ABCA1 and amphipathic apolipoproteins form high affinity molecular complexes required for cholesterol efflux

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**Running title:** ABCA1 binds amphipathic helices
Summary

Apolipoproteins, such as apolipoprotein A-I (apoA-I), can stimulate cholesterol efflux from cells expressing the ABC transporter, ABCA1. The nature of the molecular interaction between these cholesterol acceptors and ABCA1 is controversial, and models suggesting a direct protein-protein interaction or indirect association have been proposed. To explore this issue, we performed competition binding and chemical cross-linking assays using six amphipathic plasma proteins and an 18 amino acid amphipathic helical peptide. All seven proteins stimulated lipid efflux and competed the cross-linking of apoA-I to ABCA1. Cross-linking of apoA-I to ABCA1 was saturable and occurred at high affinity (Kd of 7.0±1.9 nM), as was cross-linking of apoA-II. Following binding to ABCA1, apoA-I rapidly dissociated (t1/2 25min) from the complex and was released back into the media. A mutant form of ABCA1 (W590S) that avidly binds A-I, but fails to promote cholesterol efflux, released apoA-I with similar kinetics but without transfer of cholesterol to apoA-I. Thus, a high affinity, saturable, protein-protein interaction occurs between ABCA1 and all of its amphipathic protein ligands. Dissociation of the complex leads to the cellular release of cholesterol and the apolipoprotein. However, dissociation is not dependent on cholesterol transfer, which is a clearly separable event, distinguishable by ABCA1 mutants.
**Abbreviations footnote:** ABCA1, ATP binding cassette transporter A1; apoA-I, apolipoproteinA-I; DSG, disuccinimidyl glutarate; DSP, dithiobis(succinimidylpropionato).
Introduction:

Cellular cholesterol homeostasis is critical to normal human physiology and when disrupted can lead to devastating health consequences. As part of this homeostatic mechanism, cells engage an efflux process that promotes the release of excess cellular cholesterol. This efflux mechanism involves activation of an ABC transporter, ABCA1, and transfer of cholesterol to lipid-poor acceptor apolipoproteins, such as apolipoproteinA-I (1). Following binding of apoA-I to cells, the apolipoprotein is released, along with extracted phospholipid and cholesterol, forming a nascent high density lipoprotein (HDL). As individuals harboring non-functioning mutations in both ABCA1 alleles have little or no circulating HDL, this process appears to be critical for normal lipoprotein metabolism. Given the inverse correlation of HDL levels with the risk of developing coronary artery disease, many laboratories have initiated studies to explore the mechanism by which ABCA1 stimulates the movement of lipid out of cells.

Two conceptually divergent models have recently been promulgated to account for ABCA1-mediated cholesterol efflux. The models can best be described as action at a distance vs. direct association (2-6). In the action at a distance model, ABCA1 is postulated to act by flipping phospholipids to the outer leaflet of the plasma bilayer. Subsequently, apoA-I is proposed to bind these translocated phospholipids and then extract both phospholipid and cholesterol in a process that requires no direct interaction between the apolipoprotein and ABCA1. In this model, it is the intrinsic lipid binding properties of the amphipathic helices of apo A-I that are proposed to be the driving force for the microsolubization of a small region of the lipid bilayer. In support of this hypothesis is the finding that the lipid affinity of an amphipathic helical protein positively
correlates with its ability to act as an efflux acceptor (7). Evidence for ABCA1’s role in altering the lipid environment of the plasma membrane comes from observations of enhanced phosphotidylserine expression on ABCA1-expressing cells, as well as an increased susceptibility of membrane cholesterol to oxidation by cholesterol oxidase (2, 8).

In contrast to the action at a distance model, the direct association model proposes that ABCA1 acts as a receptor to which the apoA-I ligand directly binds. This direct interaction is proposed to stimulate ABCA1’s cholesterol efflux activity, resulting in the transfer of cholesterol and phospholipids onto the acceptor apolipoprotein. Evidence for the direct association model comes from chemical cross-linking studies, performed in our laboratory and others, that indicate that apoA-I and ABCA1 are in very close proximity (< 7 angstroms) (3-5). Using mutant forms of ABCA1, we have identified a transporter that cross-links to ABCA1 but does not promote lipid efflux (5), a finding now confirmed by several laboratories (9, 10). However, we have been unable to find a mutant that effluxes cholesterol without being able to cross-link. These data suggest that a binding interaction between apoA-I and ABCA1 is necessary for cholesterol efflux to proceed. If so, the lack of primary amino acid sequence similarity between the many acceptor apolipoproteins that stimulate ABCA1-mediated cholesterol efflux raises questions about the mechanism by which these disparate ligands could functionally activate the same transporter (11).

In this report, we performed more detailed studies of the nature of the molecular interaction between ABCA1 and amphipathic apolipoprotein acceptors. Using competition binding assays coupled to cross-linking, we provide evidence that all of the amphipathic
helical apolipoproteins can compete for a shared interaction with ABCA1. Using both radiolabeled apo A-I and A-II, we show that these interactions are direct, high affinity, and saturable. We also determined that the ABCA1/apoA-I complex has a half-life of less than 30 mins and that the dissociation of this complex is not dependent on the transfer of cholesterol to the acceptor, as had been previously proposed. These data indicate that ABCA1 and its apolipoprotein acceptors form a high affinity receptor-ligand complex whose formation is dependent on the presence of the structural motif of an amphipathic helix in the acceptor. The rapid dissociation of the complex is temporally associated with cholesterol transfer in wild type ABCA1 transporters, but an ABCA1 mutant demonstrates that dissociation does not depend on the transfer of lipid.
Experimental Procedures

Materials-The DSP and DSG cross-linkers (Pierce, Rockford, IL) were used as previously described (5). Cell culture reagents were from Gibco (Rockville, MD). Apolipoproteins and annexin-V were from Calbiochem (San Diego, CA), Biodesign (Saco ME) and Biovision (Mountain View, CA). Radionucleotides were from NEN (Boston, MA).

Efflux and cross-linking assays-Cholesterol efflux assays were performed in 293 cells transfected with wild type and mutant ABCA1 cDNAs, as we reported (5). Efflux acceptors were incubated with ABCA1 expressing cells at 10ug/ml and cross-linking assays conducted as described previously (3, 5). Apo A-I and A-II were radio-labeled with $^{125}$I to a specific activity of ~ 1000 and 1300 cpm/ng, respectively, using Iodo-Beads (Pierce) according to the manufacturer’s instructions. Unincorporated radionucleotides were eliminated by gel filtration and the efficiency of the separation (>99%) was determined by trichloroacetic acid precipitation. Radio-labeled apo A-I and A-II were added to cells at 1ug/ml and unlabeled competitors were used at a 30-fold molar excess. The 18A amphipathic helical peptide (DWLKAFIGKVAKELKEAF) as well as a scramble version (18S, KDVYAFEAKKLLWEDFA) were synthesized with amino terminal acetyl and carboxy terminal amide blocking groups and were purified to greater than 95% using high pressure liquid chromatography (14). Affinity constants for complex formation between ABCA1 and apo A-I or A-II were calculated using Prism GraphPad software (San Diego, CA). Phosphor pixels of cross-linked apolipoprotein were converted to ng of apolipoprotein using equations ($r^2=0.98$) derived from curves of
known amounts of the labeled apolipoproteins imaged in parallel. To demonstrate the
temperature dependence of complex formation, $^{125}$I-apoA-I was incubated with ABCA1
expressing cells for 1h at either 37°C or 4°C, then the cells were chilled to 4°C before
treatment with the cross-linker.

**ABCA1/apo A-I dissociation assays**—To determine the dissociation rate of apoA-I from
the ABCA1/apoA-I complex, cells were incubated with $^{125}$I-apoA-I for 1h at 37°C,
rapidly washed 3X with warm PBS, and then media without apoA-I was added back to
the cell cultures. The cells were either immediately chilled and cross-linked or incubated
at 37°C for specified times prior to cross-linking. From the cross-linking data, the half-life
of complex dissociation was determined using a non-linear regression analysis and
the data was fitted with a one-phase exponential decay equation ($r^2=0.97$, Prism
GraphPad software, San Diego, CA). The amount of apoA-I released from the cells was
determined by directly measuring $^{125}$I in the media. To determine if lipid had been
transferred to the apoA-I, aliquots of media were concentrated and electrophoresed on
12% native polyacrylamide gels. A reduced electrophoretic mobility, compared to that of
delipidated apoA-I, was indicative of lipid uptake. To directly demonstrate that active
cholesterol efflux occurred under the conditions of this assay, cells were labeled with $^{14}$C-
cholesterol and treated with unlabeled apoA-I. The amount of $^{14}$C-cholesterol associated
with the released apoA-I was determined by scintillation counting.
Results:

The exchangeable apolipoproteins that contain amphipathic helices include apo A-I, A-II, C-I, C-II, C-III, and apo E. The roles of apoE and apoA-I in ABCA1-mediated cholesterol efflux have been well documented (7, 12). To confirm that the other apolipoproteins also stimulate ABCA1-mediated lipid efflux, we incubated 293 cells transfected with either a cDNA encoding wild type ABCA1 or an empty vector plasmid (mock) and measured the effect of each apolipoprotein on cholesterol transfer out of the cells (Fig. 1A). All of the apolipoproteins stimulated efflux at least 2.5 fold, comparable to the stimulation seen with apoA-I. This stimulation was seen in cells expressing ABCA1 but not in cells that were mock transfected. These results are similar to those reported by Remaley et. al., who used cells transfected with an ABCA1-green fluorescent chimeric protein (11). Our data confirm these earlier findings and demonstrate that the prior use of the ABCA1-GFP chimera did not result in a promiscuous interaction with multiple apolipoproteins. To test the importance of the amphipathic helical structure in stimulating efflux, we utilized another apolipoprotein, apoH. ApoH associates with HDL, but does so by binding phospholipids through a non-amphipathic mechanism (13). In contrast to the other exchangeable apolipoproteins, apoH was unable to stimulate ABCA1 efflux activity (Fig. 1A).

Having established that a variety of exchangeable apolipoproteins could act as stimulators of ABCA1 efflux activity, we sought to determine whether they might share a similar site of interaction with the transporter. To address this issue, a competition binding assay of apoA-I to ABCA1 was used. As it has proven difficult to measure specific binding of $^{125}$I-apoA-I to ABCA1-expressing cells at 4° C, a cross-linking assay
performed at 37°C was employed, using a thiol-soluble cross-linker, DSP (Fig. 1B). Apo A-I, A-II and C-I were found to block the cross-linking of 125I-apoA-I to ABCA1 by greater than 90%. Apo C-II, C-III and E all competed > 70% of the cross-linking. In a separate assay, the competition of unlabeled apoA-I or apoA-II for radiolabeled apoA-I cross-linking was compared to that seen with apoH (Figure 1B). Again, apo A-I and A-II reduced 125I-apoA-I cross-linking to background levels, while apoH reduced cross-linking by only 22% (Fig. 1B). Annexin-V, which has been reported to bind phospholipids that are translocated to the external leaflet of the plasma membrane by ABCA1 activity, was also tested in this assay (2). Like apoH, annexin-V caused a very modest (< 7%) inhibition of 125I-apoA-I cross-linking (Fig. 1B). Finally, we tested whether a synthetic 18 amino acid amphipathic peptide that has previously been reported to function effectively as an efflux acceptor (14) could compete for apoA-I binding to ABCA1. The amphipathic peptide (18A) competed as well as unlabeled apoA-I, whereas a scrambled peptide (18S), lacking the amphipathic helical motif, did not compete at all (Fig. 1B). These results indicate that apolipoproteins and a peptide containing amphipathic helices are all able to compete for the association of 125I-apoA-I with ABCA1. These data also demonstrate that lipid-binding proteins lacking the amphipathic helical motif, even those whose binding to cells is enhanced by ABCA1 activity (i.e., annexin-V), are not effective competitors of the apoA-I/ABCA1 interaction.

As competition by the amphipathic helical proteins for the cross linking of 125I-apoA-I to ABCA1 could result from interactions between the apolipoproteins themselves, it was important to demonstrate that apolipoproteins other than apoA-I could bind directly to ABCA1. To assay this, one of the exchangeable apolipoproteins, apoA-II, was
radiolabeled and the cross-linking assay repeated. Like $^{125}$I-apoA-I, $^{125}$I-apoA-II was readily cross-linked to ABCA1 using the cross-linking agent, DSP (Fig. 2A). This cross-linking was fully competed by unlabeled apoA-II. The nature of the interaction between apoA-I or apoA-II and ABCA1 was further explored in cross-linking experiments in which $^{125}$I-apoA-I was incubated with ABCA1-expressing cells in the absence or presence of increasing amounts of $^{125}$I-apoA-II. After cross-linking and immunoprecipitation of ABCA1, it was found that increasing the concentration of $^{125}$I-apoA-II resulted in a parallel decrease in cross-linking of $^{125}$I-apoA-I, as cross-linking of $^{125}$I-apoA-II increased (Fig. 2B). Following normalization for differences in the specific activity of the two radio-labeled proteins and their molecular weights, quantification of the cross-linking showed that apoA-II competed with apoA-I for binding to the ABCA1 in a 1 to 1 relationship (Fig. 2B).

To further investigate the specificity of the cross-linking assay, saturation binding experiments were conducted. Cells expressing ABCA1 were incubated with increasing amounts of $^{125}$I-apoA-I for 1 h at 37°C, either alone, or in the presence of a 30-fold molar excess of un-labeled apoA-I. The cells were then washed, chilled and exposed to cross-linker. As seen in Fig. 3A (top panel), the $^{125}$I-apoA-I associated with the immunoprecipitated ABCA1 could be detected at apoA-I concentrations as low as 75 ng/ml. The amount of $^{125}$I-apoA-I cross-linked to ABCA1 saturated at concentrations between 1-2 ug/ml (Fig. 3A, graph). This binding and cross-linking was fully competed by unlabeled apoA-I. In data not shown, the binding of radiolabeled apoA-II to ABCA1 was also found to saturate in the same molar range. Estimations of the apparent affinity of these proteins for ABCA1 produced similar $K_d$’s (7.0±1.9 and 16±7.1 nM, A-I & A-II, respectively).
Total cellular binding of radiolabeled apoA-I was assessed in parallel by directly counting an aliquot of the cellular lysates before immuno-precipitation of ABCA1 (Fig. 3B). While this binding did not clearly saturate at the highest apoA-I concentration used, an apparent $K_d$, assuming saturation at the highest dose, can be calculated. This apparent $K_d$ (137 nM) is approximately 20-fold higher than that found for the cross-linking interaction with ABCA1. In contrast to the cross-linking interaction, the total cellular binding assay results in a substantial amount of cell-associated apoA-I that cannot be competed by excess, unlabeled ligand (Fig. 3B). These results indicate that the binding of radiolabeled apolipoprotein directly to ABCA1 is high affinity, saturable, and readily competed by unlabeled ligand.

To explore further the relationship between cell associated of apoA-I and its association with ABCA1, additional studies were conducted. 293 cells transfected with an empty vector or the ABCA1 cDNA were again incubated with $^{125}$I-apoA-I and the washed cells were subsequently cross-linked. In these experiments, the thiol-resistant, non-reducible DSG cross-linker was employed to maintain the cross-links during SDS-polyacrylamide gel electrophoresis. Following separate immuno-precipitations of aliquots of the lysate, using either an anti-ABCA1 antibody or an anti-apoA-I antibody, SDS-PAGE was performed (Fig. 3C). When the ABCA1 antibody was used for immunoprecipitation, bands representing apoA-I in a high molecular weight complex of a size consistent for the ABCA1/apoA-I complex were identified only in the lysate of cells transfected with the ABCA1 cDNA. In contrast, use of the anti-apoA-I antibody for immunoprecipitation produced a large number of bands in both the ABCA1 and mock transfected cells. The most intense of these bands migrated at the expected molecular
weight of monomeric apoA-I (Figure 3C). While ABCA1 expression modestly increased the total amount of cell-associated apoA-I, >90% of this apoA-I migrated in its monomeric form. Thus, the vast majority of apoA-I bound to cells was not closely associated with any specific cellular protein, as assessed by chemical cross-linking (Fig. 3C).

The fate of the apoA-I/ABCA1 complexes was next examined. Previously, we had demonstrated that a mutant form of ABCA1, containing a missense mutation resulting in the substitution of serine for tryptophan at amino acid position 590, produced a transporter that had greater apoA-I cross-linking activity than wild type ABCA1. The mutant failed to efflux cholesterol normally (5). This mutant (W590S) retains the temperature dependence of cross-linking we previously demonstrated for wild type ABCA1 (Figure 4A), suggesting that its binding interaction with apoA-I mirrors that of the wild type protein. The dissociation of apoA-I from both wild type ABCA1 and ABCA1(W590S) was therefore tested. Cells expressing ABCA1, the W590S mutant, or no ABCA1 (mock) were exposed to \(^{125}\)I-apoA-I at 37°C for 1 h. The cells were then rapidly washed with warm PBS and media lacking \(^{125}\)I-apoA-I was added back to the cells. At 0 mins following the wash, a set of cells from each condition were chilled on ice and processed in the cross-linking reaction as usual. Other groups of cells were allowed to incubate for specified times at 37°C before being chilled and processed. Media from all of the cell samples were removed and retained for analysis of released \(^{125}\)I-apoA-I at the time of cell chilling. Figure 4B (top panels) shows the amount of \(^{125}\)I-apoA-I associated with wild type ABCA1 or the W590S mutant as assessed by the cross-linking assay. Although the W590S mutant was found to be associated with substantially more
radiolabeled apoA-I at time zero, its dissociation rate from the apoprotein (t\(_{1/2}\) of 30.1 mins) did not differ markedly from that measured for the wild type transporter (t\(_{1/2}\) of 24.6 min (Fig. 4B, graph).

We next determined if the dissociation of the apoA-I/ABCA1 complex was kinetically linked to the transfer of cholesterol to apoA-I. Cells expressing wild type ABCA1 released significantly more radiolabeled apoA-I compared to the mock-transfected cells (Figure 4C, and data not shown). The release of apoA-I from the cells mirrored the decay of the apoA-I/ABCA1 complex, with the release of apoA-I reaching a plateau by 240 min. 50% of the maximal amount of A-I released occurred within the first 40 min. Cells expressing the W590S mutant also released the apoA-I back into the media at a similar rate. These results, along with the measurements of the kinetics of dissociation described above, indicate that the cholesterol efflux defect in the W590S mutant cannot be accounted for by either a failure of the apoA-I/ABCA1 complex to dissociate or an ectopic release of apoA-I. Significantly, the apoA-I released from cells expressing wild type ABCA1 migrated more slowly on native gels than did the apoA-I released from cells expressing the mutant (Fig. 4D). The apoA-I released from the cells expressing the W590S mutant migrated at the expected molecular weight of lipid-depleted, monomeric apoA-I (Fig. 4D). These results suggested that the apoA-I released by cells expressing wild type ABCA1 had acquired lipid, whereas the apoA-I released from cells expressing the W590S mutant had not. To confirm this, cells were labeled with \(^{14}\)C-cholesterol and the experiment repeated using unlabeled apoA-I. As expected, \(^{14}\)C-cholesterol was found in increased amounts in the pool of apoA-I released by cells expressing the wild type transporter. Cells that were either mock-transfected or expressed
the W590S mutant had similarly low levels of $^{14}$C-cholesterol associated with apoA-I in the media (Fig. 4D, graph). As the W590S mutant had a similar impairment in its ability to transfer phosphatidylcholine to apoA-I (data not shown), the mutant does not provide a tool for dissociating the mechanisms of efflux of these two lipids.
Discussion

In this report, we have investigated the molecular interaction between ABCA1 and a series of exchangeable apolipoproteins that can stimulate cholesterol efflux from cells. Apolipoproteins that contained amphipathic helices were able to block the cross-linking of radiolabeled apoA-I to ABCA1, whereas non-amphipathic lipid binding proteins were not. These effects appear to involve competition for direct binding to ABCA1, as we were able to demonstrate direct cross-linking of another apolipoprotein, radiolabeled apoA-II, to the transporter. This cross-linking was also competed by unlabeled ligand. The cross-linking assay permitted us to measure an apparent $K_d$ of 7±1.9 nM for the interaction between apo A-I and ABCA1. The interaction of apo A-II with ABCA1 was of a similar affinity to that measured with apoA-I (apparent $K_d$ of 16±7.1 nM), with saturation of cross-linking occurring with both ligands at a concentration of ~1 ug/ml. When both radiolabeled apoA-II and apoA-I were used in a competitive cross-linking assay, the former cross-linked to ABCA1, competing the latter in a 1 to 1 molar relationship, assuming that apoA-II bound the transporter as either a dimer or two monomers. These results, taken together, provide strong evidence that the apolipoproteins compete for binding to a shared region on the transporter. The amphipathic helix itself appears to be the structural element that allows an apolipoprotein to interact with ABCA1, as an 18 amino acid peptide that has been demonstrated to adopt an amphipathic helical conformation was also able to efficiently compete for the binding of apoA-I to ABCA1 (14).

Our work indicates that the various efflux acceptors, including a synthetic 18A peptide, are able to compete for the direction interaction of apoA-I with ABCA1, despite
substantial differences in their primary amino acid sequence. Indeed, although the exchangeable apolipoproteins are thought to have evolved from a common ancestor gene they vary greatly in their overall length and only share from 20 to 30% amino acid identity (15). Despite this divergence the exchangeable apolipoproteins have strongly conserved their amphiphatic helical character. We propose that the binding of an efflux acceptor to ABCA1 relies on this amphiphatic helical structure, with its separation of hydrophilic and hydrophobic surfaces on opposite sides of the helix. That an amphiphatic helical motif can participate in protein-protein interactions that are not strongly dependent upon primary sequence has a precedent in the interaction between the regulatory subunits of cAMP-dependent protein kinase (PKA) and its anchoring proteins (16). Like ABCA1, PKA is also able to form high affinity complexes (~Kd of 4 nM) with a variety of docking proteins (17). These interactions are dependent upon the docking protein containing an amphiphatic helix. Nuclear magnetic resonance solution structures have provided structural evidence for the plasticity of this interaction by showing that the hydrophobic face of the docking protein amphiphatic helix is bound by a hydrophobic groove on the regulatory subunit of PKA (18). On the regulatory subunit of PKA the groove is formed by a X-type four helix bundle that presents a hydrophobic patch with which the hydrophobic face of the amphiphatic helix interacts. Whether ABCA1 is able to bind amphiphatic helices through a similar hydrophobic patch remains to be determined. Clearly, additional studies identifying the residues on ABCA1 involved in the cross-linking reactions will be helpful in characterizing the mechanism by which ABCA1 directly interacts with the various efflux acceptors.
To characterize how complex formation relates to the release of apoA-I and cholesterol efflux, we developed an off-rate protocol that measures the dissociation of the apoA-I/ABCA1 complex. For wild type ABCA1, we found that the complex dissociated with a half-life of 25 min. As the amount of apoA-I released reached a half maximum, by 40 min, these kinetics are consistent with the hypothesis that the released, lipid enriched apoA-I derives exclusively from the dissociation of the transporter/apoprotein complex. By 4 hours, no further release of apoA-I was detectable, consistent with the reduction of the apoA-I/ABCA1 complex by more than 95%. For cells expressing wild type ABCA1, the released apoA-I contained cholesterol, though lipid transfer was not necessary for release (see below). We also found that cell association measurements for apoA-I binding had a nearly 20-fold lower affinity (137 nM vs 7 nM, respectively) than that measured for the specific cross-linking of apoA-I to ABCA1. This measurement is in good accord with a recent report by Gillotte-Taylor, et. al. that showed the rate constant for cholesterol efflux to be 25 nM, as compared to a Kd for apoA-I cellular binding of 700 nM (19). In contrast, Remaley et. al reported a higher affinity binding site (22 nM) for apoA-I in cells expressing ABCA1 than either we or Gillote-Taylor et al could measure using standard radiolabeled binding assays (11). At present, the relationship between such high affinity binding sites and the cross-linking complex we describe in this report is unclear, as we have been unable to demonstrate formation of the cross-linking complex when the initial apoA-I cell binding assay is carried out at 4°C.

In our studies we compared the dissociation behavior of wild type ABCA1/apoA-I complexes to those formed with the W590S ABCA1 mutant. The latter is fully competent to form a complex with apoA-I but it fails to efflux cholesterol normally. The
W590S mutant, like wild type ABCA1, was not able to form a cross-linkable complex with apoA-I at 4°C. At 37°C, apoA-I dissociated from the W590S mutant at a rate similar to that measured using the wild type ABCA1 (t_{1/2} 30 vs. 25 min, respectively). However, only wild type ABCA1 was able to transfer cholesterol to the released apoA-I. These results suggest the W590S mutant binds and releases apoA-I in a normal manner, but that these processes are uncoupled from the transfer of cholesterol to the released apoA-I. Our results indicate the W590S mutant has a similar impairment in the transfer of phospholipid, and as reported by Rigot et. al, the mutant does not stimulate the translocation of phosphatidylserine to the outer leaflet of the plasma membrane (9). These results are of significance, as it has been speculated that the release of apoA-I from ABCA1 might be a consequence of the transfer of lipid to the apolipoprotein by ABCA1, with a consequent reduction in binding affinity (1). If this mechanism were operative, it seems unlikely that theapoA-I would dissociate from the W590S mutant with the kinetics we have measured. The behavior of the W590S mutant/apoA-I complex indicates that cellular release of apoA-I and transfer of cholesterol and phospholipid are separable events, and thus are likely distinct steps in the efflux mechanism.

In summary, our results support an efflux model in which ABCA1 can form specific high affinity complexes with a variety of apolipoproteins. The amphipathic helix was found to be the structural motif that determined an apolipoprotein’s ability to form a complex with ABCA1. The competition studies suggest that all of the amphipathic apolipoproteins could be directly interacting with common site on the transporter, possibly a hydrophobic patch, but more detailed structural analysis of the ABCA1 binding locus will be needed before that conclusion can be made with certainty. Our
results do not appear to be compatible with an indirect model of efflux, where ABCA1 merely flips lipids in the plasma bilayer that are subsequently micro-solublized by apoA-I (2, 7, 20). A more recently proposed hybrid model, in which apoA-I first interacts with the lipid bilayer and then, though lateral diffusion, subsequently forms a complex with ABCA1, is in better agreement with our data (6). This model could also explain our inability to measure cross-linking complexes when the apolipoprotein binding assay is done at temperatures below that which permit the apolipoprotein to diffuse in the membrane. Our work has measured the affinity of binding of apoA-I to ABCA1 and determined it to be nanomolar. We have also determined the dissociation kinetics for the apolipoprotein/transporter complex, demonstrating that its half-life is 25-30 minutes. Following complex formation, the apolipoprotein is released from wild type ABCA1 with its associated lipid. The W590S ABCA1 mutant demonstrates that this release is not dependent on cholesterol transfer to the acceptor apolipoprotein, indicating that release and cholesterol transfer are separable events in the efflux process. These results form the basis for future studies aimed at exploring the molecular mechanism of ABCA1 mediated cholesterol efflux to apolipoprotein acceptors.

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References:


Figure legends:

Fig. 1. **ABCA1 efflux acceptors require amphipathic helices and compete for the cross-linking of $^{125}$I-apoA-I to ABCA1.**

A. Various exchangeable apolipoproteins were tested for the ability to act as cholesterol acceptors of ABCA1 efflux activity. 293 cells loaded with $^3$H-cholesterol and expressing empty vector (mock) or a wild type ABCA1 cDNA (ABCA1) were incubated with 10 ug/ml of the indicated apolipoproteins for 4 h. Percent cholesterol effluxed [media counts/(media + cell associated counts)] was determined and graphed (triplicate samples, ± SD).

B. Exchangeable apolipoproteins with amphipathic helices compete for the cross-linking of $^{125}$I-apoA-I to ABCA1. Mock or ABCA1 expressing cells were incubated for 1 h at $37^\circ$C with $^{125}$I-apoA-I alone, or in the presence of a 30-fold molar excess of the indicated unlabeled apolipoproteins, annexin-V (an-V), an 18 amino acid amphipathic peptide (18A), or a 18 amino acid peptide of identical sequence that was scrambled to eliminate the amphipathic structure (18S). $^{125}$I-apoA-I associated with ABCA1 was assessed by cross-linking and immuno-precipitation of ABCA1. The associated apoA-I was visualized and quantitated by phosphor imaging (duplicate samples, ±SD). Results are representative of two or more experiments.

Fig. 2. **ApoA-II competes with apoA-I for direct binding to ABCA1 in a 1:1 molar fashion.**

A. ApoA-II directly interacts with ABCA1. Mock or ABCA1 expressing cells were incubated for 1 h at $37^\circ$C with $^{125}$I-apoA-I and $^{125}$I-apoA-II alone, or in the presence of a 30-fold molar excess of the respective unlabeled apolipoprotein. Shown is the amount of labeled apolipoprotein associated with ABCA1 as determined by cross-linking and immuno-precipitation of ABCA1.

B. ApoA-II cross-linking to ABCA1 competes the
cross-linking of apoA-I. $^{125}$I-apoA-I was incubated with ABCA1 expressing cells either alone or in the presence of increasing amounts of $^{125}$I-apoA-II. After cross-linking, the apolipoproteins associated with ABCA1 were isolated by immuno-precipitation of ABCA1 and visualized by phosphor imaging (top panel). Graphed below is the molar ratio of input apoA-II to A-I vs. the molar ratio of cross-linked apoA-II to A-I after normalization for the difference in specific activity and molecular mass of an apoA-II dimer. A linear equation with a slope of 1 fit this data with an $r^2=.9989$. Results are representative of two or more experiments.

Fig. 3. ApoA-I shows saturable binding to ABCA1. A. ApoA-I cross-linking to ABCA1 is high affinity and saturable. ABCA1 expressing cells were incubated with increasing amounts of $^{125}$I-apoA-I alone, or in the presence of a 30-fold excess of unlabeled apoA-I at 37°C for 1h. ABCA1 associated apoA-I is shown in the top panels with the amount of associated apoA-I graphed below. B. Total cell binding of apoA-I is not highly correlated with cross-linking. Total cell association of $^{125}$I-apoA-I with the ABCA1-expressing cells was determined as above by counting an aliquot of the cell lysates before immuno-precipitation of ABCA1. Graphed is the amount total cell associated $^{125}$I-apoA-I, the amount of $^{125}$I-apoA-I associated in the presence of a 30-fold molar excess of unlabeled apoA-I, and the difference between these values (specific). C. Expression of ABCA1 facilitates cell association of $^{125}$I-apoA-I but the majority of the associated apoA-I is not closely associated with any specific protein. Mock or ABCA1 expressing cells were incubated with $^{125}$I-apoA-I as above and the cells were cross-linked with the non-reducible DSG cross-linker. ApoA-I associated with ABCA1 was detected
by immuno-precipitating ABCA1 and total cell associated apoA-I was assessed by immuno-precipitating apoA-I. Specificity of the apoA-I immuno-precipitation is demonstrated by the lack of $^{125}$I-apoA-I brought down in an immuno-precipitation using normal rabbit antibody (IgG). The apoA-I/ABCA1 complex as well as monomeric apoA-I are indicated. Results are representative of two or more experiments.

Fig. 4. **ApoA-I associates with the ABCA1 W590S mutant in a temperature dependent manner and is released from the mutant complex with kinetics similar to the release from wtABCA1.**

A. ABCA1 and the W590S mutant display a similar temperature sensitivity of complex formation. WtABCA1, W590S mutant, or empty vector (mock)-expressing cells were incubated with $^{125}$I-apoA-I either at 37 or 4°C for one hour. The cells incubated at 37°C were then chilled on ice and along with the 4°C samples exposed to the DSP cross-linker for 2 h. All incubations were maintained on ice during the cross-linking reaction. ABCA1-associated $^{125}$I-apoA-I was then assessed by immuno-precipitation of ABCA1 and phosphor imaging. B. ApoA-I complexes between wild type ABCA1 and the W590S mutant turn over at a similar rate. Cells expressing ABCA1 or the W590S mutant were incubated with $^{125}$I-apoA-I at 37°C for 1 h and then washed with warm PBS. Media without apoA-I was substituted and the cells were either immediately chilled and exposed to cross-linker (0 h) or incubated for additional times at 37°C as indicated. ABCA1 associated apoA-I is shown in the top panels as determined by cross-linking and the percent of the complex remaining relative to the 0 h samples is graphed below (duplicate samples, ±SD). C. ABCA1 and W590S mutant cells release cell-associated apoA-I with similar kinetics. $^{125}$I-apoA-I released during the off-rate
experiment was quantified by scintillation counting of the media that was removed from the cells before the addition of cross-linker. Graphed is the amount of $^{125}$I-apoA-I released from wtABCA1 and W590S-expressing cells relative to the mock cells (duplicate samples, ±SD) D. Only apoA-I released from wild type ABCA1 is associated with cholesterol. The lipidation state of the released $^{125}$I-apoA-I was assessed by pooling and concentrating the media from the last three time points and electrophoresing the pooled samples on native polyacrylamide gels (left panel). Shown is a phosphor image of the resulting gel. Efflux activity was confirmed by labeling cells with $^{14}$C-cholesterol and conducting a dissociation experiment with un-labeled apoA-I. The graph shows the amount of $^{14}$C-cholesterol that was associated with the released apoA-I as determined by scintillation counting of the samples. Results are representative of two or more experiments.
Fig. 1

A

X-linked apoA-I (125I pixels X 1000)

cold competitor

apoAI

apoH

apoE

apoAI

apoH

apoE

B

percent cholesterol efflux

apoAI

apoH

apoE

apoAI

apoH

apoE

apoAI

apoH

apoE

apoAI

apoH

apoE

apoAI

apoH

apoE

apoAI

apoH

apoE

apoAI

apoH

apoE

apoAI

apoH

apoE
Figure 2
Figure 3
Figure 4