ATP-binding Cassette Transporter A1 (ABCA1) Functions as a Cholesterol Efflux Regulatory Protein*

Received for publication, March 15, 2001, and in revised form, April 16, 2001
Published, JBC Papers in Press, April 17, 2001, DOI 10.1074/jbc.M102348200

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ABCA1, an ATP-binding cassette transporter mutated in Tangier disease, promotes cellular phospholipid and cholesterol efflux by loading free apoA-I with these lipids. This process involves binding of apoA-I to the cell surface and phospholipid translocation by ABCA1. The goals of this study were to examine the relationship between ABCA1-mediated lipid efflux and apolipoprotein binding and to determine whether phospholipid and cholesterol efflux are coupled. Inhibition of lipid efflux by glibenclamide treatment or by mutation of the ATP-binding cassette of ABCA1 showed a close correlation between lipid efflux, the binding of apoA-I to cells, and cross-linking of apoA-I to ABCA1. The data suggest that a functionally important apoA-I binding site exists on ABCA1 and that the binding site could also involve lipids. After using cyclodextrin preincubation to deplete cellular cholesterol, ABCA1-mediated cholesterol efflux was abolished but phospholipid efflux and the binding of apoA-I were unaffected. The conditioned media from cyclodextrin-pretreated, ABCA1-expressing cells readily promoted cholesterol efflux when added to fresh cells not expressing ABCA1, indicating that cholesterol efflux can be dissociated from phospholipid efflux. Further, using a phototransactivatable cholesterol analog, we showed that ABCA1 did not bind cholesterol directly, even though several other cholesterol-binding proteins specifically bound the cholesterol analog. The data suggest that the binding of apoA-I to ABCA1 leads to the formation of phospholipid-apoA-I complexes, which subsequently promote cholesterol efflux in an autocrine or paracrine fashion.

Plasma high density lipoprotein (HDL)$^1$ plays a key role in maintaining cholesterol homeostasis. Epidemiological studies have shown a strong inverse relationship between HDL levels and the risk of coronary artery disease (1). Although detailed mechanisms remain unclear, one theory to explain the protective role of HDL is the reverse cholesterol transport hypothesis, which holds that HDL facilitates the transfer of cholesterol from macrophage foam cells to the liver for secretion into bile (2). The initial step in this process is the release of free cholesterol and phospholipid from the plasma membranes of macrophage foam cells to both apolipoproteins and HDL (3). Recent studies in Tangier disease (TD), an inherited HDL deficiency state characterized by cholesterol ester accumulation in macrophages, have shed light on the molecular mechanisms responsible for cellular cholesterol efflux (4–6). These studies identified ABCA1, an ATP-binding cassette transporter, as the mutated gene in TD, providing a potential explanation for the cellular defect in cholesterol and phospholipid efflux characteristic of TD subjects (7).

We and others have recently reported that ABCA1 expression in cultured cells, achieved either by introducing the ABCA1 cDNA or by cAMP treatment, increases cellular cholesterol and phospholipid efflux to apolipoprotein A-I (apoA-I) (8–12). The enhanced lipid efflux is associated with increased apoA-I binding to the cell surface. Chemical cross-linking analysis reveals complex formation between apoA-I and ABCA1 (8, 9). However, the mechanism for ABCA1-mediated lipid efflux is still poorly understood. Whereas ABCA1 has been proposed to be a phospholipid translocase/flippase (8, 12), it is not clear whether ABCA1 directly promotes cholesterol efflux or acts by an indirect mechanism.

The present study was undertaken to examine the relationship between lipid efflux and apoA-I binding to ABCA1 and the coupling of ABCA1-mediated cholesterol efflux to phospholipid efflux. The data suggest that ABCA1 acts primarily as a phospholipid translocase, an activity that is closely associated with the binding of apoA-I to ABCA1. However, cholesterol efflux can be dissociated completely from phospholipid efflux and appears to be mediated indirectly by ABCA1. These data provide the first direct evidence that ABCA1 functions as a cholesterol efflux regulatory protein.

EXPERIMENTAL PROCEDURES

Materials—Human apoA-I was obtained commercially (BioDesign). Anti-Flag antibodies, glibenclamide, 2-hydroxypropyl-β-cyclodextrin, and sodium orthovanadate were from Sigma. Dithiobis(succinimidyl-propionate) was from Pierce.

Plasmid Constructs—Plasmid construct with full-length murine ABC1 cDNA or cDNA encoding ABCA1-Flag were constructed as described (8). ABCA1-M-Flag was constructed by polymerase chain reaction-based mutagenesis, which created a missense mutation of K939M and disrupted the first Walker A motif of ABCA1.

Cell Culture and Lipid Efflux Assays—HEK293 cells were cultured in DMEM plus 10% fetal bovine serum and antibiotics. One day before transfection, the cells were plated on 6- or 24-well plates coated with collagen. The next day, cells at about 95% confluence were transfected using LipofectAMINE 2000 (Life Technologies, Inc.) and corresponding plasmid constructs. For lipid efflux assays, cells were labeled with 0.5 μCi/ml 1,2-[^3H]cholesterol or 1 μCi/ml methyl-[^3H]choline on the same day as cell transfection. 24 h after transfection and labeling, the cells were washed three times with phosphate-buffered saline, incubated at 37°C for 2 h with DMEM plus 0.2% fatty acid free bovine albumin (DMEM/BSA). The media were then replaced with fresh DMEM/BSA in the presence or absence of the indicated amount of apoA-I or HDL$_2$ and

* This work was supported by National Institutes of Health Grant HL 58948 (Specialized Center of Research in Molecular Medicine and Atherosclerosis). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: HDL, high density lipoprotein; apoA-I, apolipoprotein A-I; TD, Tangier disease; ABCA1, ATP-binding cassette transporter A1; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; CFTR, cystic fibrosis transmembrane conductance regulator; PS, phosphatidylserine; MDR, multi-drug resistance.

This paper is available on line at http://www.jbc.org

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incubated at 37 °C for 4 h. The media were collected and counted for radioactivity by liquid scintillation counting (cholesterol efflux assay) or extracted with hexane/isopropanol (3:2) solution and then counted (phospholipid efflux assay). Cells were dissolved with 0.1 N NaOH and 0.2% SDS (cholesterol efflux) or extracted with hexane/isopropanol solution (phospholipid efflux) to determine residual radioactivity remaining in cells. For lipid efflux assays with conditioned media, cells expressing ABCA1 were pretreated with 20 mM 2-hydroxypropyl-beta-cyclodextrin for 30 min, and then the washed cells were incubated with 10 μg/ml apoA-I for 4 h at 37 °C. The conditioned media were transferred to cells transfected with the control vector (mock cells) or ABCA1 vector, and cholesterol efflux during a 4-h incubation was determined.

ApoA-I Binding Assay—ApoA-I was iodinated with [125I]iodide by Iodo-Gen (Pierce) to a specific activity of ~1300 cpm/ng apoA-I. Cells grown on 24-well plates were incubated on ice or at 37 °C for 90 min in DMEM/BSA with 1.0 μg/ml labeled apoA-I in the presence or absence of 50-fold excess of unlabeled ligands. Cells were then washed rapidly four times with ice-cold DMEM/BSA and dissolved with 0.1 N NaOH and 0.2% SDS, the protein content was measured with a modified Lowry method, and bound iodinated ligands were determined by gamma counting.

Other Assays—Chemical cross-linking, immunoprecipitation, immunoblot and autoradiography. Analysis of [125I]apoA-I-ABCA1 complexes, biotinylated cell surface ABCA1, and total cellular ABCA1 were carried out as described (8). Labeling HEK293 cells with [3H]phosphocholesterol and [3H]photophosphatidylcholine as well as UV-dependent photoaffinity cross-linking were performed as described (13).

RESULTS
Glybenclamide Abolishes ABCA1-mediated Cholesterol and Phospholipid Efflux as Well as apoA-I Binding—To evaluate the relationship between the lipid efflux activities of ABCA1 and apoA-I binding and cross-linking to the transporter, we used several compounds that are known to affect ABC transporter activities (14–16). Glybenclamide, a sulfonylurea derivative, is an effective inhibitor of several ABC transporters including CFTR (15), MDR (16), and ABCA1 (14, 17). We tested the effect of this compound on ABCA1-mediated lipid efflux and apoA-I binding. Glybenclamide (1 mM) abolished ABCA1-mediated phospholipid efflux (Fig. 1A) and markedly inhibited cholesterol efflux (Fig. 1B). Vanadate, an inhibitor of MDR shown in some studies to be an inhibitor of cholesterol efflux to apoA-I (17), had no major effect on ABCA1-mediated lipid efflux. The enhanced binding of apoA-I to cells expressing ABCA1 and cross-linking of apoA-I to ABCA1 were abolished by glybenclamide treatment (Fig. 1, C and F), demonstrating a close relationship between binding and lipid efflux. Glybenclamide treatment did not alter the level of total cellular ABCA1 and the level of ABCA1 presented to the cell surface, as demonstrated by immunoblot analysis of cell samples subjected to cell surface biotinylation and ABCA1-specific immunoprecipitation (data not shown). To test the specificity of glybenclamide in ABCA1-mediated lipid efflux, we examined cholesterol efflux to HDL. ABCA1 expression failed to promote cholesterol efflux to HDL2 (Fig. 1D). HDL2-mediated cholesterol efflux was unaffected in glybenclamide-treated cells with or without ABCA1 expression (Fig. 1E), indicating specificity. These data show a
A Walker motif mutation of ABCA1 abolishes cross-linking of apoA-I to ABCA1. Cells were transfected with ABCA1-Flag or ABCA1-M-Flag, and the chemical cross-linking of [3H]apoA-I to ABCA1 and detection of the cross-linked products by immunoprecipitation and autoradiography were performed as described under “Experimental Procedures.” A, cross-linking of [3H]apoA-I to ABCA1-Flag or ABCA1-M-Flag expression in total cell lysates; B, immunoblot analysis of ABCA1-Flag or ABCA1-M-Flag expression in total cell lysates; C, biotinylated cell surface ABCA1-Flag or ABCA1-M-Flag detected by immunoprecipitation and immunoblot.

parallel inhibition of phospholipid and cholesterol efflux by glybenclamide and indicate that lipid efflux is closely related to apoA-I binding to cells and to cross-linking to ABCA1.

Walker Motif Mutant of ABCA1 Fails to Be Cross-linked to apoA-I—To further evaluate the relationship between lipid efflux and apoA-I binding to ABCA1, we made a Walker motif mutant (ABCA1-M) of ABCA1 that disrupts the first ATP binding site of the transporter. ABCA1-M was expressed and presented to the cell surface at a level similar to ABCA1, as determined by cell surface biotinylation/immunoprecipitation and immunoblot analysis (Fig. 2, B and C). However, ABCA1-M failed to promote cholesterol and phospholipid efflux as well as apoA-I binding (data not shown). Cross-linking analysis revealed no complex formation between apoA-I and ABCA1-M even though complexes between apoA-I and ABCA1 (wild type) were produced under similar conditions (Fig. 2A). These data confirm that both the phospholipid and cholesterol efflux activities of ABCA1 are closely correlated with binding of apoA-I as recently suggested (18). However, they also demonstrate that cross-linking of apoA-I to ABCA1 is abolished by the Walker motif mutation of the transporter, or by glybenclamide treatment, indicating that a functionally important binding site is created by ABCA1, which may involve ABCA1 itself.

ABCA1 has been shown to increase the presentation of phosphatidylserine (PS) at the outer leaflet of the cell membrane (12), and the enhanced presentation of PS has been suggested to play a functional role in cholesterol efflux (12, 18). To evaluate the proposed role of PS, we carried out the cholesterol efflux assay in the presence of excess amount of annexin V (5 μM), a membrane PS-binding protein (19) that has been shown to have an increased cell surface association in ABCA1 expressing cells (12). However, we did not detect any effect on ABCA1-mediated cholesterol efflux (Fig. 3), indicating that membrane PS translocation may not be involved in ABCA1-mediated cholesterol efflux. Our results are consistent with a previous report that glybenclamide blocked phosphatidyicholine, phosphatidylinositol, and sphingolipid transfer from cultured cells to apoA-I but had little effect on PS transfer (17).

Cyclodextrin Abohishes ABCA1-mediated Cholesterol Efflux but Not Phospholipid Efflux and ApoA-I Binding—The above data suggest that both the phospholipid and cholesterol efflux activities of ABCA1 are closely related to binding of apoA-I to the transporter. In an attempt to dissociate phospholipid and cholesterol efflux activities, we used 2-hydroxypropyl-β-cyclo-

dextrin, an efficient cellular cholesterol acceptor (20). Treatment of cells with 20 mM cyclodextrin for 30 min resulted in the recruitment of ~55% of total cellular cholesterol label in both ABCA1-expressing and mock-transfected cells (Fig. 4D). This treatment abolished ABCA1-mediated cholesterol efflux (Fig. 4B). In contrast, ABCA1-mediated phospholipid efflux and apoA-I binding were not affected by cholesterol depletion (Fig. 4A and C). We also determined the dose relationship between cyclodextrin treatment and cholesterol efflux. As the cyclodextrin concentration increased in the pretreatment, cellular cholesterol pools and ABCA1-mediated cholesterol efflux were proportionally decreased (Fig. 4D). These findings show that ABCA1-mediated phospholipid efflux to apoA-I can be completely dissociated from cholesterol efflux. They also indicate that the availability of cholesterol for efflux is not a requirement for apoA-I binding. Although cyclodextrin preincubation abolished ABCA1-mediated cholesterol efflux, the promotion of cholesterol efflux by cyclodextrin was independent of ABCA1, because ABCA1 overexpression did not alter cyclodextrin-mediated cholesterol efflux over a range of 10 μM to 10 mM of this reagent (data not shown). These findings are consistent with a previous report on cyclodextrin-mediated cholesterol efflux in macrophage-like J774 cells treated with a cAMP analog (10).

ABCA1 Stimulates Cholesterol Efflux by an Indirect Mechanism—To further test the relationship between phospholipid and cholesterol efflux mediated by ABCA1, we examined the ability of photoactivatable phosphatidylcholine and photoactivatable cholesterol to directly bind ABCA1 in living cells. These photoactive lipids have recently been developed as highly specific probes to identify cholesterol binding proteins (13). [3H]Photocholesterol was added to cells or [3H]photophosphatidylcholine was synthesized in cells expressing ABCA1-Flag. Photoaffinity labeling of ABCA1-Flag was activated by UV irradiation followed by the immunoprecipitation of ABCA1-Flag. The lipid binding to ABCA1 was detected by fluorography. Interestingly, although ABCA1 could promote the efflux of photoactive cholesterol (Fig. 5D), no photoactive cholesterol binding to ABCA1 was detected (Fig. 5A). Under similar conditions, the specific binding of photoactive cholesterol to scavenger receptor BI, a plasma membrane receptor involved in HDL cholesteryl ester uptake and cellular cholesterol efflux (21, 22), was readily detected as well as binding of photocholester to caveolin (13). To test the possibility that ABCA1 may bind cholesterol only in the presence of apoA-I, cells were incubated with apoA-I for 2 h before UV irradiation. Under these conditions, photoactive cholesterol binding to ABCA1 was still not detected (data not shown). In contrast to photoactive cholesterol, photoactive phospholipid bound to ABCA1 and was cross-linked by UV irradiation (Fig. 5C). However, this binding
appeared not to depend on the phospholipid translocase activity of ABCA1, because the mutant ABCA1-M bound photoactivable phospholipid as well as the wild type (Fig. 5C). These results indicate that ABCA1 is localized in a phospholipid-rich and cholesterol-poor region of the membrane.

These data could indicate that ABCA1 does not directly bind and transport cholesterol and that ABCA1-mediated cholesterol efflux occurs secondary to phospholipid efflux. To further explore this hypothesis, we used cyclodextrin preincubation to block the cholesterol efflux but not phospholipid efflux to apoA-I in HEK293 cells expressing ABCA1 and then transferred the conditioned media to a second set of cells labeled with [3H]cholesterol. The cells receiving conditioned media were either mock-transfected or transfected with ABCA1. The conditioned media readily promoted cholesterol efflux from mock cells, albeit slightly less efficiently than from ABCA1-expressing cells (Fig. 5E). These results demonstrate that the nascent apoA-I/phospholipid particles generated by ABCA1 can promote cellular cholesterol efflux in an ABCA1-independent fashion.

**DISCUSSION**

In this study we examined the relationship between ABCA1-mediated cholesterol and phospholipid efflux and the correlation between ABCA1-mediated lipid efflux and the binding of apoA-I to the cell surface. Using cyclodextrin preincubation, we demonstrated that ABCA1-mediated cholesterol efflux could be dissociated completely from phospholipid efflux. Further, we showed that cholesterol efflux could occur subsequent to phospholipid efflux even in the absence of ABCA1 expression. Direct evidence that ABCA1 does not bind cholesterol was obtained by use of a novel photoactivatable cholesterol analog, which by contrast labels selected cholesterol-binding plasma membrane proteins (13). These findings suggest that ABCA1 acts primarily as a phospholipid translocase and may not directly transport cholesterol. Together, the data suggest that ABCA1 func-
particular interest was the finding that the enhanced binding of apoA-I in fibroblasts loaded with cholesterol, a condition known to increase ABCA1 expression (17), was also not affected in the TD cells (24), while lipid efflux was markedly reduced, indicating a dissociation of ABCA1-mediated lipid efflux from apoA-I binding. These data suggest an alternative interpretation, i.e. that ATP binding/hydrolysis induces a conformational change of ABCA1 leading to apoA-I binding and phospholipid efflux. Both the Walker motif mutant and glybenclamide treatment could prevent such a conformational change. There is previous evidence that ATP binding/hydrolysis is associated with conformational changes of ABC transporters (25, 26). For instance, in the maltose transport system of *Escherichia coli*, the ATP hydrolysis cycle is coupled with a conformational change of the ABC transporter that is crucial in regulating the binding of the transporter to maltose binding protein and maltose transport (26).

The failure of ABCA1 to bind photocholesterol and the enhanced cholesterol efflux by ABCA1-conditioned media in the absence of ABCA1 expression indicate an indirect mechanism for ABCA1-mediated cholesterol efflux. This scenario is supported by the current models for certain ABC transporters. Cystic fibrosis transmembrane conductance regulator (CFTR) functions as a chloride channel but seems to have other activities, in addition to conducting chloride, that are essential for normal fluid and electrolyte transport (27, 28). Some of the effects of CFTR on regulating electrolyte transport appear to be indirectly mediated by regulating the function of other ion transporters (27, 36). Murine Mdr2, another ABC transporter, acts as a specific phospholipid translocase indirectly promoting cholesterol secretion into bile (23, 29). These precedents, as well as data herein, suggest that ABCA1 functions as a choleste-

FIG. 5. ABCA1 mediates cholesterol efflux in an indirect fashion. UV-dependent photoaffinity cross-linking of photoactive lipids to ABCA1 was carried out as described under “Experimental Procedures.” A, photoaffinity labeling of HEK293 cells expressing ABCA1-Flag with [3H]photocholesterol, UV-dependent cross-linking, and immunoprecipitation of ABCA1-Flag. No photocholesterol-ABCA1 complex was detected. B, immuno blot analysis of immunoprecipitated ABCA1-Flag from the sample of panel A. C, photoaffinity labeling of HEK293 cells expressing ABCA1-Flag or ABCA1-M-Flag with [3H]photophosphatidylcholine. Both ABCA1-Flag and ABCA1-M-Flag were labeled by photophospholipid. D, ABCA1-facilitated [3H]photocholesterol efflux to apoA-I; the assay was similar to cholesterol efflux assay except that [3H]cholesterol was replaced by [3H]photocholesterol. E, mock- or ABCA1-transfected cells were pretreated with 20 mM cyclodextrin for 30 min, and then apoA-I (10 μg/ml) was added and incubated for 4 h. The media from mock cells (designated apoA-I) or from ABCA1 expressing cells (designated as conditioned media) were then transferred to a separate set of [3H]cholesterol-labeled mock or ABCA1-expressing cells and incubated for 4 h, and cholesterol efflux from these recipient cells was determined.
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terol efflux regulatory protein and promotes cholesterol efflux in an indirect fashion. Dissociation of ABCA1-mediated cholesterol efflux from phospholipid efflux suggests that these two processes can be regulated independently. One potential physiological implication of these findings could be related to the generation of phospholipid-rich, cholesterol-poor nascent HDL by liver and small intestine (30–32). Recent studies demonstrate that hepatocytes express ABCA1 (33). Hepatic ABCA1 may be involved in nascent HDL production by facilitating transfer of phospholipids to apoA-I. Once they reach peripheral tissues, these nascent HDL particles could promote cholesterol efflux.

Prior to the discovery of ABCA1, Li et al. (34) suggested that phospholipid and cholesterol efflux mediated by apoA-I may be distinctly regulated. A two-step mechanism for cholesterol and phospholipid efflux has also been proposed in which the authors suggest that apoA-I/phospholipid particles promote cholesterol efflux from caveolae (17). However, we have observed that co-transfection of HEK293 cells with ABCA1 and caveolin-1 blocked ABCA1-mediated lipid efflux by an unknown mechanism.3 Although an indirect mechanism of ABCA1-mediated cholesterol efflux was indicated by our study, the efflux of cholesterol promoted by the conditioned media from mock cells was slightly less efficient than the efflux from ABCA1-expressing cells (Fig. 4F), suggesting that ABCA1 may have an additional role in coordinating cholesterol and phospholipid efflux.

ABCA1 expression promotes cholesterol efflux to apoA-I but not to HDL₃ or cycloextrin, indicating specificity of the transporter-mediated cholesterol efflux. One layer of the specificity probably comes from the specific transfer of phospholipid to apoA-I. Earlier in vitro studies demonstrate that phospholipids are required for apoA-I to form complexes with cholesterol (35). ABCA1-mediated specific phospholipid efflux to apoA-I is likely to prime apoA-I for subsequent cholesterol efflux.

In summary, data from this study indicate that ABCA1 functions primarily as a phospholipid translocase. Although ABCA1-mediated PS translocation might be important for phagocytosis of apoptotic cells (18), it does not appear to be required for cholesterol efflux. ATP binding/hydrolysis on ABCA1 probably induces a conformational change of the transporter, which leads to the binding of apoA-I and phospholipid translocation. The binding site may consist of phospholipids and ABCA1 itself. Recent studies suggest that an N-terminal ~600-amino acid segment of ABCA1, with clustered loss of function mutations in TD subjects, is located extracellularly (37). Potentially, this segment could be involved in apoA-I binding. Once apoA-I recruits phospholipids translocated by ABCA1, the nascent complexes promote cholesterol efflux in an autocrine, paracrine, or even endocrine fashion, perhaps from specific pools of cellular cholesterol.

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3 N. Wang, D. L. Silver, and A. R. Tall, unpublished observation.