Mechanism of ATP-binding Cassette Transporter A1-mediated Cellular Lipid Efflux to Apolipoprotein A-I and Formation of High Density Lipoprotein Particles*

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The ATP-binding cassette transporter A1 (ABCA1) plays a critical role in the biogenesis of high density lipoprotein (HDL) particles and in mediating cellular cholesterol efflux. The mechanism by which ABCA1 achieves these effects is not established, despite extensive investigation. Here, we present a model that explains the essential features, especially the effects of ABCA1 activity in inducing apolipoprotein (apo) A-I binding to cells and the compositions of the discoidal HDL particles that are produced. The apo A-I/ABCA1 reaction scheme involves three steps. First, there is binding of a small regulatory pool of apo A-I to ABCA1, thereby enhancing net phospholipid translocation to the plasma membrane exofacial leaflet; this leads to unequal lateral packing densities in the two leaflets of the phospholipid bilayer. Second, the resultant membrane strain is relieved by bending and by creation of exovesiculated lipid domains. The formation of highly curved membrane surface promotes high affinity binding of apo A-I to these domains. Third, this pool of bound apo A-I spontaneously solubilizes the exovesiculated domain to create discoidal nascent HDL particles. These particles contain two, three, or four molecules of apo A-I and a complement of membrane phospholipid classes together with some cholesterol. A key feature of this mechanism is that membrane bending induced by ABCA1 lipid translocase activity creates the conditions required for nascent HDL assembly by apo A-I. Overall, this mechanism is consistent with the known properties of ABCA1 and apo A-I and reconciles many of the apparently discrepant findings in the literature.

Since the discovery that mutations in the ABCA12 gene are responsible for Tangier disease and low circulating levels of HDL, the ABCA1 system has been the subject of extensive research activity. Much has been learned about the ways in which this membrane transporter contributes to the biogenesis of HDL particles (1–7). HDL is anti-atherogenic, at least in part, because it can participate in reverse cholesterol transport, the process by which excess cholesterol in peripheral cells is returned to the liver for catabolism (5, 6, 8–10). It is now apparent that liver and intestinal ABCA1 are responsible for producing essentially all circulating HDL particles (11, 12). However, ABCA1 expressed in macrophages is critical for mediating the efflux of excess cholesterol from the cells and preventing the formation of the foam cells characteristic of fatty streak atherosclerotic lesions (7, 13). Despite the intense interest, a major question that has remained unresolved is the mechanism by which ABCA1 interacts with apo A-I, the principal protein of HDL, to remove cellular lipids and create nascent HDL particles. Knowledge of this mechanism will provide a basis for systematic manipulation of the process.

The mechanism of the active ABCA1-mediated efflux of cellular lipids to apo A-I has been proposed variously to involve 1) binding of apo A-I either directly to ABCA1 or indirectly to a lipid site created by ABCA1 activity, 2) either simultaneous or sequential release of membrane phospholipid and cholesterol to apo A-I, and 3) assembly of nascent HDL particles either at the cell surface or at intracellular sites during the retroendocytosis of ABCA1 (for reviews see Refs. 2–5, 9). More recent models have centered on a two-step mechanism in which there is initial formation of an apo A-I complex followed by lipidation of this bound apo A-I and dissociation of the resultant apo A-I/lipid complex (14–17). However, at this stage, a generally accepted model for the mechanism by which apo A-I/ABCA1 interaction creates nascent HDL particles does not exist. Here we propose a reaction scheme for the ABCA1/apo A-I system that integrates key findings from the literature and our laboratory. A central feature of this mechanism is that membrane phospholipid translocation via ABCA1 induces bending of the membrane bilayer to create high curvature sites to which apo A-I can bind and solubilize membrane phospholipid and cholesterol to create nascent HDL particles. Experimental evidence that supports the novel features of the model is presented.

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2 The abbreviations used are: ABCA1, ATP-binding cassette transporter A1; apo, apolipoprotein; DMPC, dimyristoyl phosphatidylcholine; HDL, high density lipoprotein; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, phosphatidylcholine; SUV, small unilamellar vesicles; WT, wild type.
Mechanism of ABCA1-mediated Lipid Efflux to Apo A-I

EXPERIMENTAL PROCEDURES

Apo A-I—Apo A-I was isolated from human plasma HDL as described before (18). The construction of the plasmids for expressing WT apo A-I and its mutants and the isolation and purification of these proteins have been described before (19, 20). Apo A-I samples were trace radiolabeled by reductive methylation with [14C]formaldehyde (21).

Cellular Cholesterol Efflux—Cholesterol efflux to apo A-I and its variants from monolayers of J774 mouse macrophages in which ABCA1 expression was up-regulated was measured as described previously (16, 21, 22).

Immunogold Labeling and Electron Microscopy—Immunogold labeling of J774 cells was performed essentially as described previously (23). Briefly, J774 cells in which ABCA1 was up-regulated with cAMP and control cells were incubated with apo A-I (10 μg/ml) at 37 °C for 4 h, after which they were washed with phosphate-buffered saline and pre-fixed with paraformaldehyde (4%) in phosphate-buffered saline (pH 7.4) for 30 min at room temperature. After washing with phosphate-buffered saline, the cells were treated with either goat anti-human apo A-I serum (Academy Bio-Medical Company) or goat anti-human apo A-I antibody (Novus Biologicals) overnight at 4 °C. The cells were then treated with donkey anti-goat IgG conjugated with 10 nm colloidal gold (Aurion) for 1 h at room temperature and post-fixed with 2.5% glutaraldehyde for 2 h. The fixed cells were then dehydrated and embedded with EPON, and ultrathin sections were stained with uranyl acetate and examined in a transmission electron microscope.

Binding of Apo A-I to Phospholipids—The binding isotherms for apo A-I to egg PC unilamellar vesicles were determined by gel filtration chromatography as described (20, 24). Approximately 20-nm-diameter SUV were prepared by sonication (24), and 100- or 200-nm-diameter LUV were prepared by extrusion through polycarbonate filters (25). Enthelopes of apo A-I binding to unilamellar egg PC vesicles were measured by isothermal titration calorimetry as described (20, 24).

Solubilization of Phospholipid Vesicles by Apo A-I—The kinetics of solubilization of DMPC MLV by apo A-I and its variants at 24 °C to form small discoidal apo A-I/PC complexes were measured by monitoring the decreases in absorbance at 325 nm, as described before (26). The solubilization by apo A-I at 37 °C of MLV prepared from a membrane phospholipid mixture was monitored in a similar fashion. To mimic the mixture of membrane lipids incorporated into the nascent HDL particles created by the activity of ABCA1 in J774 cells (22), the following lipids (purity >99%) were purchased from Avanti Polar Lipids (Birmingham, AL) and used to prepare MLV. The membrane lipid mixture comprised: 55% (w/w total phospholipid) bovine liver PC, 12% porcine brain sphingomyelin, 5% egg lyso PC, 10% bovine liver phosphatidylethanolamine, 8% porcine brain phosphatidylserine, 10% bovine liver phosphatidylinositol. Cholesterol was added at the level of 8% w/w total phospholipids. This membrane lipid mixture was dissolved in chloroform, and after removal of the solvent, the lipids were dispersed as multilamellar vesicles in deoxygenated Tris-buffered saline (10 mM Tris, 0.01% EDTA, 0.15 M NaCl, 0.01% sodium azide, pH 7.4) by vortexing and stored at 4 °C overnight under nitrogen before use in the solubilization assay. The incubations of MLV with apo A-I were conducted under nitrogen.

RESULTS AND DISCUSSION

Because it is impossible to define the mechanism of an enzyme reaction without defining the products of such a reaction, we have previously performed a detailed characterization of the products of the ABCA1/apo A-I interaction using both the J774 macrophage cell line and primary human skin fibroblasts (21, 22). These products include discoidal HDL particles containing two, three, or four apo A-I molecules/particle. The nascent HDL particles are heterogeneous with respect to size and lipid content, and their phospholipids comprise all of the major classes present in mammalian cell membranes. In addition, some monomeric apo A-I species associated with three to four phospholipid and one to two cholesterol molecules can be produced. Any proposed apo A-I/ABCA1 reaction scheme has to be consistent with the formation of the above products. Another important requirement of a model of the reaction is that it accounts for the ABCA1-induced binding of apo A-I to cells. We have recently resolved some of the ambiguities in the literature on this topic by showing that ABCA1 activity creates two cell surface binding sites for apo A-I (27). Binding occurs to a low capacity site created by direct apo A-I/ABCA1 interaction and also to a much higher capacity site that involves apo A-I/lipid interactions. The existence of these two binding sites implies that, contrary to current thought, the binding of apo A-I to ABCA1 and the binding of lipid molecules to apo A-I are uncoupled events. A successful reaction scheme has to account for these important features.

We believe that the integrated reaction scheme summarized in Fig. 1 explains the above phenomena and is consistent with the known properties of ABCA1 and apo A-I. Furthermore, as will be argued below, there is strong experimental evidence supporting the novel features of the model.

Step 1: Apo A-I Binding to ABCA1—Cross-linking studies from several laboratories have established that apo A-I can bind directly to the ABCA1 molecule when the latter is in its active conformation (14, 15, 27–29). The binding site on ABCA1 is apparently a hydrophobic region in its largest extracellular loop (30). The motif in apolipoproteins that is recognized by this site is apparently the amphipathic α-helix, which explains the low specificity of the apolipoprotein/ABCA1 interaction. In the case of human apo A-I, the dissociation constant Kd of binding as measured in several laboratories is in the range of 10^{-7} M (14, 27, 31). An important consequence of the binding of apo A-I to ABCA1 is that the transporter becomes stabilized at the cell surface because of signaling responses mediated by Janus kinase 2 (32, 33) and modulations in phosphorylation that inhibit ABCA1 degradation by caspases (32, 34–36). The active form of ABCA1 seems to be oligomeric (37, 38),

3 Duong, P. T. and Phillips, M. C., unpublished observations.
Mechanism of ABCA1-mediated Lipid Efflux to Apo A-I

Step 1: Apo A-I Binding to ABCA1

The reaction in which apo A-I binds to ABCA1 and membrane lipids to create discoidal nascent HDL particles contains three steps. Step 1 involves the high affinity binding of a small amount of apo A-I to ABCA1 located in the plasma membrane phospholipid bilayer; this regulatory pool of apo A-I up-regulates ABCA1 activity, thereby enhancing the active translocation of membrane phospholipids from the cytoplasmic to exofacial leaflet. This translocase activity leads to lateral compression of the phospholipid molecules in the exofacial leaflet and expansion of those in the cytoplasmic leaflet. Step 2 involves the bending of the membrane to relieve the strain induced by the unequal molecular packing density across the membrane and the formation of an exovesiculated domain to which apo A-I can bind with high affinity. This interaction with the highly curved membrane surface involves apo A-I/membrane lipid interactions and creates a relatively large pool of bound apo A-I. Step 3 involves the spontaneous solubilization by the bound apo A-I of membrane phospholipids and cholesterol in the exovesiculated domain to create discoidal HDL particles containing two, three, or four apo A-I molecules/particle. See text for further details.

Although this self-association is apparently unaffected by apo A-I binding (37).

Any stabilization of ABCA1 in the cell plasma membrane will lead to an enhancement of net phospholipid translocase activity. By analogy to bacterial ABC lipid transporters, the active translocation of phospholipid occurs in a chamber created by the two-transmembrane helical domains in the ABCA1 molecule (Fig. 1). The preferred substrate for translocation by ABCA1 has not been established unambiguously, although it is clear that phosphatidylserine can be transported (39–41). ABCA1 translocase activity also leads to an increase in non-raft microdomains in the plasma membrane (42) where cholesterol becomes accessible to cholesterol oxidase and removal by apolipoproteins (43). Regardless of the exact nature of the lipid molecules translocated, the net accumulation of lipid molecules in the exofacial leaflet of the membrane will lead to lateral compression of the phospholipid molecules in this half of the bilayer (Fig. 1). Concomitantly, the net depletion of phospholipid molecules in the cytoplasmic leaflet will lead to expansion of the phospholipid lateral packing. The resultant asymmetric phospholipid packing across the bilayer created by local ABCA1 activity induces strain in the membrane that has to be relieved.

Step 2: Apo A-I Binding to Exovesiculated Membrane Domains—The strain in the membrane induced by phospholipid translocation can be relieved by bending of the phospholipid bilayer. Given that the direction of ABCA1-induced net phospholipid transport in the membrane is to the exofacial leaflet, it follows that the lateral phospholipid packing density in the two halves of the membrane can be equalized by bulging of the membrane in the direction of the extracellular space. The differences in radius of curvature of the inner and outer bilayer leaflets in a highly curved exovesiculated domain will relieve the membrane strain induced by ABCA1 phospholipid translocase activity (see Step 2 in Fig. 1). In agreement with this concept, plasma membrane protrusions have been observed in cells expressing ABCA1 (28). It is noteworthy that the perturbed lipid environment associated with the exovesiculated domain is not a direct result of phospholipid translocation via ABCA1 but rather an indirect effect caused by induced membrane curvature.

Induction of high curvature into a phospholipid bilayer, such as is created in 20-nm-diameter SUV, disorders the molecular packing and creates space between the phospholipid polar groups into which apolipoprotein amphipathic α-helices can penetrate (20, 24, 44). As a consequence, apo A-I is able to bind with high affinity ($K_d = 0.1 \mu M$ (20, 44)) to such a surface (see the binding isotherm for SUV in Fig. 2A). A similar $K_d$ is observed for ABCA1-induced binding of apo A-I to lipid sites in the cell surface (27). The strong influence of phospholipid bilayer curvature is demonstrated by the comparison in Fig. 2A of the apo A-I-binding isotherms for egg PC SUV (20-nm diameter) and LUV (200-nm diameter). The maximum level of apo A-I binding is reduced ~8-fold for the LUV presented at the same total exposed surface area, consistent with the idea that apo A-I does not bind well to relatively planar phospholipid bilayers. Confirmation of the promotion of high affinity apo A-I binding by the induction of high curvature into a phospholipid bilayer is provided by the isothermal titration calorimetry experiment summarized in Fig. 2B. It is apparent that binding of apo A-I to SUV is accompanied by a large exothermic heat ($-93 \text{ kcal/mol}$); a large part of this favorable enthalpy of interaction arises from the increase in apo A-I α-helix content upon binding (24, 45). Consistent with much reduced apo A-I binding to the less curved LUV surface, the enthalpy is reduced
Some 25-fold when apo A-I is mixed with the same amount of exposed bilayer surface present as LUV.

The electron micrographs presented in Fig. 3 provide direct evidence for the existence of the protruding exovesiculated domains depicted in Step 2 in Fig. 1. It is apparent that the immunogold label of apo A-I bound to the surface of J774 cells in which ABCA1 is active is clustered on structures that protrude from the plasma membrane (Fig. 3, A and B); these structures could not be detected in control J774 cells in which ABCA1 was not up-regulated (Fig. 3C). Our results are in agreement with those of Lin and Oram (23), who previously characterized the morphology of the apo A-I-binding sites on ABCA1-expressing cells. They saw 10–60-nm “mushroom-like” protrusions from the surfaces of human fibroblasts and THP-1 macrophages to which apo A-I was bound. The formation of such protrusions required energy expenditure, and they were not apparent in Tangier disease cells where ABCA1 was inactive. Apo E also participates effectively in the ABCA1 reaction to create nascent HDL particles (Ref. 46 and references contained therein), and as expected from the reaction scheme (Fig. 1), apo E has been shown to bind to the exovesiculated domains created by ABCA1 activity (23).

Overall, it is apparent that formation of a highly curved membrane bilayer surface to which apo A-I can bind with high affinity explains the ABCA1-induced formation of high capacity lipid-binding sites at the cell surface. The binding of apo A-I to the exovesiculated domains (Fig. 1) creates conditions for formation of nascent HDL particles. It is of note that such binding of apo A-I to the highly curved membrane surface may be enhanced by the presence of phosphatidylserine molecules translocated to the exofacial leaflet by ABCA1; the presence of phosphatidylserine enhances vesicle curvature (47) and the binding of apo A-I to SUV (data not shown). The nascent HDL particles contain apo A-I as the sole detectable protein (22). Plasma membrane proteins (including ABCA1) are presumably excluded from the exovesiculated domains because they are clustered into islands attached to the cytoskeleton (48). These observations...
suggest that the exovesiculated lipid domains are formed from regions of protein-free plasma membrane that are likely to be relatively fluid. This inference of higher membrane fluidity is supported by the following observations: 1) ABCA1 translocase activity creates a pool of cholesterol that is accessible to cholesterol oxidase (43), and 2) cholesterol in more fluid membranes is the preferred substrate for cholesterol oxidase (49).

**Step 3: Solubilization of Exovesiculated Membrane Lipid Domains by Apo A-I**—Step 3 requires the spontaneous solubilization of the membrane lipid exovesiculated domain to create nascent HDL. We have defined this process, which leads to the simultaneous release of cellular phospholipid and cholesterol (50, 51), as membrane microsolubilization (50, 52, 53). It has been known for some three decades that apo A-I can rapidly solubilize MLV formed from DMPC in a matter of minutes at 24 °C. This process involves insertion of apo A-I amphipathic α-helices into lattice defects that exist at the gel to liquid crystal phase transition of the DMPC bilayer (26, 54, 55). This reaction is extremely rapid with DMPC because the bilayer is relatively unstable because of the presence of short, 14-carbon atom, acyl chains in the PC molecule. The reaction leads to the formation of discoidal apo A-I/DMPC complexes that contain either two or three apo A-I molecules/particle (55). An important question that has not been addressed is whether or not apo A-I can spontaneously solubilize more stable bilayers comprised of the phospholipids typically found in the plasma membrane of mammalian cells. In this case, no gel to liquid crystal phase transition to create lattice defects is apparent, although the presence of sphingomyelin, which creates a heterogeneous interface, can accelerate solubilization (56). The data in Fig. 4 show that MLV prepared from a phospholipid and cholesterol mixture representing the lipids released into nascent HDL particles (22) are indeed spontaneously solubilized by apo A-I. The rate is slower than that typically observed with DMPC; under the experimental conditions used, the absorbance decreased by ~10% in the first hour of incubation at 37 °C. This rate is significantly faster than the rate of ABCA1-mediated efflux of cellular phospholipid to apo A-I, which is typically ~1%/h (16). The products of the reaction described in Fig. 4 are discoidal particles as visualized by negative stain electron microscopy (Fig. 5); cross-linking studies (data not shown) indicate that these particles predominantly contain two or three apo A-I molecules/particle (some poorly lipidated, monomeric apo A-I is also produced). The number of apo A-I molecules/discoidal particle is the same as that observed in the spontaneous solubilization of DMPC bilayers by apo A-I (55) and in the nascent HDL particles created by ABCA1-mediated efflux of cellular lipids (22). Clearly, the apolipoprotein content of the latter discoidal HDL particles is determined by apo A-I/phospholipid bilayer interactions in which ABCA1 is not directly involved and by the structural properties of the apo A-I molecule. The solubilization process requires penetration of apo A-I helices into the phospholipid bilayer with the hydrophobic C-terminal α-helices playing a key role (19, 45, 52). This effect is demonstrated by the result in Fig. 4 for apo A-I Δ190–243, which is unable to effectively solubilize the membrane phospholipid bilayers to create discoidal HDL particles.

Prior investigations of the mechanism of ABCA1-mediated efflux of cellular lipids have suggested that the step in which apo A-I acquires lipid is rate-limiting (14, 16). On this basis, it is expected that Step 3 in the reaction scheme summarized in Fig. 1 is the slowest and therefore rate-limiting. If this idea is correct, then it is to be expected that factors that modulate the rate of membrane bilayer solubilization by apo A-I will have a similar effect on the rate of phospholipid and cholesterol efflux from cells via ABCA1. To test this concept experimentally, we employed a series of apo A-I mutants and examined the effects of the apo A-I structural changes on
both the rate of cellular cholesterol efflux via ABCA1 (Fig. 6) and on the rate of solubilization of DMPC MLV (Fig. 7). The fact that there is a strong correlation between these two parameters (Fig. 8) indicates that the third step (Fig. 1) involving the solubilization of the phospholipid bilayer by apo A-I and the creation of the discoidal nascent HDL particles is rate-limiting for the overall reaction depicted in Fig. 1. Furthermore, the mechanism of membrane microsolubilization is very likely the same as that of DMPC bilayer solubilization via apo A-I. In both cases, adsorption of apo A-I molecules into lattice defects present in the surface of the phospholipid bilayer leads to the eventual destabilization of the membrane and rearrangement to form discoidal apo A-I/phospholipid complexes (26). The hydrophobic C-terminal \( \alpha \)-helix in apo A-I initiates the adsorption of the protein to a lipid surface (45), and its removal (16, 19) or modification (57) drastically slows the solubilization process, whether it is a consequence of ABCA1 activity or the addition of apo A-I to DMPC MLV. As expected from the reaction mechanism (Fig. 1), removal of the C-terminal \( \alpha \)-helix from apo A-I reduces the ability of the protein to bind to the exovesiculated lipid domain created by ABCA1 activity (27). Differences in dependence on apo A-I concentration are likely for ABCA1-mediated lipid efflux and DMPC MLV solubilization (Figs. 6 and 7) because of variations in membrane curvature and lipid composition (e.g. cholesterol content (58)) that modify apo A-I binding in the two systems.

In conclusion, the ABCA1/apo A-I reaction scheme presented in Fig. 1 explains in a comprehensive fashion for the first time 1) the contributions of apo A-I/ABCA1 and apo A-I/lipid interactions and 2) the nature of the HDL products that are formed. The heterogeneity in lipid composition of
Mechanism of ABCA1-mediated Lipid Efflux to Apo A-I

the different nascent discoidal HDL particles produced (22) is probably due to solubilization of exovesiculated domains created by ABCA1 molecules in different membrane environments.

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FIGURE 8. Correlation of the relative efficiencies of human apo A-I variants in mediating efflux of cellular cholesterol via ABCA1 and in solubilizing DMPC MLV. The measurements of relative cholesterol efflux from J774 cells as a function of apo A-I variant concentration (Fig. 6) were used to determine the concentrations of apo A-I variants required to achieve a relative cholesterol efflux of 0.1. The concentration of WT apo A-I required to give a relative cholesterol efflux of 0.1 was 0.3 mg/ml and was normalized to 1.0. The measurements of rates of clearance of turbidity of DMPC MLV as a function of apo A-I variant concentration (Fig. 7) were used to determine the concentrations relative to that for WT apo A-I (10 µg/ml normalized to 1.0) required to reduce the absorbance at 325 nm by 0.1 units in 10 min. The data were fitted by linear regression that was forced through the origin ($r^2 = 0.70$).
Mechanism of ABCA1-mediated Lipid Efflux to Apo A-I

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