

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

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**Abstract** Apolipoproteins, such as apolipoprotein A-I (apoA-I), can stimulate cholesterol efflux from cells expressing the ATP binding cassette transporter A1 (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 inhibited the cross-linking of apoA-I to ABCA1. Cross-linking of apoA-I to ABCA1 was saturable and occurred at high affinity ( $K_d$  of  $7.0 \pm 1.9$  nM), as was cross-linking of apoA-II. After binding to ABCA1, apoA-I rapidly dissociated (half-life of 25 min) from the complex and was released back into the medium. A mutant form of ABCA1 (W590S) that avidly binds apoA-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.—Fitzgerald, M. L., A. L. Morris, A. Chroni, A. J. Mendez, V. I. Zannis, and M. W. Freeman. ABCA1 and amphipathic apolipoproteins form high-affinity molecular complexes required for cholesterol efflux. *J. Lipid Res.* 2004. 45: 287–294.

**Supplementary key words** ATP binding cassette transporter A1 • Tangier Disease • high density lipoprotein

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 the ATP binding cassette transporter A1 (ABCA1) and transfer of cholesterol to lipid-poor acceptor apolipoproteins, such as apolipoprotein A-I (apoA-I) (1). After binding of apoA-I to cells, the apolipoprotein is released, along with extracted phospholipid and cholesterol, forming a nascent HDL. As individuals harboring nonfunctioning 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 versus 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 apoA-I that are proposed to be the driving force for the microsolvubilization 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 phosphatidylserine expression on ABCA1-expressing cells as well as an increased

Abbreviations: ABCA1, ATP binding cassette transporter A1; apoA-I, apolipoprotein A-I; DSG, disuccinimidyl glutarate; DSP, dithiobis(succinimidylpropionate).

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Manuscript received 20 August 2003 and in revised form 23 October 2003.

Published, JLR Papers in Press, November 16, 2003.  
DOI 10.1194/jlr.M300355-JLR200

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 binds directly. 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 ( $<7 \text{ \AA}$ ) (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 apoA-I and apoA-II, we show that these interactions are direct, of high affinity, and saturable. We also determined that the ABCA1/apoA-I complex has a half-life of less than 30 min 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 dithiobis(succinimidylpropionate) (DSP) and disuccinimidyl glutarate (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

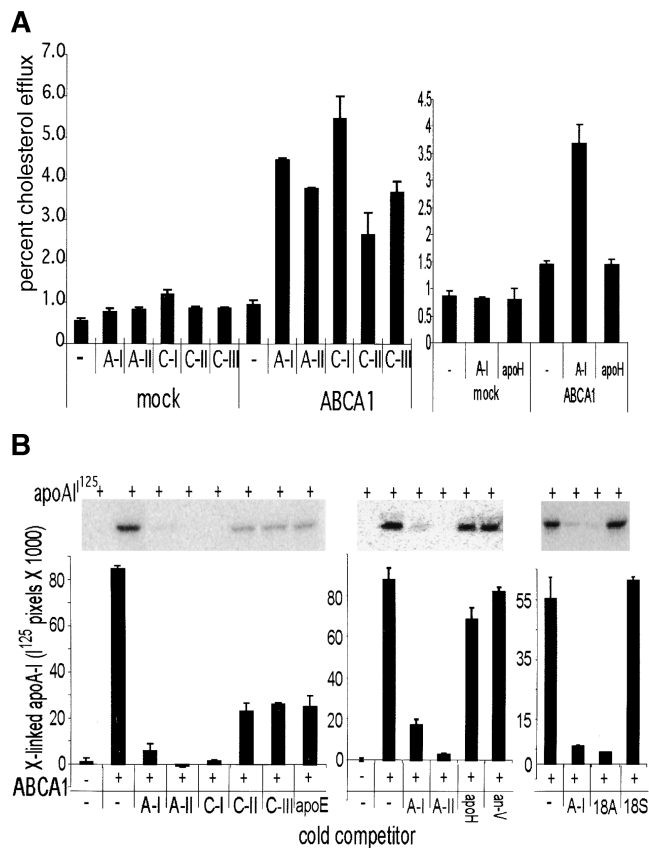
(5). Efflux acceptors were incubated with ABCA1-expressing cells at  $10 \mu\text{g/ml}$ , and cross-linking assays conducted as described previously (3, 5). ApoA-I and apoA-II were radiolabeled with  $^{125}\text{I}$  to a specific activity of  $\sim 1,000$  and  $1,300 \text{ cpm/ng}$ , respectively, 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. Radiolabeled apoA-I and apoA-II were added to cells at  $1 \mu\text{g/ml}$ , and unlabeled competitors were used at a 30-fold molar excess. The 18A amphipathic helical peptide (DWLKAIFYDKVAEKLKEAF) as well as a scramble version (18S; KDVYAFEAKKKLLWEDFA) were synthesized with amino-terminal acetyl and carboxyl-terminal amide blocking groups and were purified to greater than 95% using HPLC. Affinity constants for complex formation between ABCA1 and apoA-I or apoA-II were calculated using Prism GraphPad software (San Diego, CA). Phosphor pixels of cross-linked apolipoprotein were converted to nanograms 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,  $^{125}\text{I}$ -apoA-I was incubated with ABCA1-expressing cells for 1 h at either  $37^\circ\text{C}$  or  $4^\circ\text{C}$ , and the cells were chilled to  $4^\circ\text{C}$  before treatment with the cross-linker.

### ABCA1/apoA-I dissociation assays

To determine the dissociation rate of apoA-I from the ABCA1/apoA-I complex, cells were incubated with  $^{125}\text{I}$ -apoA-I for 1 h at  $37^\circ\text{C}$  and rapidly washed three times with warm PBS, and then medium without apoA-I was added back to the cell cultures. The cells were either immediately chilled and cross-linked or incubated at  $37^\circ\text{C}$  for specified times before cross-linking. From the cross-linking data, the half-life of complex dissociation was determined using nonlinear regression analysis, and the data were fitted with a one-phase exponential decay equation ( $r^2 = 0.97$ ; Prism GraphPad). The amount of apoA-I released from the cells was determined by directly measuring  $^{125}\text{I}$  in the medium. To determine if lipid had been transferred to the apoA-I, aliquots of medium were concentrated and electrophoresed on 12% native polyacrylamide gels. A reduced electrophoretic mobility, compared with 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  $^{14}\text{C}$ cholesterol and treated with unlabeled apoA-I. The amount of  $^{14}\text{C}$ cholesterol associated with the released apoA-I was determined by scintillation counting.

## RESULTS

The exchangeable apolipoproteins that contain amphipathic helices include apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, and apoE. 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 compared with 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. (11), who used cells transfected

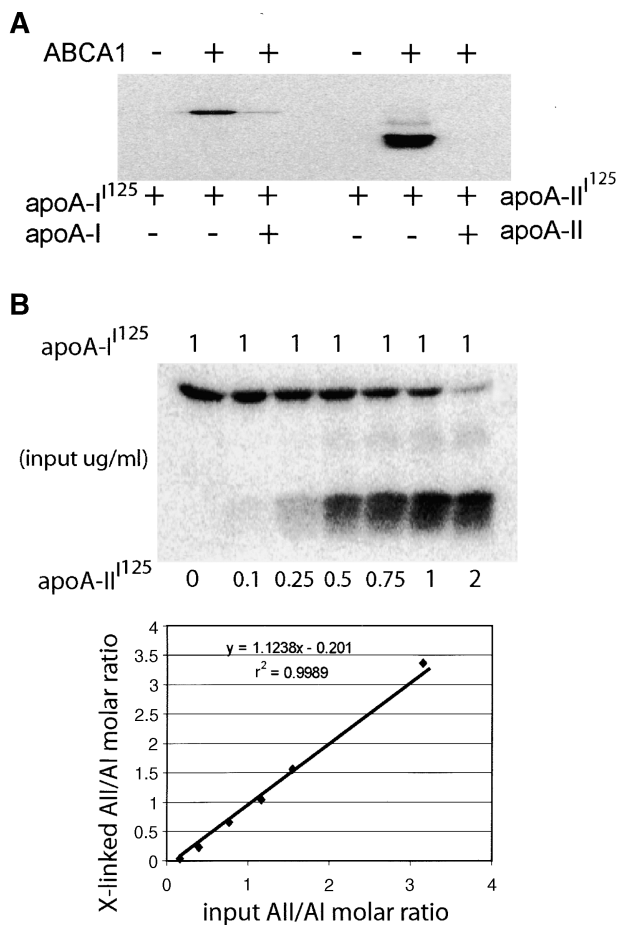


**Fig. 1.** ATP binding cassette transporter A1 (ABCA1) efflux acceptors require amphipathic helices and compete for the cross-linking of  $^{125}\text{I}$ -apolipoprotein A-I ( $^{125}\text{I}$ -apoA-I) to ABCA1. **A:** Various exchangeable apolipoproteins were tested for the ability to act as cholesterol acceptors of ABCA1 efflux activity. A: 293 cells loaded with [ $^3\text{H}$ ]cholesterol and expressing empty vector (mock) or a wild-type ABCA1 cDNA (ABCA1) were incubated with 10  $\mu\text{g}/\text{ml}$  of the indicated apolipoproteins for 4 h. Percentage cholesterol effluxed [media counts/(media + cell-associated counts)] was determined and graphed (triplicate samples,  $\pm\text{SD}$ ). **B:** Exchangeable apolipoproteins with amphipathic helices compete for the cross-linking of  $^{125}\text{I}$ -apoA-I to ABCA1. Mock or ABCA1-expressing cells were incubated for 1 h at  $37^\circ\text{C}$  with  $^{125}\text{I}$ -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 an 18-amino acid peptide of identical sequence that was scrambled to eliminate the amphipathic structure (18S).  $^{125}\text{I}$ -apoA-I associated with ABCA1 was assessed by cross-linking and immunoprecipitation of ABCA1. The associated apoA-I was visualized and quantitated by phosphorimaging (duplicate samples,  $\pm\text{SD}$ ). Results are representative of two or more experiments.

with an ABCA1-green fluorescent chimeric protein. Our data confirm these earlier findings and demonstrate that the prior use of the ABCA1-green fluorescent protein chimera did not result in a promiscuous interaction with multiple apolipoproteins. To test the importance of the amphipathic helical structure in stimulating efflux, we used another apolipoprotein, apoH. ApoH associates with HDL but does so by binding phospholipids through a nonamphipathic 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 the specific binding of  $^{125}\text{I}$ -apoA-I to ABCA1-expressing cells at  $4^\circ\text{C}$ , a cross-linking assay was performed at  $37^\circ\text{C}$  using a thiol-soluble cross-linker, DSP (Fig. 1B). ApoA-I, apoA-II, and apoC-I were found to block the cross-linking of  $^{125}\text{I}$ -apoA-I to ABCA1 by greater than 90%. ApoC-II, apoC-III, and apoE all competed for more than 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 with that seen with apoH (Fig. 1B). Again, apoA-I and apoA-II reduced  $^{125}\text{I}$ -apoA-I cross-linking to background levels, whereas 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  $^{125}\text{I}$ -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  $^{125}\text{I}$ -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  $^{125}\text{I}$ -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  $^{125}\text{I}$ -apoA-I,  $^{125}\text{I}$ -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  $^{125}\text{I}$ -apoA-I was incubated with ABCA1-expressing cells in the absence or presence of increasing amounts of  $^{125}\text{I}$ -apoA-II. After cross-linking and immunoprecipitation of ABCA1, it was found that increasing the concentration of  $^{125}\text{I}$ -apoA-II resulted in a parallel decrease in cross-linking of  $^{125}\text{I}$ -apoA-I, as cross-linking of  $^{125}\text{I}$ -apoA-II increased (Fig. 2B). After normalization for differences in the specific activity of the two radiolabeled proteins and their molecular weights, quantification of the cross-linking showed that apoA-II competed with



**Fig. 2.** ApoA-II competes with apoA-I for direct binding to ABCA1 in a 1:1 molar ratio. **A:** ApoA-II directly interacts with ABCA1. Mock or ABCA1-expressing cells were incubated for 1 h at 37°C with <sup>125</sup>I-apoA-I and <sup>125</sup>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 immunoprecipitation of ABCA1. **B:** ApoA-II cross-linking to ABCA1 competes for the cross-linking of apoA-I. <sup>125</sup>I-apoA-I was incubated with ABCA1-expressing cells either alone or in the presence of increasing amounts of <sup>125</sup>I-apoA-II. After cross-linking, the apolipoproteins associated with ABCA1 were isolated by immunoprecipitation of ABCA1 and visualized by phosphorimaging (top panel). Graphed below is the molar ratio of input apoA-II to A-I versus the molar ratio of cross-linked apoA-II to apoA-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 these data ( $r^2 = 0.9989$ ). Results are representative of two or more experiments.

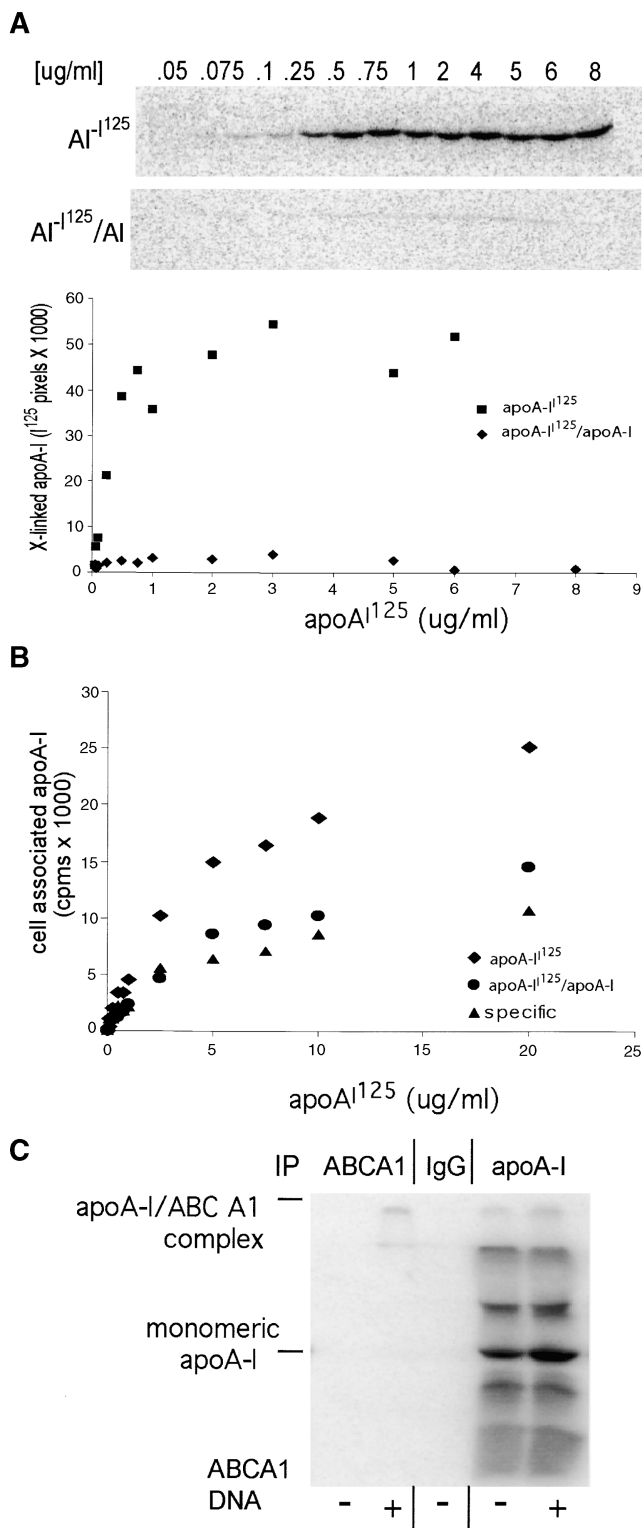
apoA-I for binding to the ABCA1 in a 1:1 relationship (Fig. 2B).

To further investigate the specificity of the cross-linking assay, saturation binding experiments were conducted. Cells expressing ABCA1 were incubated with increasing amounts of <sup>125</sup>I-apoA-I for 1 h at 37°C either alone or in the presence of a 30-fold molar excess of unlabeled apoA-I. The cells were then washed, chilled, and exposed to cross-linker. As seen in **Fig. 3A** (top panel), the <sup>125</sup>I-apoA-I associated with the immunoprecipitated ABCA1 could be detected at apoA-I concentrations as low as 75 ng/ml. The

amount of <sup>125</sup>I-apoA-I cross-linked to ABCA1 saturated at concentrations between 1 and 2  $\mu\text{g/ml}$  (Fig. 3A, graph). This binding and cross-linking were fully competed by unlabeled apoA-I. The binding of radiolabeled apoA-II to ABCA1 was also found to saturate in the same molar range (data not shown). Estimations of the apparent affinity of these proteins for ABCA1 produced similar  $K_d$  values ( $7.0 \pm 1.9$  nM and  $16 \pm 7.1$  nM for apoA-I and apoA-II, respectively). Total cellular binding of radiolabeled apoA-I was assessed in parallel by directly counting an aliquot of the cellular lysates before immunoprecipitation of ABCA1 (Fig. 3B). Although 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  $\sim 20$ -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 of high affinity, saturable, and readily competed by unlabeled ligand.

To explore further the relationship between cell-associated apoA-I and its association with ABCA1, additional studies were conducted. A total of 293 cells transfected with an empty vector or the ABCA1 cDNA were again incubated with <sup>125</sup>I-apoA-I, and the washed cells were subsequently cross-linked. In these experiments, the thiol-resistant, nonreducible DSG cross-linker was used to maintain the cross-links during SDS-PAGE. After separate immunoprecipitations 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 with 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 (Fig. 3C). Although ABCA1 expression modestly increased the total amount of cell-associated apoA-I, more than 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 (**Fig. 4A**), suggesting that its binding interaction

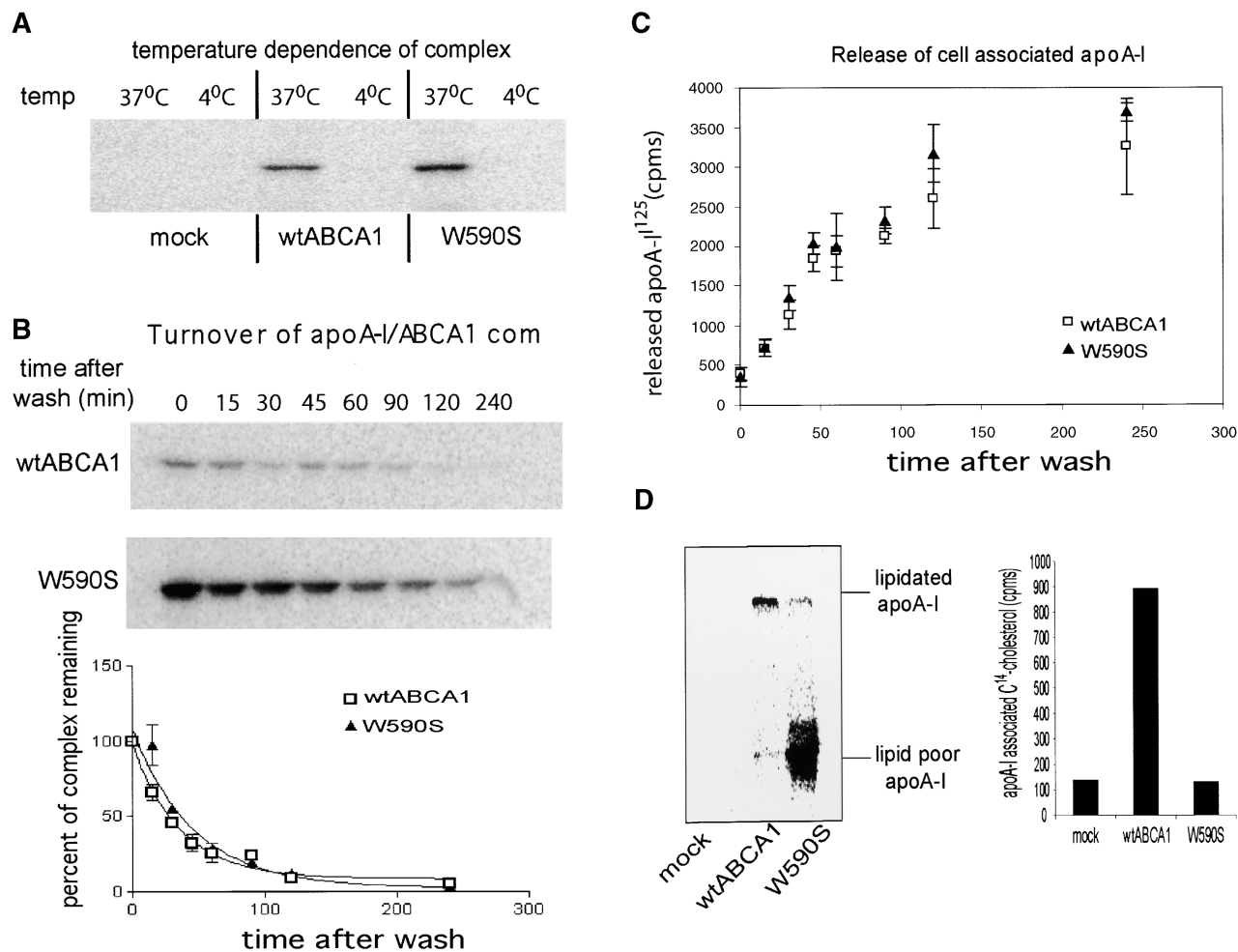


**Fig. 3.** ApoA-I shows saturable binding to ABCA1. **A:** ApoA-I cross-linking to ABCA1 is of high affinity and saturable. ABCA1-expressing cells were incubated with increasing amounts of <sup>125</sup>I-apoA-I alone or in the presence of a 30-fold excess of unlabeled apoA-I at 37°C for 1 h. ABCA1-associated 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 <sup>125</sup>I-apoA-I with the ABCA1-expressing cells was determined as in **A** by counting an aliquot of the cell lysates before immunoprecipitation of ABCA1. Graphed is the amount of total cell-associated <sup>125</sup>I-apoA-I, the amount of <sup>125</sup>I-apoA-I associated

with apoA-I mirrors that of the wild-type protein. Thus, the dissociation of apoA-I from both wild-type ABCA1 and ABCA1 (W590S) was tested. Cells expressing ABCA1, the W590S mutant, or no ABCA1 (mock) were exposed to <sup>125</sup>I-apoA-I at 37°C for 1 h. The cells were then rapidly washed with warm PBS, and medium lacking <sup>125</sup>I-apoA-I was added back to the cells. At 0 min after 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 <sup>125</sup>I-apoA-I at the time of cell chilling. Fig. 4B (top panels) shows the amount of <sup>125</sup>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 apolipoprotein (half-life of 30.1 min) did not differ markedly from that measured for the wild-type transporter (half-life 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 with the mock-transfected cells (Fig. 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. Fifty percent of the maximal amount of apoA-I released was released within the first 40 min. Cells expressing the W590S mutant also released the apoA-I back into the medium 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

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 the cell association of <sup>125</sup>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 <sup>125</sup>I-apoA-I as in **A**, and the cells were cross-linked with the nonreducible disuccinimidyl glutarate cross-linker. ApoA-I associated with ABCA1 was detected by immunoprecipitating ABCA1, and total cell-associated apoA-I was assessed by immunoprecipitating apoA-I. The specificity of the apoA-I immunoprecipitation is demonstrated by the lack of <sup>125</sup>I-apoA-I brought down in an immunoprecipitation 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. IP, immunoprecipitant.



**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 wild-type ABCA1 (wtABCA1). **A:** ABCA1 and the W590S mutant display a similar temperature sensitivity of complex formation. Wild-type ABCA1, the W590S mutant, or empty vector (mock)-expressing cells were incubated with <sup>125</sup>I-apoA-I at either 37°C or 4°C for 1 h. The cells incubated at 37°C were then chilled on ice and, along with the 4°C samples, exposed to the dithio-bis(succinimidylpropionate) cross-linker for 2 h. All incubations were maintained on ice during the cross-linking reaction. ABCA1-associated <sup>125</sup>I-apoA-I was then assessed by immunoprecipitation of ABCA1 and phosphorimaging. **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 <sup>125</sup>I-apoA-I at 37°C for 1 h and then washed with warm PBS. Medium 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 as determined by cross-linking is shown in the top panels, and the percentage of the complex remaining relative to the 0 h samples is graphed below (duplicate samples, ±SD). **C:** ABCA1 and the W590S mutant cells release cell-associated apoA-I with similar kinetics. <sup>125</sup>I-apoA-I released during the off-rate experiment was quantified by scintillation counting of the medium that was removed from the cells before the addition of cross-linker. Graphed is the amount of <sup>125</sup>I-apoA-I released from wild-type ABCA1 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 <sup>125</sup>I-apoA-I was assessed by pooling and concentrating the medium from the last three time points and electrophoresing the pooled samples on native polyacrylamide gels (left panel). Shown is a phosphorimage of the resulting gel. Efflux activity was confirmed by labeling cells with [<sup>14</sup>C]cholesterol and conducting a dissociation experiment with unlabeled apoA-I. The graph shows the amount of [<sup>14</sup>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.

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 [<sup>14</sup>C]cholesterol and the experiment was repeated using unlabeled apoA-I. As expected, [<sup>14</sup>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 that expressed the W590S mutant had similarly low levels of [<sup>14</sup>C]cholesterol associated with apoA-I in the medium (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 apoli-

poproteins 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 nonamphipathic 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  $7 \pm 1.9$  nM for the interaction between apoA-I and ABCA1. The interaction of apoA-II with ABCA1 was of a similar affinity to that measured with apoA-I (apparent  $K_d$  of  $16 \pm 7.1$  nM), with saturation of cross-linking occurring with both ligands at a concentration of  $\sim 1$   $\mu$ g/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 1:1 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 of 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 direct interaction of apoA-I with ABCA1, despite substantial differences in their primary amino acid sequences. Indeed, although the exchangeable apolipoproteins are thought to have evolved from a common ancestor gene, they vary greatly in their overall length and share only from 20% to 30% amino acid identity (15). Despite this divergence, the exchangeable apolipoproteins have strongly conserved their amphipathic helical character. We propose that the binding of an efflux acceptor to ABCA1 relies on this amphipathic helical structure, with its separation of hydrophilic and hydrophobic surfaces on opposite sides of the helix. That an amphipathic helical motif can participate in protein-protein interactions that are not strongly dependent on 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 ( $K_d$  of  $\sim 4$  nM) with a variety of docking proteins (17). These interactions are dependent on the docking protein containing an amphipathic 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 amphipathic helix is bound by a hydrophobic groove on the regulatory subunit of PKA (18). This groove is formed by an  $\alpha$ -type four-helix bundle that presents a hydrophobic patch with which the hydrophobic face of the amphipathic helix interacts. Whether ABCA1 is able to bind amphipathic helices through a sim-

ilar 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/apolipoprotein complex. By 4 h, 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, although 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 versus 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 Gilotte-Taylor et al. (19) that showed the rate constant for cholesterol efflux to be 25 nM, compared with a  $K_d$  for apoA-I cellular binding of 700 nM. In contrast, Remaley et al. (11) reported a higher affinity binding site (22 nM) for apoA-I in cells expressing ABCA1 than either we or Gilotte-Taylor et al. (19) could measure using standard radiolabeled binding assays. 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 the 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 with those formed with the W590S ABCA1 mutant. The latter is fully competent to form a complex with apoA-I but 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 (half-life of 30 versus 25 min, respectively). However, only wild-type ABCA1 was able to transfer cholesterol to the released apoA-I. These results suggest that 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 that the W590S mutant has a similar impairment in the transfer of phospholipid, and as reported by Rigot et al. (9), the mutant does not stimulate the translocation of phosphatidylserine to the outer leaflet of the plasma membrane. 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 the apoA-I would dissociate from the W590S mutant with the kinetics we have measured. The behavior of the W590S mutant/apoA-I complex indicates that the cellular release of apoA-I and the transfer of cholesterol and phospholipid are separable events and thus 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 interact directly with a 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, in which ABCA1 merely flips lipids in the plasma bilayer that are subsequently microsolvubilized by apoA-I (2, 7, 20). A more recently proposed hybrid model, in which apoA-I first interacts with the lipid bilayer and then, through 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 those that 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 min. After 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. **■**

The authors acknowledge the technical assistance of Jennifer Manning and Susan Bell as well as the helpful comments of Hank Kronenberg and Kathryn Moore on the manuscript.

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