ABCA1 Mediates Unfolding of Apolipoprotein AI N Terminus on the Cell Surface Before Lipidation and Release of Nascent High-Density Lipoprotein

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Objective—To gain insight into the mechanism by which ABCA1 generates nascent high-density lipoprotein.

Approach and Results—HEK293 cells were stably transfected with ABCA1 vectors, encoding wild type, and the W590S and C1477R Tangier disease mutation isoforms, along with the K939M ATP-binding domain mutant. Apolipoprotein AI (ApoAI) binding, plasma membrane remodeling, cholesterol efflux, apoAI cell surface unfolding, and apoAI cell surface lipidation were determined, the latter 2 measured using novel fluorescent apoAI indicators. The W590S isoform had decreased plasma membrane remodeling and lipid efflux activities, and the C1477R isoform had decreased apoAI binding, and lipid efflux activities, whereas the K939M isoform did not bind apoAI, remodel the membrane, or efflux cholesterol. However, all ABCA1 isoforms led to apoAI unfolding at the cell surface, which was higher for the isoforms that increased apoAI binding. ApoAI lipidation was not detected on ABCA1-expressing cells, only in the conditioned medium, consistent with rapid release of nascent high-density lipoprotein from ABCA1-expressing cells.

Conclusions—We identified a third activity of ABCA1, the ability to unfold the N terminus of apoAI on the cell surface. Our results support a model in which unfolded apoAI on the cell surface is an intermediate in its lipidation and that, once apoAI is lipidated, it forms an unstable structure that is rapidly released from the cells to generate high-density lipoprotein. (Arterioscler Thromb Vasc Biol. 2013;33:1197-1205.)

Key Words: ABCA1 ■ apolipoprotein AI unfolding ■ nascent high-density lipoprotein ■ reverse cholesterol transport

A high level of high-density lipoprotein (HDL) is an independent protective factor against cardiovascular disease, which may in part be mediated through the role of HDL in promoting reverse cholesterol transport. The first step in the reverse cholesterol transport pathway is the biogenesis of nascent HDL (nHDL) from extracellular lipid-free apolipoprotein AI (apoAI) and cellular lipids in a process mediated by ABCA1. However, the mechanism of nHDL assembly is not understood at the molecular level. One recent model from Phillips proposes that ABCA1 shuttles phospholipids from the inner to extracellular face of the plasma membrane, resulting in membrane bulges with high curvature that are sufficient to allow apoAI penetration and nHDL formation.

Mutations in ABCA1 lead to Tangier disease and familial hypoalphalipoproteinemia, characterized by very low levels of plasma HDL-cholesterol. Functional studies of certain Tangier disease mutations that are properly trafficked to the plasma membrane demonstrate that ABCA1 seems to have at least 2 distinct activities: apoAI binding and plasma membrane remodeling, the latter demonstrated through phosphatidylserine (PS) translocation to the outer leaflet of the plasma membrane. The W590S mutation in the first extracellular domain of ABCA1 is defective in PS translocation, but competent for apoAI binding. In contrast, the C1477R mutation in the second extracellular domain of ABCA1 is defective in apoAI binding, but competent for PS translocation. Further demonstration of the ability of ABCA1, and the defect of W590S isoform, to remodel the plasma membrane was provided by Nagao et al., who used sodium taurocholate (NaTC) as a weak detergent extracellular lipid acceptor; wild type (WT) but not W590S ABAC1 mediates increased lipid efflux to NaTC. Thus, the lipid translocation and apoAI-binding activities of ABCA1 are independent of each other and seem to be dependent on different extracellular domains of this large membrane protein. Although not discovered in a Tangier disease subject, the K939M mutation in the first ATP-binding domain has been shown to be defective in phosphatidylserine translocation, apoAI binding, and cholesterol efflux.

In addition to nHDL, apoAI can spontaneously form reconstituted HDL (rHDL) particles in vitro when incubated with dimyristoylphosphatidylcholine (DMPC) dispersions or liposomes but not when incubated with the physiologically

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ABCA1 expression. As previously described, WT, W590S, were screened by confocal fluorescence microscopy and flow cytometry to select lines for further study with equivalent levels of ABCA1 expression (Figure 1B). GFP levels in the 4 selected stably transfected cell lines were equivalent, demonstrating similar expression levels of WT, W590S, C1477R, and K939M ABCA1-GFP fusions (Figure 1A). Furthermore, GFP levels in the 4 selected stably transfected cell lines were equivalent, demonstrating similar expression levels of ABCA1 (Figure 1B).

The ability of nontransfected HEK (control) cells and the 3 ABCA1 isoform specific cell lines to bind Alexa647-labeled apoAI at 23°C was measured by flow cytometry (Figure 2A). We confirmed the previously identified apoAI-binding activity of the WT and W590S-ABCA1 isoforms, with both having ≈6-fold higher apoAI binding than the control cells (P<0.001 by ANOVA posttest). In contrast, the C1477R and K939M isoform-expressing cells had no significant increase in apoAI binding compared with control cells (by ANOVA posttest). However, even the control HEK cells bound low levels of apoAI nonspecifically (6.4-fold above background; P<0.001 by t test). We were able to take advantage of this nonspecific apoAI binding to the control and the C1477R and K939M isoform-expressing cells in cell studies described below.

**Results**

Lipid translocation and apoAI-binding activities of ABCA1 are independent of each other and segregate with mutations in the first and second large extracellular domains.

HEK293 cells were stably transfected with different murine ABCA1-green fluorescent protein (GFP) fusion vectors encoding WT, K939M, W590S, and C1477R isoforms, the latter 2 identified as Tangier disease-causing mutations in the first and second large extracellular domains, respectively. Several clonally derived cell lines from each construction were screened by confocal fluorescence microscopy and flow cytometry to select lines for further study with equivalent ABCA1 expression. As previously described, WT, W590S, C1477R, and K939M ABCA1-GFP fusions were processed correctly in cells and expressed on the plasma membrane (Figure 1A). Furthermore, GFP levels in the 4 selected stably transfected cell lines were equivalent, demonstrating similar levels of ABCA1 expression (Figure 1B).

The ability of nontransfected HEK (control) cells and the 3 ABCA1 isoform specific cell lines to bind Alexa647-labeled apoAI at 23°C was measured by flow cytometry (Figure 2A). We confirmed the previously identified apoAI-binding activity of the WT and W590S-ABCA1 isoforms, with both having ≈6-fold higher apoAI binding than the control cells (P<0.001 by ANOVA posttest). In contrast, the C1477R and K939M isoform-expressing cells had no significant increase in apoAI binding compared with control cells (by ANOVA posttest). However, even the control HEK cells bound low levels of apoAI nonspecifically (6.4-fold above background; P<0.001 by t test). We were able to take advantage of this nonspecific apoAI binding to the control and the C1477R and K939M isoform-expressing cells in cell studies described below.

To gain insight into the molecular process by which ABCA1 assembles cellular lipids to generate nHDL, we use the above-mentioned ABCA1 mutations and novel apoAI indicators of apoAI folding and lipidation state. Either the apoAI binding or the plasma membrane remodeling activity of ABCA1 was sufficient to mediate cholesterol efflux to apoAI, albeit at reduced levels versus WT ABCA1, whereas the ATP-binding domain mutant had no efflux activity. The ABCA1 mutant with retained apoAI-binding activity promoted apoAI unfolding at the cell surface to the same extent as WT ABCA1, whereas the mutants in the ATP-binding domain or with defective apoAI binding displayed reduced levels of cell surface apoAI unfolding. Also, we could not detect lipidated apoAI on the surface of ABCA1-expressing cells, implying that once apoAI enters the lipid bilayer, nHDL is rapidly assembled and released from the cell. We discuss the implications of these findings on ABCA1 mechanism and apoAI structure.

**Material and Methods**

Materials and Methods are available in the online-only Supplement.

**Cholesterol Efflux Activities of WT and Mutant ABCA1 Isoforms**

We then examined the cholesterol efflux activity of these cell lines in the absence of exogenous acceptor, in the presence of the ABCA1-specific acceptor apoAI, and in the presence of the nonspecific acceptor NaTC, which is a weak detergent capable of extracting cell membrane lipids (Figure 2C). In the absence of any acceptor, WT ABCA1 increased basal 1H cholesterol efflux by 32% (P<0.05 by ANOVA posttest versus HEK), which has previously been shown to be attributable to increased microparticle release.14 The C1477R-ABCA1 isoform also had increased basal cholesterol efflux activity (34% increase; P<0.05); however, efflux from the W590S-ABCA1 isoform cell line was not significantly different from the control HEK cells. In the presence of apoAI, the HEK cells had basal cholesterol efflux of 0.63%, and the WT ABCA1 cell line had 8.43-fold higher cholesterol efflux
compared with the control HEK cells. Interestingly, both Tangier disease mutations supported partial efflux to apoAI with 4.27- and 4.02-fold increases above the control HEK cells for the W590S and C1477R isoforms, respectively. All 4 cell lines have significantly different efflux to apoAI (P<0.001 by ANOVA posttest), except for efflux from the W590S and C1477R cells that were not different from each other. In a separate study, we found that the K939M cells had efflux to apoAI similar to the nontransfected control HEK cells, thus they had no detectable ABCA1 activity as had been previously determined.5 In the presence of the weak detergent NaTC, the control HEK cells increased cholesterol efflux to 2.62% (a 5.36-fold increase versus absence of acceptor). The WT ABCA1 cell line had 6.21% cholesterol efflux in the presence of NaTC. The W590S-ABCA1 isoform, which can mediate binding of apoAI, yielded a similar cholesterol efflux as the HEK cells at 2.57%. However, the C1477R-ABCA1 isoform yielded 5.04% cholesterol efflux, more similar to the WT ABCA1 isoform. Thus, both in the absence of acceptor or in the presence of NaTC, cholesterol efflux followed PS floppase activity (highest for WT and C1477R isoforms), and we confirmed the use of NaTC acceptor as an independent indicator of ABCA1-mediated membrane remodeling.

Thus, our findings show that ABCA1 mutations that disrupt either plasma membrane remodeling (W590S) or apoAI binding (C1477R) are still competent to mediate cholesterol efflux to apoAI, albeit at reduced efficiency.

**ABCA1 Mediates N-Terminal Unfolding of ApoAI on the Cell Surface**

We designed a novel apoAI indicator to follow apoAI unfolding based on the N-terminal hairpin fold shown in the x-ray crystal structure of the C-terminal–deleted human apoAI.15 We mutated L38 and M112, predicted to be separated by 6.4 angstroms (Figure II in the online-only Data Supplement), to cysteine residues, and labeled these residues with the proximity self-quenching fluorophore Bodipy-tetramethylrhodamine (TMR). To use this indicator independent of apoAI concentration, we also lightly labeled the lysine residues with Alexa647, allowing us to measure the Bodipy-TMR/Alexa647 ratio as an indicator of apoAI unfolding. We validated this indicator by guanidine denaturation. ApoAI has 4 tryptophan residues, which are largely protected from the aqueous environment, and aqueous exposure of the tryptophan residues induced by increasing concentrations of guanidine can be assessed by a red shift in the peak fluorescent emission wavelength. As the guanidine concentration was increased from 0 to 3 mol/L, Bodipy-TMR/Alexa647-labeled apoAI exhibited a wavelength of maximum fluorescence shift from 342.4 to 352.9 nm, with an EC50 of 1.6 mol/L guanidine (Figure 3A). We also measured the Bodipy-TMR/Alexa647 ratio and found that increasing guanidine increased this ratio from 0.117 to 0.218, with an EC50 of 1.8 mol/L guanidine (Figure 3B). The observed difference in guanidine sensitivity is minor, and the different shape of these 2 curves may represent the altered exposure of the 4 tryptophan residues versus the separation of the dyes at positions 38 and 112. A comparison of the guanidine denaturation of WT apoAI, the 38/112 double cysteine mutant, and the labeled double mutant is shown in Figure VI in the online-only Data Supplement.

We next investigated whether N-terminal unfolding occurs on apoAI lipidation. The apoAI unfolding indicator was incubated with either DMPC or POPC multilamellar vesicles, because apoAI is known to spontaneously form rHDL with DMPC, but not with POPC. Over a 45-minute time course,
incubation of the unfolding indicator with DMPC led to an increase in the Bodipy-TMR/Alexa647 ratio from 0.14 to 0.37 (2.64-fold increase), whereas incubation with POPC failed to increase this ratio (Figure 3C). In a control study, we determined that the free Bodipy-TMR fluorophore was not sensitive to a hydrophobic environment by measuring fluorescence intensity in increasing concentrations of methanol (data not shown). Thus, we demonstrated that the N terminus of apoAI unfolds when apoAI is lipidated to form HDL.

The apoAI unfolding indicator was then incubated with live cells at 23°C (binding occurs without internalization) to determine whether the apoAI N terminus is unfolded on the cell surface, and whether this activity is ABCA1 dependent. Here, we took advantage of the high sensitivity of flow cytometry and our observation that even control HEK cells and cells expressing the apoAI-binding defective C1477R and K939M-ABCA1 isoforms bound apoAI nonspecifically, enabling us to determine the Bodipy-TMR/Alexa647 ratio of each cell. Incubation of Bodipy-TMR/Alexa647-labeled apoAI with control HEK cells yielded a cellular Bodipy-TMR/Alexa647 ratio peak of 0.7 (Figure 4A). However, on incubation of this indicator with the WT and W590S ABCA1-expressing cells, there was a rightward shift to a higher cellular Bodipy-TMR/Alexa647 ratio peak of 1.3 to 1.4 (1.86–2.0-fold increase). The C1447R and K939M apoAI-binding deficient mutants displayed intermediate activity with Bodipy-TMR/Alexa647 ratio peaks of 1.0 (1.43-fold increase versus control HEK cells). Thus, WT ABCA1 has 3 activities: apoAI binding, apoAI unfolding, and plasma membrane remodeling. The partially active W590S isoform has 2 activities: apoAI binding and apoAI unfolding. The other partially active C1477R isoform has 2 activities: apoAI unfolding (albeit reduced) and plasma membrane remodeling. Finally, the defective K939M isoform retains only one activity: apoAI unfolding (also reduced). We repeated this study using RAW264.7 macrophages in which endogenous ABCA1 expression is dependent on induction by cyclic adenosine monophosphate analogs.16 We observed a 1.5-fold increase in the peak Bodipy-TMR/Alexa647 ratio in ABCA1-induced versus control-treated RAW264.7 cells (Figure 4B), thus validating this cell surface apoAI unfolding assay. One caveat of these findings is that we cannot determine the fraction of apoAI that is unfolded on the cell surface by use of apoAI unfolding indicator, because we cannot make a standard curve of differentially unfolded apoAI on the cell surface for detection by flow cytometry. However, we can roughly estimate this fraction by examining the fold increase in the Bodipy-TMR/Alexa647 ratio in the condition that yielded the highest, 2.64-fold increase, the formation of rHDL by incubation with DMPC (Figure 3C). We observed a range of 1.4- to 2.0-fold increase in the fluorescence ratio on the surface of ABCA1-expressing cells. This corresponds to an estimated range of ≈53% to 76% of unfolded apoAI on the cell surface. However, the confidence of this estimate is limited because it is based on fluorescent measurements by 2 different instruments (flow cytometer and fluorescence plate reader), and thus the baseline fluorescence ratios are not comparable, although the fold increase in the ratios on each instrument may still be worth comparing.

We also examined the Bodipy-TMR/Alexa647 ratio, by spectrofluorometry, in the conditioned media after incubation for 10 hours of the apoAI unfolding indicator with control HEK cells and cells transfected with the WT and mutant ABCA1 isoforms (Figure 4C). Medium recovered from control HEK cells and the defective K939M-ABAC1 isoform...
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**ABCA1 Mediates apoAI N-Terminal Unfolding**

had Bodipy-TMR/Alexa647 ratios that were not different from the ratio of medium conditioned in the absence of cells (not significant by ANOVA posttest). Medium recovered from the WT ABCA1 cell line had a 1.8-fold higher Bodipy-TMR/Alexa647 ratio compared with the control HEK cells ($P<0.001$ versus control cells by ANOVA posttest). The W590S and C1447R mutant isoforms had intermediate ratios ≈1.4 and 1.2-fold higher than that observed from the control HEK cells ($P<0.001$ and $P<0.05$, respectively, versus control cells by ANOVA posttest). Thus, the Bodipy-TMR/Alexa647 ratio of the conditioned media increased only in cells capable of mediating efflux to apoAI, with the highest increase for WT ABCA1-expressing cells, which also have the highest cholesterol efflux to apoAI (Figure 2C). We attribute the intermediate fluorescence ratio levels in the conditioned media from the W590S and C1447R transfected cells to be a result of fewer lipidated particles released into the conditioned media, rather than to less unfolding of apoAI per particle.

**Lipidated ApoAI Not Detected at the Surface of ABCA1-Expressing Cells**

The apoAI unfolding indicator demonstrated N-terminal unfolding of apoAI on the surface of ABCA1-expressing cells; however, this indicator could not distinguish whether or not this unfolded protein was lipidated on the cell surface. To investigate this, we created a ratiometric fluorescent apoAI lipidation indicator using the lipid sensitive and insensitive dyes 7-nitrobenz-2-oxa-1,3-diazole (NBD) and Alexa647, respectively. This lipidation indicator was validated through a time course incubation with DMPC and POPC vesicles, yielding a large increase in the NBD/Alexa647 ratio from 0.141 to 0.797 (5.65-fold increase) for DMPC, but no increase for POPC (Figure 5A).

To determine whether apoAI is lipidated on the surface of ABCA1-expressing cells, we used RAW264.7 cells because the emission spectrum of NBD overlaps with that of GFP fluorescence (present in the HEK cells expressing ABCA1-GFP fusion proteins). RAW264.7 cells, pretreated in the presence or absence of 8-Br-cyclic adenosine monophosphate, were incubated with or without the fluorescent apoAI lipidation indicator, and the fluorescence intensity of NBD and Alexa647 on the cell surface was measured by flow cytometry (Figure 5B). After subtracting the background in the absence of the apoAI indicator, the ABCA1-induced RAW264.7 cells incubated with the apoAI lipidation indicator, compared with these uninduced cells, had 3.0-fold increase in Alexa647 fluorescence, whereas the NBD fluorescence only increased by 2.30-fold. Had the apoAI indicator been lipidated at the surface of the induced RAW264.7 cells, we should have observed a larger fold increase in the NBD fluorescence versus the Alexa647 fluorescence; and, because this was not observed, we find no evidence for the accumulation of lipidated apoAI on the surface of ABCA1-expressing cells. In contrast, the conditioned media from ABCA1-induced RAW264.7 cells incubated with the lipidation indicator for 24 hours had a significantly higher NBD/Alexa647 ratio than the media conditioned with uninduced cells, the latter of which was not different from media conditioned in the absence of cells (Figure 5C). The Alexa647 fluorescence was examined to evaluate total apoAI recovery in the conditioned media, and although incubation of the fluorescent apoAI lipidation indicator with cells for 24 hours led to 20% decrease in Alexa647 recovery, there was no difference from the WT ABCA1 cell line that had a 1.8-fold higher Bodipy-TMR/Alexa647 ratio compared with the control HEK cells ($P<0.001$ versus control cells by ANOVA posttest). The W590S and C1447R mutant isoforms had intermediate ratios ≈1.4 and 1.2-fold higher than that observed from the control HEK cells ($P<0.001$ and $P<0.05$, respectively, versus control cells by ANOVA posttest). Thus, the Bodipy-TMR/Alexa647 ratio of the conditioned media increased only in cells capable of mediating efflux to apoAI, with the highest increase for WT ABCA1-expressing cells, which also have the highest cholesterol efflux to apoAI (Figure 2C). We attribute the intermediate fluorescence ratio levels in the conditioned media from the W590S and C1447R transfected cells to be a result of fewer lipidated particles released into the conditioned media, rather than to less unfolding of apoAI per particle.

**Figure 4.** ABCA1 mediates apoAI unfolding on the cell surface. A, Control HEK or stably transfected ABCA1-green fluorescent protein (GFP) cells were incubated with 1 μg/mL Bodipy-tetramethylrhodamine (TMR)/Alexa647-labeled apoAI at room temperature followed by flow cytometry to measure the Bodipy-TMR/Alexa647 ratio as indication of apoAI unfolding. Plot shows the frequency histogram of the Bodipy-TMR/Alexa647 ratio for the different cell lines (representative of 3 similar assays). B, RAW264.7 cells were incubated in the absence or presence of 0.3 mmol/L 8-Br-cyclic adenosine monophosphate (cAMP) to induce ABCA1 expression, and cell surface apoAI unfolding as determined by the Bodipy-TMR/Alexa647 ratio was assayed as described above (representative of 3 similar assays). C, ApoAI unfolding indicator was incubated without cells or with control HEK or stably transfected ABCA1-GFP cells for 10 hours, and the Bodipy-TMR/Alexa647 ratio of the conditioned media was measured (n=3; mean±SD; different numbers above the bars show $P<0.05$, by ANOVA posttest).
between the amount of Alexa647 fluorescence in the conditioned media from cells in the presence or absence of ABCA1 induction by 8-Br-cyclic adenosine monophosphate (Figure VII in the online-only Data Supplement). Thus, these data show that lipidated apoAI is below the level of detection on the cell surface but detectable in the media. These data imply that once apoAI is lipidated on the cell surface, it is immediately released into the medium as nHDL.

**Discussion**

ABCA1 is a large protein with 12 transmembrane domains and 2 large extracellular domains between transmembrane segments 1 and 2, and 7 and 8, respectively.7,17 Although ABCA1 can be specifically cross-linked to apoAI, its substrate for nHDL assembly, the site of cross-linking of apoAI on ABCA1 has not been identified. We chose to further study 2 specific mutations in the first (W590S) and second (C1477R) extracellular domains, respectively, based on their previously identified distinct activities. Fitzgerald et al17 examined 5 Tangier disease mutations that mapped to the 2 large extracellular domains, and reported that only the W590S mutation in the first extracellular domain was still competent to mediate apoAI cross-linking, whereas other mutations in the first (R587W and Q597R) and second (C1477R and S1506L) extracellular domains could not mediate apoAI cross-linking. Although the flag-tagged R587W and Q597R variants were reported to be expressed on the plasma membrane in transfected cells,17 2 other independent groups reported that these 2 variants have impaired processing and decreased cell surface expression5,8,18; but all agree that the W590S is expressed on the plasma membrane similarly to the WT isoform and can mediate apoAI binding. Our findings reproduce that the W590S isoform has plasma membrane localization and WT levels of apoAI-binding activity; and that it has partial cholesterol efflux activity to extracellular apoAI compared with the WT isoform, as previously demonstrated.5,7,8,18

We chose to study the C1477R mutation in the second extracellular domain because it is processed correctly to the plasma membrane and has defective apoAI binding,5,7,17 but it retains its PS translocase activity and partial efflux activity,7 all findings that we confirmed in the current study, where we found ≈50% cholesterol efflux activity to apoAI. Nagao et al8 first demonstrated the use of NaTC as a nonpeptide acceptor of cellular lipids, and that WT ABCA1 increased lipid efflux to this weak detergent, and that the W590S mutation abolished this activity. Here, we compared the acceptor activity of NaTC for cells expressing WT, W590S, and C1477R-ABCA1 isoforms, and found that the C1477R mutant has equivalent efflux to NaTC as the WT isoform, whereas the W590S mutant has no detectable efflux to NaTC above nontransfected cells. The NaTC efflux activities of these ABCA1 isoforms is similar to the PS translocase activity, and thus both of these assays are evidence that the WT and C1477R isoforms can remodel the plasma membrane, whereas the W590S isoform is mostly deficient in this activity. The simplest explanation for these findings is that the first extracellular domain is critical for plasma membrane remodeling, whereas the second extracellular domain is critical for apoAI binding, although the presence of interdomain disulfide bonds is a caveat to these attributed activities. However, neither activity is fully required for cholesterol efflux to apoAI, because both of these isoforms retain partial activity.

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Lipidated apoAI is not detectable on the surface of ABCA1-expressing cells. A. Characterization of NBD/Alexa647 doubly labeled fluorescent ratiometric apoAI lipidation indicator. Evidence of apoAI indicator lipidation during spontaneous nHDL formation after incubation with dimyristoylphosphatidylcholine (DMPC) but not palmitoyloleoylphosphatidylcholine (POPC) multilamellar vesicles (MLVs; lipid:apoAI mole ratio = 200:1) as detected by an increased NBD/Alexa647 ratio. B, RAW264.7 cells with or without 8-Br-cAMP pretreatment were incubated with or without 1 μg/mL NBD/Alexa647-labeled apoAI at room temperature followed by flow cytometry to measure the NBD and Alexa647 fluorescence (n=3; mean±SD). Background fluorescence in the absence of the apoAI indicator was subtracted from both channels. If apoAI was lipidated on the cell surface, then a larger fold increase in NBD vs Alexa647 fluorescence would be expected. C, ApoAI lipidation indicator was incubated with RAW264.7 cells pretreated in the presence or absence of 8-Br-cAMP for 24 hours, and the NBD/Alexa647 ratio of the conditioned media was measured (n=3; mean±SD; different numbers above the bars show P<0.01, by ANOVA posttest).
Through the use of an N-terminal apoAI unfolding indicator, we observed what we estimate to be \( \approx 75\% \) unfolding of apoAI on the surface of HEK cells expressing either WT or W590S-ABCA1 isoforms, both of which have full apoAI-binding activity (Figure 4A). The slight rightward shift of the W590S isoform may be as a result of defective membrane remodeling in the W590S-expressing cells, leading to the unfolded apoAI having fewer opportunities to form rHDL and be released from the cell. Interestingly, the 2 ABCA1 isoforms with impaired apoAI binding, C1447R and K939M, also displayed some, albeit reduced, apoAI unfolding activity compared with nontransfected HEK cells (Figure 4A). Because the K939M isoform is also deficient in plasma membrane remodeling, this partial unfolding activity cannot be attributed to this membrane remodeling. We speculate that this apoAI unfolding activity is a third distinct activity of ABCA1. We also speculate that function of ABCA1 to be increased by, but does not require, the high-affinity apoAI binding. This apoAI unfolding activity of ABCA1 could be attributable to the presence of a separate low-affinity apoAI-binding site, not distinguishable from the nonspecific binding observed in cells lacking ABCA1 expression. This low-affinity site on ABCA1 could transiently interact with apoAI and act as a chaperone to mediate apoAI N-terminal unfolding. The presence of the high-affinity apoAI-binding site in WT and W590S-ABCA1 isoforms would promote apoAI proximity to the low-affinity binding site and therefore increase apoAI unfolding.

The finding of unfolded apoAI on the cell surface of ABCA1-expressing cells may help us understand the features of apoAI structure and function, as well as about the mechanism of ABCA1-mediated nHDL assembly. The transformation of lipid-free apoAI into nHDL, or rHDL in cell-free systems, is accompanied by many changes in apoAI structure, including increased helicity, measured by circular dichroism, changes in apoAI intramolecular and intermolecular cross-linking, and alignment of specific amino acid residues determined by fluorescence resonance energy transfer. Our proximity quenching apoAI unfolding indicator data show that during rHDL formation from apoAI and DMPC vesicles there is an unfolding of the N-terminal hairpin, separating residues 38 and 112 from each other. Furthermore, our data show that ABCA1 mediated apoAI unfolding on the cell surface, without the detection of apoAI lipidation on the cell surface, implying that there is an unfolded apoAI intermediate in nHDL formation.

Although the x-ray crystal structure of holo-apoAI was published, serious doubts have been raised about its validity; however, an N-terminal hairpin is clear from the x-ray structure of the C-terminal-deleted apoAI. The roles of the N and C termini in apoAI for rHDL and cellular nHDL biogenesis have been well studied, showing that (1) the WT and N-terminal deletions can form rHDL and nHDL; (2) the C-terminal deletion is not competent to form rHDL or nHDL; and (3) the double N- and C-terminal deletion restores HDL formation capacity. In addition, our previous equilibrium and kinetic analysis of apoAI folding and unfolding in guanidine hydrochloride offers some insight into the roles of these termini. Compared with the holoprotein, the C-terminal deletion unfolds in 2.5 mol/L guanidine at about half the rate, whereas the N-terminal deletion unfolds at 1.5× the rate, and the double deletion unfolds at \( \approx 1.3\times \) the rate. Thus, the N terminus is a strong stabilizing feature of the holoprotein, whereas the C terminus is a destabilizing feature. Thus, the deletion of the C terminus would inhibit apoAI unfolding that is required for rHDL and nHDL formation. In the double deletion, apoAI unfolding occurs unhampered, allowing HDL formation. Our detection of ABCA1-mediated N-terminal unfolded, but nonlipidated, apoAI on the cell surface points to the critical role that this unfolding may play to permit apoAI lipidation. Previously, Remaley has shown that class-A amphipathic helical peptide mimetics of apoAI possess ABCA1-dependent lipid acceptor activity; however, unlike apoAI that only has ABCA1-dependent acceptor activity, these peptides at high concentrations have nonspecific detergent activity and can strip lipids from cells in the absence of ABCA1 expression. We speculate that apoAI (and other exchangeable apolipoproteins) and ABCA1 have coevolved to minimize their nonselective detergent activity, and only expose their amphipathic helices to cells in the presence of ABCA1, which can specifically catalyze apolipoprotein unfolding. ApoAI is one of the most abundant plasma proteins (1–2 mg/mL), and if its detergent like activity was promiscuous, then it could lead to cellular membrane disruption; thus, the ABCA1 dependency of its detergent activity allows membrane lipid efflux to be tightly regulated.

A model for the mechanism of ABCA1 action from Phillips states that phospholipid translocation produces membrane protuberances that in themselves because of their small radius and surface packing are sufficient to spontaneously interact with apoAI and form nHDL. However, the observation that the W590S-ABCA1 isoform is not competent for phospholipid translocation and membrane remodeling but is still able to mediate HDL assembly, albeit at reduced efficiency, does not support this model. Instead, we propose that ABCA1 has 3 distinct activities and that each plays a role in catalyzing the formation of nHDL from lipid-free apoAI. ABCA1 was previously known to mediate apoAI binding and PS translocation/plasma membrane remodeling, and our findings add a novel activity of ABCA1, the ability to unfold the N terminus of apoAI on the cell surface. It is of interest that both the apoAI binding and the plasma membrane remodeling activities are disrupted in the K939M isoform without totally abolishing this apoAI unfolding activity. However, either the apoAI binding or plasma membrane remodeling activity is required to observe some apoAI lipidation and nHDL production.

We propose an apoAI reaction coordinate model to illustrate mechanistic insights from our findings, although the free energy levels are not data based (Figure 6). The initial state of lipid-free apoAI is in its folded (F) state. On binding specifically or nonspecifically to ABCA1-expressing cells, ABCA1 catalyzes the unfolding of apoAI into an intermediate (Int) state that can spontaneously resolve to a cell surface unfolded state (U). This U state is relatively stable and can be easily detected on ABCA1-expressing cells. The levels of apoAI in the Int and U states are increased by specific binding to ABCA1, but these apoAI states are still present in cells expressing the C1447C and K939M-ABCA1 isoforms that lack high-affinity apoAI binding. The next transition is
However, this L\textsubscript{C} state is not stable and rapidly resolves deleted apoAI versus the holoprotein, which may explain acyl chains of the plasma membrane to yield the lipidated cell sharply uphill and leads to the insertion of apoAI into the fatty apoAI; Int, intermediate in ABCA1-mediated apoAI unfolding; LC, mediated nascent HDL biogenesis. F indicates folded lipid-free apoAI on the cell surface; nHDL, nascent HDL released from the cell; and U, unfolded apoAI on the cell surface. Free energy levels are arbitrary and not data based.

Figure 6. Model of apoAI reaction coordinate during ABCA1-mediated nascent HDL biogenesis. F indicates folded lipid-free apoAI; Int, intermediate in ABCA1-mediated apoAI unfolding; L\textsubscript{C}, lipidated apoAI on the cell surface; nHDL, nascent HDL released from the cell; and U, unfolded apoAI on the cell surface. Free energy levels are arbitrary and not data based.

In conclusion, we identified a new activity of ABCA1, namely the ability to unfold the N terminus of apoAI on the cell surface. We also observed that lipidated apoAI is not easily detectable on the cell surface. We demonstrate that ABCA1 mutants with defects in apoAI binding or plasma membrane remodeling are still partially or totally competent, respectively, to unfold apoAI, and to promote lipid efflux at reduced efficiency, thus showing that plasma membrane remodeling is not absolutely required for ABCA1 activity. Together, these findings increase our insight into the mechanism of apoAI lipidation. Our findings suggest the importance of apoAI unfolding in its cellular lipidation may have implications for the roles of the N and C termini of apoAI in mediating finely regulated lipid efflux.

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Disclosures
None.

References
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Significance

ABCA1 mediates the assembly of lipid-free apolipoprotein Al (apoAI) with cellular phospholipids and cholesterol to generate nascent high-density lipoprotein that is released from the cells in the first step of the reverse cholesterol transport pathway; however, the mechanism of high-density lipoprotein assembly is not known. ABCA1 was previously known to have 2 distinct activities, specific binding of apoAI and plasma membrane remodeling with increased cell surface phosphatidylserine. We demonstrate here a novel third activity of ABCA1, the ability to unfold the N terminus of apoAI on the cell surface. After unfolding, lipidated apoAI is not detectable on the cell surface, implying that lipidated apoAI in the cell membrane is an unstable intermediate that is rapidly released as nascent high-density lipoprotein. Thus, these studies give new insights into ABCA1 activities and the mechanism of high-density lipoprotein biogenesis.
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To make the apoAI lipidation indicator, human apoAI was doubly-labeled on free amines with NBD, whose fluorescence increases in a hydrophobic environment, and Alexa647. Human apoAI was dissolved in 0.1M sodium bicarbonate buffer and incubated with a 6:1 dye:protein mole ratio of NBD chloride (Molecular probes, dissolved in DMSO) and Alexa647 carboxlic acid succinimidyl ester (Molecular Probes, dissolved in DMSO) at for 1 hour at room temperature. The reaction was stopped by adding 0.1ml of freshly prepared 1.5M hydroxylamine, pH8.5, and further incubation for 1 hour. The conjugate was purified by extensive dialysis. The fluorescence ratio of NBD (excitation at 460 nm and emission at 540nm) to Alexa647 serves as an indicator of apoAI lipidation. The NBD/Alexa labeled apoAI lipidation indicator retained full cholesterol acceptor activity compared to unmodified apoAI, as shown in Supplemental Figure V.

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