ABCA1-Induced Cell Surface Binding Sites for ApoA-I


Objective—The purpose of this study was to understand the interactions of apoA-I with cells expressing ABCA1.

Methods and Results—The binding of wild-type (WT) and mutant forms of human apoA-I to mouse J774 macrophages was examined. Analysis of total binding at 37°C of 125I-WT apoA-I to the cells and specifically to ABCA1, as determined by covalent cross-linking, revealed saturable high affinity binding in both cases. Determination of the level of cell-surface expression of ABCA1 showed that only about 10% of the apoA-I associated with the cell surface was bound directly to ABCA1. Furthermore, when 125I-apoA-I was cross-linked to ABCA1-upregulated cells and examined by SDS-PAGE, the major (∼90%) band migrated as monomeric apoA-I. In contrast to WT apoA-I, the C-terminal deletion mutants Δ190 to 243 and Δ223 to 243 that have reduced lipid affinity, exhibited marked reductions (50 and 70%, respectively) in their abilities to bind to the surface of ABCA1-upregulated cells. However, these C-terminal deletion mutants cross-linked to ABCA1 as effectively as WT apoA-I.

Conclusions—This study demonstrates that ABCA1 activity creates 2 types of high affinity apoA-I binding sites at the cell surface. The low capacity site formed by direct apoA-I/ABCA1 interaction functions in a regulatory role, whereas the much higher capacity site generated by apoA-I/lipid interactions functions in the assembly of nascent HDL particles. (Arterioscler Thromb Vasc Biol. 2007;27:1603-1609.)

Key Words: ABCA1 ■ apoA-I ■ phospholipids ■ binding

High density lipoprotein (HDL) is known to possess pleiotropic biological properties which are able to protect against the development of cardiovascular disease.1–3 An important aspect of HDL function is its role in reverse cholesterol transport (RCT), a process that mediates the transport of cholesterol from peripheral tissues, including the arterial wall, to the liver for biliary secretion.1,4,5 Cellular lipid efflux mediated by apolipoprotein (apo) A-I, the major HDL protein constituent, is one of the earliest events in RCT. Recent studies have identified the ATP-binding cassette transporter A1 (ABCA1) as a critical factor in the formation of HDL, and lack of a functional ABCA1 leads to cholesterol accumulation in peripheral tissues and low plasma HDL.6 The importance of ABCA1 has been demonstrated by the fact that mutations in the ABCA1 gene lead to Tangier disease and familial HDL deficiency.7 These patients are generally characterized by an accumulation of excess cellular cholesterol, low levels of HDL, and an elevated risk of atherosclerosis.8 Indeed, a direct correlation between cell surface expression of ABCA1 and apoA-I–mediated cellular lipid efflux has been revealed.4

Although it is known that the interaction of apoA-I with functional ABCA1 results in the lipiddation of apoA-I and formation of HDL, the mechanism of this interaction is not resolved. Prior studies9–11 have established that the initial binding of apoA-I to the surface of cells in which ABCA1 is upregulated directs a sequence of events leading to the transfer of cellular phospholipids and cholesterol to the apolipoprotein. However, the exact nature of this ABCA1-induced interaction of apoA-I with the cell surface remains a matter of debate. Chemical cross-linking studies have shown that apoA-I forms a complex with ABCA1 in cells expressing this transporter.12–14 The cell surface-associated apoA-I binds within 3Å of ABCA1, suggesting protein–protein interactions between apoA-I and ABCA1.15 On the other hand, some studies16–18 have proposed that apoA-I binds to a modified plasma membrane lipid domain created by ABCA1. An important limitation of the prior studies is that largely qualitative experiments were conducted to study apoA-I binding to ABCA1. The lack of quantitative comparison of the potential sites to which apoA-I binds in ABCA1-expressing cells has made it difficult to distinguish the relative importance of protein–protein and protein–lipid interactions during the reaction. Hence, it is apparent that there is unresolved complexity in understanding the mechanism of apoA-I interaction with ABCA1-expressing cells.

In the current study, we have addressed some of these questions by characterizing the sites to which apoA-I binds during its interaction with ABCA1-expressing cells. Cell surface binding sites activated by this interaction have been

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studied using engineered variants of apoA-I. Importantly, ABCA1 activity creates 2 binding sites on the cell surface for apoA-I: a low capacity site created by direct apoA-I/ABCA1 interaction and a much higher capacity site that involves apoA-I/lipid interactions. Studies of the cross-linking of apoA-I and its variants with ABCA1 have revealed that formation of the apoA-I/ABCA1 complex is not dependent on a particular structural domain within the apoA-I molecule. However, there is a strong correlation between the lipid binding affinity of the various mutants of apoA-I and effective binding to the major site on the cell membrane created by ABCA1 activity.

Methods

Preparation of ApoA-I

ApoA-I was isolated from fresh plasma of normolipidemic donors as described earlier.19,20 The construction of plasmids for expressing wild-type (WT) apoA-I and its mutants and the isolation and purification of these proteins have been described in detail previously.21

Cell Culture and Binding Experiments

ApoA-I was iodinated by the iodine monochloride method22 to a specific activity of 250 to 1200 cpm/ng. Binding of apoA-I to ABCA1-expressing J774 and Baby hamster kidney (BHK) cells was determined as described previously.14,18,23 Competition binding assays were performed as described in detail earlier.14 Chemical cross-linking and immunoprecipitation of bound apoA-I to ABCA1 expressing J774 and BHK cells was carried out as described before.14,15 Biotinylation of cell surface proteins in J774 cells was performed as described previously.24

Please see the supplemental materials, available online at http://atvb.ahajournals.org, for more methodological details.

Results

Binding of ApoA-I to ABCA1-Upregulated Cells

In the current study, binding of 125I apoA-I was examined in control and ABCA1-expressing J774 and BHK cells. As shown in Figure 1A, increasing the concentration of apoA-I resulted in a marked increase in the apoA-I binding to stimulated J774 cells. Significant binding was also observed in unstimulated cells, possibly because of the basal level of ABCA1 expression and nonspecific binding of apoA-I to other cell surface sites such as the extracellular matrix.25 The isotherm for specific binding of apoA-I to ABCA1 was obtained by subtracting the binding values for unstimulated cells from the corresponding values for stimulated cells. The binding parameters Bmax (105 ± 12 ng apoA-I/mg cell protein) and Kd (1.2 ± 0.7 μg apoA-I/mL) were calculated from fitting the hyperbolic specific binding curve to a 1-site binding model. A 2-site binding model did not fit the data satisfactorily. It is apparent that apoA-I associates with the J774 cell surface with high affinity (Kd = 0.04 μmol/L) to give saturable binding (cf.26). The specific binding was also evaluated by incubating stimulated cells with different concentrations of 125I apoA-I for 2 hours at 37°C in the absence or presence of a 30-fold excess of unlabeled apoA-I to block nonspecific binding. The values of Kd (2.6 ± 0.9 μg apoA-I/mL) and Bmax (135 ± 20 ng apoA-I/mg of cell protein) from this analysis were similar to those derived from Figure 1A. The above binding parameters are consistent with the results of similar experiments conducted with different cell types.14,27

To establish that the specific binding parameters obtained above are associated with an increase in binding of apoA-I to ABCA1 in stimulated cells, 125I apoA-I was examined for its

Figure 1. Cell association and cross-linking of 125I apoA-I to ABCA1: J774 cells (A and B) stimulated or not with cAMP and human-ABCA1 expressing and mock -transfected BHK cells (C and D) were incubated for 2 hours at 37°C with increasing concentrations of 125I apoA-I (0.5, 1, 2, 5, 10, and 20 μg/mL). After binding, cells were incubated with the crosslinker (DSP) for 30 minutes at room temperature, lysed, and radioactivity determined from aliquots of the cell lysate. A and C, The specific binding curve (ABCA1 specific [□]) was determined by subtracting the binding values for the control cells (●) from the corresponding values for ABCA1-expressing cells (■). The values for total apoA-I binding are the mean ± SD from at least 2 independent experiments performed in triplicate. B and D, Aliquots of the cell lysate were immunoprecipitated with an anti-ABCA1 antibody, separated on SDS-PAGE, transferred, and analyzed by phosphorimager. The values for apoA-I cross-linked to ABCA1 in D are representative from 2 independent experiments performed in triplicate, and the values shown in D are single values from 1 of 3 representative experiments.
ability to crosslink to ABCA1. Stimulation of cells with cAMP increased both cellular ABCA1 expression and apoA-I/ABCA1 cross-linking compared with unstimulated cells (data not shown). Treatment with increasing concentrations of radioiodinated WT apoA-I enhanced the cross-linking of apoA-I, and maximum cross-linking (~3 ng/mg cell protein) was attained at a concentration of 20 μg apoA-I/mL (Figure 1B). It is important to note that values for total (B_max = 105±12 ng/mg cell protein) and cross-linked (B_max = 3.6±0.4 ng/mg cell protein) apoA-I binding are clearly markedly different by some 30-fold, indicating that only a minor part of the cell-associated apoA-I apparently binds directly to ABCA1. Analysis of the binding isotherm in Figure 1B showed that the K_d value for cross-linking with WT apoA-I was 5.3±1.5 μg apo/mL, which is higher than the apparent K_d attained from Figure 1A.

It is apparent from the above results that ABCA1 activity in J774 cells creates 2 types of binding sites for apoA-I. To establish that these binding sites were caused only by ABCA1 activity and were not attributable to any other factor induced by cAMP, the binding and cross-linking experiments described above were repeated in mock and human ABCA1-transfected BHK cells. As is evident from Figure 1C, 125I-apoA-I exhibited concentration-dependent binding to ABCA1-transfected BHK cells; the B_max (70±3 ng/mg cell protein) and the K_d (0.06±0.04 μg apoA-I/mL) values for the specific binding originating from ABCA1 activity were obtained as described above. The total amount of apoA-I bound to the cell and cross-linked to ABCA1 (3.0±0.3 ng/mg cell protein) (Figure 1D) were significantly different, indicating once again that a major part of the cell-associated apoA-I is bound to another site created by the activity of ABCA1. It is important to note that the reaction conditions (see Methods) were selected to give quantitative cross-linking of apoA-I/ABCA1 complexes present at the cell surface. This condition was essentially achieved because cross-linking and immunoprecipitation with anti–apoA-I removed about one-third of the total cell ABCA1 (data not shown) that is consistent with the fraction of transporter present at the cell surface at any given time (see below).

**Cell Surface Expression of ABCA1**

To determine the amount of ABCA1 present at the cell surface relative to the total amount of ABCA1 in the cells, biotinylation experiments were carried out on J774 cells upregulated or not with cAMP. Western blot analysis was conducted on whole cell protein extracts to detect ABCA1 that migrated at a molecular mass ~220kDa (Figure 2, lane C). Densitometric scanning showed that treatment with cAMP increased the expression of total cellular ABCA1 by almost 8-fold (cf. Figure 2, lanes C and D). As earlier studies have indicated that ABCA1 may be most active at the plasma membrane, the all surface proteins in J774 cells were nonspecifically biotinylated using sulfo-NHS-SS-biotin. These biotinylated proteins were then isolated with streptavidin immobilized on agarose beads and analyzed by immunoblotting with a polyclonal antibody to ABCA1. It is evident from Figure 2 (lane B) that cAMP augments the expression of ABCA1 at the cell surface. The ABCA1 appeared as a doublet consistent with earlier reports. Comparison of the expression of ABCA1 at the cell surface with that in the total cell lysate by densitometric scanning (cf. Figure 2, lanes B and C) indicates that about 30% to 40% of the total cellular ABCA1 is present at the cell surface at any given time.

To further confirm the relative surface expression of ABCA1, displacement experiments were carried out with J774 cells stimulated with cAMP. After binding 125I-apoA-I (2 μg/mL) for 2 hours at 37°C, the cell monolayers were kept on ice, washed, and treated with excess unlabeled apoA-I (100 μg/mL medium). The cells were then incubated for 6 hours at 4°C, and other cells were treated in parallel at 37°C. The displacement of cell-associated 125I-apoA-I followed monoexponential decay kinetics and, as shown in Figure 3, about 30% of labeled apoA-I was displaced from cells placed at 4°C whereas about 80% was displaced from cells held at 37°C. Because internalization of the transporter was not possible for cells kept at 4°C, only labeled apoA-I bound to the cell surface as a result of ABCA1 activity was displaced by excess unlabeled apoA-I. The observation that only 30% of the 125I-apoA-I was displaced is in good agreement with the finding from the biotinylation experiment that 30% to 40% of total cellular ABCA1 is present at the cell surface at any given time. On the other hand, for the cells held at 37°C, radioiodinated apoA-I associated with ABCA1 cycling between the cell interior and the cell surface was displaced by unlabeled apoA-I.

**Binding of ApoA-I to Other Sites in Cells**

To further explore the nature of the binding sites of the cell-associated apoA-I, J774 cells expressing ABCA1 were treated with 20 μg/mL of 125I-apoA-I for 2 hours to ensure saturation of all sites and then cross-linked with DSS. This nonreducible cross-linker was used so that the cross-links between apoA-I and any cellular protein were maintained during subsequent SDS-PAGE under reducing conditions. Aliquots of the cell lysate were either directly analyzed by SDS-PAGE or were first immunoprecipitated with anti–apoA-I antibody and then analyzed by SDS-PAGE; the resultant blots were analyzed by phosphorimager (Figure 4). Immunoprecipitation with anti–apoA-I brought down a lot more 125I counts (≈80%) of the total compared with precipitation with anti–ABCA1 (≈15% of the total) consistent with
the relative bound apoA-I pool sizes in Figure 1A and 1B. When the cell lysates were analyzed directly by SDS-PAGE, only a single major band representing the monomeric form of apoA-I was identified (Figure 4A). Also, compared with control cells there was a 2- to 3-fold increase in the intensity of the apoA-I band in ABCA1-expressing cells. Similar results were also obtained with mock-transfected and ABCA1-expressing BHK cells cross-linked to apoA-I (data not shown). It is important to note that after immunoprecipitation with anti–apoA-I, the major band (band 1, in Figure 4B) migrated at the molecular weight (28 000) equivalent to that of monomeric apoA-I. The ABCA1/apoA-I complex migrated at the expected molecular weight (≈250 000; band 4 in Figure 4B). Bands 2 and 3 corresponded to the cross-linked dimeric and trimeric forms of apoA-I. Thus, it is apparent from Figure 4 that the apoA-I bound to the J774 cells as a consequence of ABCA1 activity was not associated with any other cellular protein, as evaluated by cross-linking. Indeed, while ABCA1 expression increased the cell-association of apoA-I considerably to 100 ng/mg cell protein more than 90% of this apoA-I was present in its monomeric form. For comparison, using control cells in which ABCA1 was not upregulated, band 4 was not apparent (data not shown).

**Effects of N- and C-Terminal Mutations in ApoA-I on Cellular and Lipid Interactions**

To explore the concept that the majority of the apoA-I bound to cells as a consequence of ABCA1 activity is located on lipid sites, we used apoA-I variants with altered lipid binding properties. Deletion and proline insertion mutants of apoA-I were incubated with either ABCA1-stimulated or -unstimulated J774 cells, and their efficacies of binding were compared. The specific binding curves for the various mutants were obtained as described earlier for WT apoA-I, and the results are depicted in Figure 5A. Deletion of the C-terminal domain (Δ190 to 243) and the C-terminal α-helix (Δ 223 to 243) led to 50% and 70% reductions in B_max (Table), respectively, relative to the total cell binding of WT apoA-I. These results are in agreement with an earlier study in which the C-terminal mutant (Δ187 to 243) showed a 58% reduction in total binding to THP-1 macrophages compared with WT apoA-I. In contrast, disruption of the N-terminal domain by inserting a proline residue in that region (apoA-I Y18P) had no effect on total cell binding (Table).

To analyze the effects of the alterations in apoA-I structure on the ability of apoA-I to bind directly to ABCA1, the C-terminal truncation mutants of apoA-I were assessed for their capacity to crosslink to ABCA1. As depicted in Figure 5B, these mutants and WT apoA-I cross-linked similarly to ABCA1. The B_max values for the N- and C-terminal apoA-I mutants were around the value of 3 ng/mg cell protein observed with WT apoA-I (Table). These results show that the structural requirements for binding of apoA-I to ABCA1 are not highly stringent; apparently the presence of amphipathic α–helices is sufficient to allow apoA-I to bind to ABCA1. In agreement with this idea, peptides of identical sequences containing either D or L enantiomers have been shown to have the same ability to bind to ABCA1 revealing that there is no stereoselective requirement for binding.

Figure 5C compares the binding isotherms for WT apoA-I, Δ190 to 243 and Δ223 to 243 to egg PC small unilamellar vesicles (SUV). It is evident from the binding isotherms that the abilities of the C-terminal deletion mutants to bind to a phospholipid bilayer are greatly reduced compared with WT apoA-I. This effect occurs because apoA-I initially binds to lipid surfaces via its C-terminal domain. Importantly, deletion of the C-terminal region caused parallel reductions in the ability to bind to ABCA1-expressing cells and egg PC bilayers (Figure 5D). This correlation is consistent with the idea that the second site for the apoA-I bound to cells is a membrane lipid site that is created by the expression of active

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**Figure 3.** Displaceability of cell-associated apoA-I: J774 cells after upregulation with cAMP were incubated with 125I-apoA-I (2 μg/ml) for 2 hours at 37°C, and the cells were split into 2 sets. One set was placed at 4°C (∘) while the other was placed at 37°C (□), and both were incubated with excess unlabeled WT apoA-I (100 μg/ml) for up to 6 hours. At specific time points, cells were washed, lysed, and the cell-associated radioactivity determined from aliquots of the cell lysate. The symbols represent the mean of triplicate measurements ±SD.

**Figure 4.** Immunoprecipitation of cell-associated apoA-I: J774 cells were either upregulated or not upregulated with cAMP and incubated with 125I-apoA-I for 2 hours and treated with the non-reducible crosslinker DSS (500 μmol/L) for 30 minutes at room temperature as described in the legend to Figure 1. A. Aliquots of the cell lysate were analyzed directly by SDS-PAGE and the 125I-apoA-I detected by phosphorimager analysis. B. Aliquots of the cell lysate were immunoprecipitated with anti–apoA-I antibody and analyzed by SDS-PAGE to determine the fraction of cell-associated apoA-I that was cross-linked to cellular proteins. Results are representative of three independent experiments.
ABCA1. Indeed, earlier studies have shown that ABCA1 can modify the lipid distribution in a cell plasma membrane.16,18 To further evaluate the importance of the lipid-binding properties of apoA-I in its ability to associate with ABCA1-expressing cells, we assessed the capabilities of the N- and C-terminal mutants of apoA-I to compete for the binding of 125I apoA-I to ABCA1-upregulated cells. As expected, WT apoA-I competes effectively with 125I apoA-I, such that 75% to 80% of the 125I apoA-I binding was inhibited at concentrations of unlabeled apoA-I/H11006 0.5 g/mL (supplemental Figure I). The lack of complete inhibition was probably attributable to a combination of nonspecific binding of the radiolabeled ligand to the cells and to the plastic tissue culture plates. The EC50 value for WT apoA-I was 2.9±1.2 g apo/mL (equivalent to about 0.1 μmol/L apoA-I; supplemental Table I). In contrast, the C-terminal deletion mutants (Δ190 to 243, Δ223 to 243) were less effective competitors; the EC50 values were almost 30-fold and 180-fold higher for the Δ190 to 243 and Δ223 to 243 variants, respectively (supplemental Table I). These results are consistent with the protein binding data shown in Figure 5A and Table; the apoA-I C-terminal mutants with impaired lipid binding properties have reduced capacities both to bind to ABCA1-upregulated cells and compete with WT apoA-I for binding to such cells. Significantly, the N-terminal mutant (Y18P), which has similar lipid-binding properties to WT apoA-I,32 competed for the binding of 125I-apoA-I almost as effectively as WT apoA-I (supplemental Table I).

**Discussion**

The ABCA1 transporter is critical for the biogenesis of HDL through its ability to facilitate apoA-I-mediated removal of phospholipids and cholesterol from peripheral and hepatic cells.1,3 ApoA-I is catabolized rapidly in the absence of functional ABCA1 leading to free cholesterol accumulation in peripheral tissues and low levels of plasma HDL.4,34 Hence, it is apparent that ABCA1 plays a vital role in the lipiddation of apolipoproteins, such as apoA-I. To achieve the important goal of elucidating the mechanism of interaction between apoA-I and ABCA1, it is necessary to understand the cellular binding sites for apoA-I created by ABCA1 activity. Many theories have been proposed to explain the mechanism of interaction of apoA-I with ABCA1-expressing cells. Some studies35,36 have pointed to the importance of apoA-I/ABCA1

### Table: Binding and Cross-Linking Parameters for Interaction of ApoA-I Variants With Cells or ABCA1

<table>
<thead>
<tr>
<th>ApoA-I</th>
<th>Bmax for ApoA-I Binding to Cells* (ng apoprotein/mg cell protein)</th>
<th>Bmax for Apo A-I Binding to ABCA1* (ng apoprotein/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>105±12</td>
<td>3.6±0.4</td>
</tr>
<tr>
<td>Δ190–243</td>
<td>53±7</td>
<td>1.8±0.9</td>
</tr>
<tr>
<td>Δ223–243</td>
<td>34±5</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>Y18P</td>
<td>99±38</td>
<td>3.8±0.8</td>
</tr>
</tbody>
</table>

*Mean±SD Bmax values were derived by fitting the isotherms describing the concentration-dependence of binding and cross-linking of apo A-I to a one-site binding equation.
interactions, whereas others\(^{16,17}\) have postulated the importance apoA-I/lipid interactions for the overall binding process. However, so far a consensus has not been reached and the mechanism of this reaction is still unresolved. Furthermore, the mechanism of formation of nascent HDL particles during ABCA1-mediated lipid efflux is still not clearly understood. Current thinking is that it involves 2 steps.\(^{15,23}\)

The first step involves the formation of a complex between apoA-I and ABCA1, and the second step involves the lipidation of apoA-I and dissociation of the nascent HDL particle from the transporter. This model implies that all of the cell-associated apoA-I is bound to ABCA1 where it becomes transformed into nascent HDL particles. In the current study, we present quantitative binding information showing that ABCA1 activity creates 2 binding sites for apoA-I.

**Cell-Surface Pool of ApoA-I Bound to ABCA1**

As summarized in the Results Section, apoA-I exhibits saturable high affinity binding to ABCA1. The \(K_d\) for binding to ABCA1 in J774 cells is \(\approx 3\) ng apoA-I/mg of cell protein (Table); this interaction is not very specific and apparently involves interaction of apoA-I amphipathic \(\alpha\)-helices with the transporter. This concept is supported by the data in the Table showing that all of apoA-I mutants (both N- and C-terminal domain) studied bind equally well to ABCA1. This interaction of apoA-I with ABCA1 requires signaling responses mediated by Janus kinase 2\(^{29}\) and is associated with stabilization of ABCA1 at the cell surface by protecting it from proteolytic degradation.\(^{5,9,37}\) Thus, the low capacity apoA-I binding site on the ABCA1 molecule has regulatory functions (Figure 6).

**Nature of Second ApoA-I Binding Site**

The size of the second binding site equals the total amount of \(^{125}\)I-apoA-I bound to the cell (100 ng/mg cell protein) minus the amount of protein bound specifically to ABCA1 (3 ng/mg of cell protein). However, from the biotinylation (Figure 2) and apoA-I displacement (Figure 3) experiments, it is apparent that only around 35% of the ABCA1 is present on the surface of the cells. Hence, the actual amount of apoA-I associated with the second binding site on the surface is equal to about 30 ng of apoA-I/mg cell protein [(100 ng \(\cdot\) 0.35)–3 ng]]. It follows that the amount of apoA-I associated with the second site is some 10-fold greater than that bound to ABCA1 indicating that the second site is a high capacity binding site (Figure 6), as has been postulated previously.\(^4\)

Cross-linking of bound apoA-I followed by immunoprecipitation with anti–apoA-I has demonstrated that, apart from ABCA1, apoA-I does not bind to any other cellular protein (Figure 4B). In fact, \(\approx 90\%\) of the bound apoA-I is monomeric after cross-linking, consistent with it being located on a lipid site. The \(K_d\) value for the binding of WT apoA-I to the cell surface is around 1 \(\mu\)g/mL. Interestingly, the \(K_d\) for binding of WT apoA-I to egg PC SUV, a surrogate for the phospholipid bilayer of a cell membrane, is in the same range.\(^{32}\) This similarity in \(K_d\) values lends strong support to the idea that the other binding site for apoA-I generated by ABCA1 activity is a lipid site. In support of this interpretation, ABCA1 expression in BHK cells redistributes cholesterol to cell-surface domains\(^{18}\) and causes a change in the overall lipid packing of the plasma membrane.\(^{36}\) Furthermore, removal of either the strongly hydrophobic C-terminal domain (\(\Delta 190\) to 243) or the last helix (\(\Delta 223\) to 243) of WT apoA-I reduces the level of binding to cells by 50 and 70% respectively, compared with WT apoA-I. The reason that these mutants associate poorly with the cells is their inability to bind well to the second site created by ABCA1 activity. This notion is further supported by the observation that the C-terminal mutants compete badly for binding to the cells compared with WT apoA-I (supplemental Table 1). Deletion of the C-terminal region of apoA-I results in low affinity non-saturable binding to egg PC SUV as compared with WT apoA-I which exhibits high affinity saturable binding (Figure 5C, e\(^{35}\)). It is apparent that the hydrophobic C-terminal \(\alpha\)-helices of apoA-I are needed for effective insertion into the phospholipid bilayer and high affinity binding.\(^{32,33}\) The fact that removal of these functional \(\alpha\)-helices from the apoA-I molecule leads to similar reductions in binding to both phospholipid vesicles and the second cell surface binding site created by ABCA1 activity (Figure 5D) is consistent with the latter binding site being a lipid site. The affinity of the N-terminal apoA-I (Y18P) mutant for the second cell binding site is essentially the same as that of WT apoA-I (Table) implying that the N-terminal helix-bundle domain in apoA-I\(^{33}\) does not play a major role in lipid binding. This finding is consistent with the fact that the binding affinity of apoA-I Y18P to phospholipid SUV is similar to that of WT apoA-I.\(^{32}\) Because the high capacity apoA-I binding site created by ABCA1 activity is a lipid site it follows that this site functions in the assembly of nascent HDL particles.

It is generally thought that the ABCA1 molecule itself is the major binding site for apoA-I in ABCA1-expressing cells. However, our study shows that 2 binding sites for apoA-I are created at the surface of cells containing functional ABCA1. Furthermore, the major binding site is the modulated lipid site orchestrated by the ABCA1 activity. Hence, it is evident that there is a need to reevaluate the current theories on the formation of nascent HDL particles during apoA-I/ABCA1 interaction.

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DISCLOSURES

None.

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Materials and Methods

Materials
Fetal bovine serum (FBS), gentamicin, 8-(4-chlorophenythio)(cpt)-cAMP, dithiobis(succinimidyl-propionate) (DSP), disuccinimidylsuberate (DSS) and protease inhibitor cocktail were purchased from Sigma (St.Louis, MO). Bovine serum albumin (BSA) was obtained from Intergen (Purchase, NY). Na\textsuperscript{125}I was obtained from Perkin Elmer Life Sciences (Boston, MA). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL). Sulfo-NHS-SS-biotin and ultra-link immobilized streptavidin were obtained from Pierce (Rockford, IL). RPMI 1640 and phosphate-buffered saline were purchased from CellGro (Herndon, VA). Rabbit polyclonal antibody to ABCA1 and goat polyclonal antibody to apo A-I were purchased from Novus Biologicals (Littleton, CO). Minimum Essential medium buffered with 25mM Hepes, pH 7.4 (MEM-Hepes) was obtained from BioWhittaker (Walkersville, MD). The acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Pfizer CP-113, 818, was a gift from Pfizer Inc. (Groton, CT).

Methods

Preparation of Apo A-I - HDL was isolated from fresh plasma of normolipidemic donors by sequential ultracentrifugation as described earlier\textsuperscript{1}. Human HDL was delipidated in ethanol/diethyl ether and apo A-I was isolated by anion exchange chromatography \textsuperscript{2}. Prior to use, the purified protein stored in lyophilized form at –20°C was resolubilized in guanidine hydrochloride (6M) and dialyzed extensively against Tris buffer (10mM Tris, 150mM NaCl,
1.0mM EDTA, pH 7.4). The protein concentration was determined by measuring its absorbance at 280nm; the mass extinction coefficient (ml/(mg.cm)) of apo A-I is 1.13.

Expression and purification of Apo A-I mutants – The construction of plasmids for expressing wild type (WT) apo A-I and its mutants, and the isolation and purification of these proteins have been described in detail previously. The following mutants of human apo A-I were used in this study: Y18P, Δ190-243 and Δ223-243.

Apo A-I binding to J774 and BHK cells expressing ABCA1 - Apo A-I was iodinated by the iodine monochloride method to a specific activity of 250-1200 cpm/ng. Binding of apo A-I to ABCA1-expressing cells was determined as described previously. Briefly, J774 cells were seeded in 12-well plates and grown to 80-90% confluence as described before. The cells were washed with MEM-HEPES and incubated with or without 0.3mM cpt-cAMP for 14h in RPMI medium containing 0.2% (w/v) BSA and 2µg/ml CP-113,818 ACAT inhibitor. These cells, in which ABCA1 expression was either upregulated or not, were then washed and incubated at 37°C for 2h with increasing concentrations of 125I-apo A-I in RPMI/HEPES in the presence or absence of a 30-fold excess of unlabeled apo A-I. The cells were then rapidly washed twice with ice-cold PBS/BSA (1mg/ml), two more times with PBS and lysed in 0.1N NaOH. The lysates were centrifuged and the amount of bound 125I-apo A-I was determined from an aliquot of the lysate by γ-counting. An aliquot of the lysate was used to determine the cellular protein concentration by the Markwell-Lowry protein assay.

Baby hamster kidney (BHK) cells expressing human ABCA1 and mock-transfected cells (generously provided to Dr. Sean Davidson by Dr. John Oram) were generated as described in detail before. Cells were grown and maintained in DMEM containing 10% fetal bovine serum and ABCA1 was induced by incubating the cells for 14 h in DMEM with 1 mg/ml fatty acid-free
BSA and 10 nM mifepristone. Binding experiments with $^{125}\text{I}$-apo A-I were carried out as described in detail above.

Competition binding assays were performed as described in detail previously. J774 cells were upregulated with cAMP and then incubated at 37°C with 2µg/ml of $^{125}\text{I}$-apo A-I together with various concentrations of unlabeled WT or apo A-I variant. The amount of cell-associated $^{125}\text{I}$-apo A-I was determined as described above. 100% binding of $^{125}\text{I}$-apo A-I corresponds to the level in the absence of a competitor. The abilities of the mutant forms of apo A-I to compete for binding were assessed from the percentage decreases in binding of $^{125}\text{I}$-apo A-I in the presence of the competitor. The IC$_{50}$ values were determined using Graph Pad Prism software.

To assess the degree to which $^{125}\text{I}$-apo A-I bound to the surface of J774 cells can be displaced, cells with bound $^{125}\text{I}$-apo A-I were treated with 100µg/ml of unlabeled apo A-I in RPMI/HEPES. One set of cells was then placed at 4°C while another was placed at 37°C. At specific times, cells were washed thoroughly, lysed and the radioactivity remaining with the cells were determined by $\gamma$-counting. The value that corresponds to the binding of $^{125}\text{I}$-apo A-I (2µg/ml) to cells before the addition of unlabeled apo A-I was set at 100%.

**Chemical cross-linking and Immunoprecipitation:** Chemical cross-linking of bound apo A-I to ABCA1 was carried out as described before. $^{125}\text{I}$-apo A-I was bound to J774 and BHK cells as described above and the cells were then placed on ice for 15 min and washed thrice with ice-cold PBS. Immediately prior to use, DSP was dissolved in DMSO and diluted to 500µM in PBS. One ml of this DSP solution was then added to each well and the cells were incubated for 30 min at room temperature. Consistent with these conditions giving efficient cross-linking of apo A-I/ABCA1 complexes present at the cell surface, the amount of cross-linked apo A-I was unaffected by changes in DSP concentration (250-750µM), reaction time (30-120 min) and apo A-I concentration (10-20µg/ml). The fact that the interacting apo A-I and ABCA1 molecules can
be cross-linked readily is consistent with a rapid, specific and saturable binding reaction\textsuperscript{5,9,10}. After the crosslinking reaction was complete, the medium was removed and the cells were washed thrice with ice-cold PBS. Cell were then lysed with radioimmunoprecipitation buffer (RIPA) (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing a mixture of protease inhibitors and allowed to stand for 30 min at 4°C. The mixture was then centrifuged and the radioactivity of an aliquot of the lysate was determined by $\gamma$-counting. For immunoprecipitation, cell lysates (150-200 $\mu$g cell protein) were initially treated with 20ul of protein-A for 45 min on a rotating platform at 4°C and any non-specifically bound proteins were separated by pelleting the sepharose beads. The supernatant cell lysate was then incubated overnight at 4°C with either 10µl of rabbit polyclonal antibody to ABCA1 or goat polyclonal antibody to apo A-I. Subsequently, the cell lysate was incubated for an additional 2h with protein-A sepharose and centrifuged. The supernatant was treated again with the ABCA1 antibody and the immunoprecipitation was repeated to ensure that the antibody pulled down all the ABCA1 in the cell lysate. The immunoprecipitates were washed thrice with RIPA buffer and once with PBS and boiled in lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) containing DTT for 15 min. The samples were centrifuged and the proteins present in the supernatant were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The iodinated protein was visualized in a phosphorimaging STORM system and the band intensities were quantized using Imagequant software. Phosphor pixels of the crosslinked iodinated protein were converted to ng of apo protein using equations derived from a standard curve of known amounts of $^{125}$I-apo A-I imaged in parallel.

**Cell Surface Biotinylation:** Biotinylation of cell surface proteins was performed as described before\textsuperscript{11}. J774 cells were grown to confluence and either upregulated or not with cAMP for 12h. Cells were then treated with 10ug/ml of apo A-I in RPMI for 2h at 37°C. After binding of apo A-
I to the cells was complete, the cells were placed on ice and washed thrice with ice-cold PBS. Surface proteins were biotinylated by incubating the cells with 1mg/ml sulfo-NHS-SS-biotin in PBS for 30 min at 4°C on a platform rotator. Cells were then washed twice with ice-cold quench buffer (50mM Tris, 0.1mM EDTA, 150mM NaCl) and twice with ice-cold PBS. Cells were lysed at 4°C with RIPA buffer in the presence of a protease inhibitor cocktail and incubated on ice for 30 min. The cells were then centrifuged at 4°C and aliquots were taken for the determination of protein concentration. 200-250µg/ml of cell protein was added to 75 ul of ultra-link plus immobilized streptavidin gel and incubated overnight on a platform rotator at 4°C. The solution was centrifuged and the pellet was washed four times with lysis buffer and once with PBS. The pellets were then boiled in LDS sample buffer containing DTT for 15 min. The samples were centrifuged and the proteins present in the supernatant were separated by 4-12% SDS-PAGE and transferred to a nitrocellulose membrane. To assess total ABCA1, an aliquot of the cell lysate was loaded onto the 4-12% SDS PAGE. After transfer and blocking, blots were incubated with anti-ABCA1 antibody overnight at 4°C, washed and then incubated with the anti-rabbit secondary antibody for 1h at room temperature. Blots were developed using a chemiluminescence kit (Perkin Elmer), according to the manufacturer’s instructions.
References


Figure Legends

Figure I: Competition binding between $^{125}$I-apo A-I and apo A-I mutants to ABCA1-upregulated J774 cells: J774 cells were incubated for 2h at 37°C with 2µg/ml of $^{125}$I–apo A-I plus or minus unlabeled WT or variant apo A-I. The binding of $^{125}$I–apo A-I to the cells was measured at increasing concentrations of the competitors: WT apo A-I (∆), Δ190-243 (●), Δ223-243 (■) and Y18P (▼). Data represent the mean of triplicate measurements ± SD.
Figure I

![Graph showing the binding of 125I-apo A-I to cells as a percentage of control against the log of the concentration of competitor.](image-url)
Table II

Competition between WT apo A-I and its mutants for
binding sites on the surface of J774 cells

<table>
<thead>
<tr>
<th>Apo-A-l</th>
<th>( \text{EC}_{50}^a ) (( \mu g ) of apoprotein/ml)</th>
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<tr>
<td>WT</td>
<td>2.9± 1.2</td>
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<tr>
<td>( \Delta )190-243</td>
<td>83 ± 3.5</td>
</tr>
<tr>
<td>( \Delta )223-243</td>
<td>540 ± 55</td>
</tr>
<tr>
<td>Y18P</td>
<td>6.4± 1.4</td>
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\( ^a \)EC\(_{50}\) values were derived from competitive binding experiments (mean ± SD, Fig.6) by fitting the % of bound apoprotein at different competitor concentrations to a one-site competition equation.