutations L38G and K40G are colored in red.

Figure 1 ApoA-I mutant L38G/K40G designed to destabilize the N-terminal hinge. A. ApoA-I
Figure 2 In-solution characterization of apoA-I and mutant L38G/K40G. Protein concentration used in this experiment was between 0.02 mg/ml to 0.2 mg/ml, freshly-refolded. A. Far-UV CD spectra (190 nm – 250 nm) of apoA-I mutant L38G/K40G and WT. B. Thermal unfolding of apoA-I WT and mutant L38G/K40G. $\theta_{222}$ signal was recorded from 5 °C to 90 °C. Melting temperature was calculated as the peak of the first derivative of the sigmoidal unfolding curve. C. ANS fluorescence assay to measure exposed hydrophobic surface. D. DMPC clearance assay to measure lipid binding ability.
Figure 3 nHDL formation and cholesterol efflux from ABCA1-transfected HEK293 cells. A.
Nascent HDL formation by apoA-I WT and mutant L38G/K40G (4 µg/ml). ABCA1-transfected groups were in triplicate; EV: empty vector mock-transfected cells. No acceptor: neither mutant nor WT apoA-I was added. B. Expression of ABCA1 in HEK293 cells with L38G/K40G or WT apoA-I added in the media. Cell lysates were resolved by SDS-PAGE followed by transfer onto a membrane. The membrane was probed with antibodies to ABCA1(top) and subsequently probed with antibodies to actin (bottom).
C. Quantification of bands of nHDL particles formed by mutant L38G/K40G and WT apoA-I (depicted in panel A) using ImageJ. Total signal from WT particles was taken as 100%. N=3, **: P<0.01. D. Comparison of cholesterol efflux between mutant L38G/K40G and WT apoA-I. WT efflux was taken as 100%. No acc: no acceptor added group. E. Western blot of nHDL formation at time points 0 h, 2.5 h, 5 h, 7.5 h, 12 h, and 24 h. F. Cholesterol efflux promoted by mutant L38G/K40G and WT apoA-I was plotted over time. Percent efflux at each time point was normalized to maximum efflux level at 24 h (taken as 100%). Data for each time point were in triplicate. G. nHDL formation from mutant L38G/K40G and WT apoA-I plotted over time. Quantification of bands was done using ImageJ. For both
L38G/K40G and WT apoA-I, intensity of total band signal, I_{int} (including both lipidated apoA-I and free apoA-I) after 24 h incubation was taken as 100%.

**Figure 4** Lipid analysis of efflux media. A. TLC of total lipids from efflux media after 24 h incubation. Marker 1 (top to bottom: phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA)); marker 2 (top to bottom: cholesteryl ester (CE), free cholesterol (FC), sphingomyelin (SM)); marker 3, BODIPY-Cholesterol. Lipids were visualized by iodine stain. B. Immunoblot of efflux media probed for cell-death marker LDH. Sodium azide at concentrations 0.01%, 0.05%, and 0.5% were added to the media of ABCA1-transfected cells together with apoA-I and incubated 20-24 h. C. Immunoblot of density gradient fractions probed by anti-apoA-I antibody. D, E, F. Comparison of cholesterol, GM1, and
apoA-I distribution from density gradient fractions of efflux media from mutant L38G/K40G and WT apoA-I. Fraction densities (g/ml): Fraction 1: <1.06; Fraction 2: 1.060; Fraction 3: 1.073; Fraction 4: 1.097; Fraction 5: 1.129; Fraction 6: 1.153; Fraction 7: 1.179; Fraction 8: 1.184; Fraction 9: 1.219; Fraction 10: 1.228; Fraction 11: 1.247; Fraction 12: 1.271; 1.293.

**Figure 5. Negative-stain electron micrograph of density gradient fractions.**

A. Fraction 1 (apoA-I-free fraction) of ABCA1-transfected, apoA-I added group. B. Fraction 1 of ABCA1-transfected, no acceptor added group. C. Fraction 1 of empty vector-transfected, apoA-I added group. D. Fraction 1 of 0.5% azide-killed HEK293 cells. E. Fraction 1 of labeling media containing BODIPY-cholesterol/cholesterol/cyclodextrin complexes. F. Fraction 3-6 (nHDL fractions) of ABCA1-transfected, apoA-I added group.
Figure 6 Mutant L38G/K40G and WT apoA-I cross-linking to HEK293 cells. A. Cross-linking of apoA-I to HEK293 cells containing different level of cholesterol. The no-apoA-I-added group was subtracted as baseline and WT apoA-I cross-linked to ABCA1-expressing cells with basal cholesterol level was taken as 100%. N=3, ***: P<0.001. B. ABCA1 expression in HEK293 cells containing different levels of cholesterol. ABCA1 was resolved on 4-15% Native-PAGE followed by transfer onto membrane and probing with anti-ABCA1 antibody. Ch: cholesterol. In cholesterol-elevated groups, cells were treated with cholesterol/cyclodextrin complexes (24 µg/ml cholesterol). Under these conditions cellular cholesterol level was elevated by 13.6% ± 1.3%.
Figure 7 Binding of apoA-I to purified ABCA1 in solution. A. Immunoblot of nHDL particles formed from ABCA1-rho1d4-expressing HEK293 cells after 20-24 h incubation. B. SDS-PAGE of rho1d4-antibody-purified ABCA1 from HEK293F cells. Two fractions were eluted from the rho1d4-antibody-conjugated beads by 500 µM rho1d4 peptide. C. Immunoblot (following SDS-PAGE) of elution fractions containing purified ABCA1 probed by anti-ABCA1 antibody. D. Native-PAGE of purified ABCA1 from
HEK293F cells followed by staining with Denville Blue protein stain. E. ATPase activity of amphipol solubilized- ABCA1 at ~ 500 µg/ml concentration. ATP hydrolysis driven by ABCA1 was taken as 100%. Control: basal level ATP self-decay in buffer only. N=3, **: P<0.01. F. ApoA-I binding to ABCA1 solubilized by amphipol. Lane numbers with apostrophe represent the same blot probed with anti-ABCA1 antibody after stripping with 0.1 M glycine, pH = 2. The following protein concentrations were added: apoA-I, 10 µg/ml, ABCA1, 100 µg/ml, ATP, 20 µM.
Figure 8 Model for the mechanism of nHDL biogenesis by apoA-I and ABCA1.

Structure of ABCA1 in the model was based on the cryo-EM structure from 2017 (44).

**ApoA-I-independent pathway:** Step 1. Without apoA-I, ABCA1 at its basal activity translocates cholesterol and other lipids between membrane leaflets, creating bulge-like structures on the membrane and/or release of apoA-I-free particles into the media.

**ApoA-I-dependent pathway:** Step 2. ApoA-I docks to the cell membrane through its hydrophobic C-terminus that was rearranged by ABCA1, while its N-terminus interacts with ABCA1. The N-terminal bundle unfolds and allows apoA-I to dimerize. Step 3. Dimerized apoA-I recruits phospholipid and cholesterol transported by ABCA1 and forms small pre-β nHDL particles. Step 4. Small pre-β nHDL particles gain more lipids, including GM1, from apoA-I-free particles produced in the apoA-I-independent pathway or from membrane bulges via passive diffusion to form larger pre-β nHDL species.