Mutations of the ABC1 transporter have been identified as the defect in Tangier disease, characterized by low HDL and cholesterol ester accumulation in macrophages. A full-length mouse ABC1 cDNA was used to investigate the mechanisms of lipid efflux to apoA-I or HDL in transfected 293 cells. ABC1 expression markedly increased cellular cholesterol and phospholipid efflux to apoA-I but had only minor effects on lipid efflux to HDL. The increased lipid efflux appears to involve a direct interaction between apoA-I and ABC1, because ABC1 expression substantially increased apoA-I binding at the cell surface, and chemical cross-linking and immunoprecipitation analysis showed that apoA-I binds directly to ABC1. In contrast to scavenger receptor BI (SR-BI), another cell surface molecule capable of facilitating cholesterol efflux, ABC1 preferentially bound lipidd-free apoA-I but not HDL. Immunofluorescence confocal microscopy showed that ABC1 is primarily localized on the cell surface. In the absence of apoA-I, cells over-expressing ABC1 displayed a distinctive morphology, characterized by plasma membrane protrusions and resembling echinocytes that form when there are excess lipids in the outer membrane hemileaflet. The studies provide evidence for a direct interaction between ABC1 and apoA-I, but not HDL, indicating that free apoA-I is the metabolic substrate for ABC1. Plasma membrane ABC1 may act as a phospholipid/cholesterol flipase, providing lipid to bound apoA-I, or to the outer membrane hemileaflet.

Epidemiological studies have demonstrated a strong inverse correlation between the levels of plasma HDL and the risk of coronary heart disease (1). It has been proposed that HDL promotes reverse cholesterol transport by facilitating transfer of cholesterol from peripheral tissues to the liver for disposal (2). However, the molecular mechanisms for transfer of cholesterol from peripheral cells to HDL are incompletely understood. A breakthrough in our understanding of this process came recently from studies of Tangier disease and familial HDL deficiency, in which the molecular defect was shown to be mutations in the ATP binding cassette transporter 1 (ABC1) gene (3–5). Tangier disease is a rare recessive genetic disorder characterized by extremely low HDL levels, accumulation of cholesterol esters in macrophages, and, in some cases, premature coronary heart diseases (6). ABC1 is a 240-kDa protein belonging to a large family of conserved transmembrane proteins that transport a wide variety of substrates, including ions, drugs, peptides, and lipids, across cell membranes (7). The finding that fibroblasts from Tangier disease patients have a marked defect in efflux of cholesterol and phospholipids to apoA-I (8–10) suggests that ABC1 mediates or regulates the efflux of cellular cholesterol and phospholipids to apoA-I.

Another membrane protein that binds HDL and mediates HDL cholesterol ester (HDL CE) uptake (11) and cellular unesterified cholesterol efflux (12, 13) is scavenger receptor class B type I (SR-BI). SR-BI is primarily expressed in liver and steroidogenic tissues and mediates HDL CE and free cholesterol uptake (11, 14). SR-BI also has been reported to be expressed in atherosclerotic lesion macrophages (15–17). However, the function of SR-BI in macrophages is yet to be defined.

In the present study, we characterized the ABC1 cDNA in human embryonic kidney 293 (HEK 293) cells. In contrast to the published ABC1 cDNA (GenBank accession numbers X75926 and NM005502), we found an additional 60-amino acid peptide at the amino terminus of ABC1 that is required for it to mediate cellular cholesterol and phospholipid efflux. We also demonstrate, by binding and chemical cross-linking studies, that apoA-I directly interacts with ABC1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human apoA-I was commercially obtained (BioDesign). Anti-FLAG antibodies were from Sigma (St. Louis, MO) and anti-SR-BI antibodies were from Novus Biologicals (Littleton, CO). Dithiobis(succinimidylpropionate) (DSP) was from Pierce (Rockford, IL).

**ABC1 cDNA and Plasmid Constructs**—Murine ABC1 cDNA was obtained by reverse transcription-PCR using murine RAW cell total RNA and cloned into a pcDNA3.1 vector (Invitrogen, CA). The 5′-rapid amplification of cDNA ends PCR of human ABC1 mRNA of THP1 cells, isolation, and characterization of novel ABC1 transcripts, and isolation and determination of human ABC1 promoter and exon/intron structures of human ABC1 gene have been described (18). The pcDNA3.1/mABC1Δ had a murine ABC1 cDNA from bp 254 to 6869 according to the published mouse ABC1 mRNA sequence (GenBank accession number X75926). pcDNA3.1/mABC1Δ had the murine ABC1 cDNA from bp 49 to 6869. Compared with ABC1Δ, this results in a putative extra amino-terminal 60-amino acid sequence (MACWQPQLRLWLWNLNTRRRQCTQLLLEAVWPL-FIPNIISVRSWPPYEQHECFFPNKA). ABC1 with the FLAG epitope incorporated at its carboxyl terminus was constructed using PCR and primers encoding the amino acid sequence of the FLAG epitope (DYKDDDDK).

**Cell Culture and Lipid Efflux Assays**—HEK 293 cells were cultured in DMEM media plus 10% fetal bovine serum and antibiotics. 1 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., Rockville, MD) and corresponding plasmid constructs. For lipid efflux assays, cells were labeled with 0.5 μCi/ml 1,2-3H]cholesterol or 1 μCi/ml [methyli- 
3]H]choline on the same day as cell transfection. 24 h after transfection and labeling, the cells were washed three times with PBS, incubated at 37 °C for 2 h with DMEM media 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 HDL₃, and incubated at 37 °C for 4 h. The media were collected and counted for radioactivity by liquid scintillation counting in cholesterol efflux assay or extracted with hexane/isopropanol solution in phospholipid efflux assay. Cells were dissolved with 0.1 N NaOH and 0.2% SDS in cholesterol efflux assays or extracted with hexane/isopropanol solution in phospholipid efflux assays and isolated from residual radioactivity remaining in cells was determined.

ApoA-I and HDL₃ Binding Assay—ApoA-I or HDL₃ (d = 1.12–1.21 g/ml) isolated from human plasma were iodinated with [125I]iodide by IODO-GEN (Pierce) to a specific activity of ~1295 cpm/ng apoA-I or ~1246 cpm/ng HDL₃ protein. Cells grown on 24-well plates were incubated on ice for 2 h in DMEM/BSA with 1.0 μg/ml labeled apoA-I or HDL₃ in the presence or absence of a 50-fold excess of unlabeled ligands. Cells were then washed rapidly four times with ice-cold DMEM/BSA. Cells were dissolved with 0.1 N NaOH and 0.2% SDS. Protein content was measured with a modified Lowry method, and bound iodinated ligands were determined by gamma counting.

Chemical Cross-linking and Immunoprecipitation Analysis—Cells grown on 6-well plates were incubated at 37 °C for 1 h with 1 μg/ml iodinated apoA-I or HDL₃ in DMEM/BSA in the presence or absence of 50-fold excess of unlabeled ligands. Cells were then placed on ice for 15 min and washed three times with PBS. DSP was dissolved immediately before use in dimethyl sulfoxide and diluted to 250 μM with PBS, and 1.5 ml was added per well. Cells were incubated at room temperature for 1 h; the medium was removed, and the cells were washed twice with PBS. Cells were lysed at 4 °C with radioimmunoprecipitation buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, protease inhibitor mixture (Roche Molecular Biochemicals, GmbH), 1 mM phenylmethylsulfonyl fluoride). For immunoprecipitation, the cell lysates were centrifuged at top speed in a microfuge for 15 min. The supernatant was collected, 40 μg of normal goat IgG and 10 μl of Protein AG Plus-agarose (Santa Cruz Biotechnology, CA) were added to it, and the mixture was rotated at 4 °C for 3 h. After a brief spin, 15 μg of monoclonal anti-FLAG M2 antibody or 50 μg of polyclonal anti-SR-BI antibody was added to the preassembled cell lysates and incubated by rotating at 4 °C overnight. Protein AG Plus-agarose was then added, and incubation was continued for 2 h by rotating the tubes. The samples were centrifuged briefly, and the pellet was washed twice with radioimmunoprecipitation buffer and twice with PBS. The bound proteins were eluted from the agarose beads by incubation and boiling with Laemmli sample buffer in the presence or absence of 5% 2-mercaptoethanol. The eluted proteins were loaded onto 4–20% gradient SDS-polyacrylamide gel electrophoresis gels for electrophoresis followed by electrotransfer to a nitrocellulose sheet for autoradiography and Western analysis.

Immunofluorescence Confocal Microscopy—Cells were fixed with 3% formaldehyde for 10 min and then incubated with 0.1% Triton X-100 in PBS for 2 min. After washing with PBS, cells were incubated with primary antibody in 4 ng/ml normal goat globulin, 0.1% saponin in PBS at room temperature for 30 min. Alexa 488-labeled goat anti-ribbon IgG or Alexa 568-labeled goat anti-mouse IgG was used as the secondary antibody. After washing and postfixing with 3% formaldehyde, cells were examined by confocal microscopy as described previously (19).

RESULTS

ABC1 Promotes ApoA-I-mediated Cholesterol Efflux—During recent studies to identify the promoter of the human ABC1 gene, we found a 5′-extension of the cDNA sequence, compared with published cDNA (GenBank accession numbers X75992 and NM0055502). (18). This contained a new upstream start codon with a strong Kozak consensus sequence (20) and is in-frame with the previously published ATG located 60 amino acids downstream (21). The strong homology of this new amino-terminal amino acid sequence of ABC1 with the amino-terminal amino acid sequences of ABCR and ABC5, two other ABC1 family members (22–24), and the prediction of a signal peptide with a cleavage site between residues 45 and 46 (using the program SignalP (25), suggested that this newly identified upstream start codon is the authentic translation initiation site for ABC1. To test this hypothesis, we made plasmid constructs expressing ABC1 with or without the putative amino-terminal 60 amino acids (pcDNA3.1/ABC1 versus pcDNA3.1/ABC1Δ). To facilitate expression analysis, a FLAG epitope was incorporated into the carboxyl terminus of ABC1 or ABC1Δ. Cellular cholesterol efflux mediated by apoA-I was determined in HEK 293 cells transfected with the expression constructs. Addition of apo-A-I in the control cells only slightly increased cholesterol efflux (mock versus mock + apo-A-I; Fig. 1). In contrast, apo-A-I markedly increased cholesterol efflux in ABC1-transfected cells. The apo-A-I-specific enhancement of cholesterol efflux (apo-A-I present minus apo-A-I absent) increased 10.5-fold (0.4% efflux in mock cells versus 4.2% in ABC1-transfected cells). ABC1-FLAG was as equally effective as ABC1 in promoting cholesterol efflux, showing that the FLAG epitope does not interfere with the function of ABC1 (Fig. 1). SDS-polyacrylamide gel electrophoresis and Western analysis showed that the ABC1-FLAG migrated with a Mᵦ ~ 240 kDa (data not shown). In contrast, expression of ABC1Δ had no effect on apo-A-I-mediated cholesterol efflux, indicating that the amino-terminal 60 amino acids of ABC1 (18) are essential for function. To test for the expression of ABC1Δ, we also transfected HEK 293 cells with ABC1Δ-FLAG. Immunofluorescence microscopy detected the expression of ABC1Δ-FLAG in the transfected cells (data not shown) even though this construct failed to increase apo-A-I-mediated cholesterol efflux.

ABC1 Expression Markedly Increases ApoA-I but Not HDL-mediated Lipid Efflux—Some previous studies have suggested that both apoA-I- and HDL-mediated cholesterol efflux are impaired in fibroblasts from Tangier disease patients (8, 26). Thus, we compared the ability of apoA-I and HDL to mediate cholesterol efflux in ABC1-transfected or control cells. ApoA-I markedly increased cholesterol-dependent cholesterol efflux, in a process that was saturated at low apoA-I concentrations (Fig. 2a). The EC₅₀ for apoA-I-mediated cholesterol efflux in ABC1-transfected cells was approximately 2.0 μg/ml. HDL₃ did promote cellular cholesterol efflux in the control cells (Fig. 2b). However, this effect was largely ABC1-independent, because ABC1 expression only slightly increased cholesterol efflux by HDL₃. These results indicate that apoA-I is the preferred acceptor for ABC1-mediated cholesterol efflux.
We also examined ApoA-I- and HDL₃-mediated phosphatidylcholine (PC) efflux. ABC1 transfection substantially increased apoA-I-mediated PC efflux (Fig. 2c). However, HDL₃-mediated PC efflux was only slightly increased in ABC1-transfected cells relative to the control (mock + HDL₃).

**ABC1 Expression Increases the Cell Surface Binding of ApoA-I but Not the Binding of HDL₃**—To determine if ABC1 increases binding of ligands to the cell surface, [¹²⁵I]apoA-I and [¹²⁵I]HDL₃ binding to HEK 293 cells was examined. ABC1 transfection markedly increased the binding of apoA-I to cells at 4 °C (Fig. 3a). However, HDL₃ binding to the cell surface did not change in ABC1-transfected cells relative to the control (Fig. 3b). In contrast to ABC1, SR-BI expression in HEK 293 cells was found to increase both apoA-I and HDL binding (Fig. 3), as previously reported (27, 28).

**ApoA-I Directly Interacts with ABC1**—The increased binding of apoA-I in HEK 293 cells overexpressing ABC1 could be due to direct interactions between apoA-I and ABC1, or it could be secondary to other changes in the cell membrane resulting from ABC1 overexpression. To address this, we performed chemical cross-linking studies using [¹²⁵I]apoA-I or [¹²⁵I]HDL₃ and the homobifunctional cross-linker DSP. To facilitate the isolation of the cross-linked complexes by immunoprecipitation, we transfected HEK293 cells with the ABC1-FLAG cDNA, which was indistinguishable from the ABC1 cDNA in promoting cellular cholesterol efflux (Fig. 1). [¹²⁵I]apoA-I was co-immunoprecipitated with anti-FLAG from the lysate of ABC1-FLAG-transfected cells and migrated as a broad band at an apparent molecular mass of approximately 260–320 kDa when cross-linked with DSP (Fig. 3c, lane 2), whereas the control cells did not yield any detectable band (Fig. 3c, lane 1). This indicates that apoA-I directly binds to ABC1. The size of the complex suggests that ABC1 is monomeric in vivo. In the absence of cross-linker (lane 3) or in the presence of 50-fold excess of unlabeled apoA-I (lane 4), this high molecular mass band was abolished. Reducing agents such as 2-mercaptoethanol can break the -S-S- bridge of DSP and therefore separate the components cross-linked by DSP. Upon 2-mercaptoethanol treatment the [¹²⁵I]apoA-I, migrating as a high molecular mass band in lane 2, now migrated at the position of monomeric apoA-I (Fig. 3c, lanes 5 and 6). Corroborating a recent report showing that apoA-I binds directly to SR-BI (28), we found that [¹²⁵I]apoA-I bound and cross-linked to SR-BI (Fig. 3c, lane 7) and migrated as a high molecular mass broad band. This is consistent with the hypothesis that SR-BI forms homodimers in vivo (28). In contrast to apoA-I, [¹²⁵I] HDL₃ was not co-immunoprecipitated with anti-FLAG from ABC1-FLAG-transfected cells treated with DSP (Fig. 3d, lane 2), although the lipoprotein particles were bound and cross-linked to SR-BI (Fig. 3d, lane 7). These results demonstrate that apoA-I directly interacts with ABC1, whereas HDL does not.

**ABC1 Is Localized on the Cell Surface**—Confocal microscopy was used to determine the localization of ABC1 with anti-FLAG antibody in permeabilized ABC1-FLAG-transfected HEK 293 cells. Confocal microscopy revealed a strong cell surface signal (Fig. 4), indicating that ABC1 is localized primarily on the cellular plasma membrane. There were also a few intracellular punctate structures with positive FLAG signals. They probably represent ABC1 at intracellular sites as reported previously (29). Frequently we found that FLAG-positive cells had long thin projections from the cell body, containing strong immunofluorescent signals for ABC1 (Fig. 4b). Approximately 25% of FLAG-positive cells displayed such morphological changes. In contrast, the percentage was reduced to ~4% when 40 µg/ml apoA-I was added during transfection (data not shown). apoA-I did not affect the transfection efficiency, because the expression of green fluorescence protein was unchanged in the presence of apoA-I.

**DISCUSSION**

In this study we have demonstrated that ABC1 facilitates apoA-I-mediated cellular cholesterol and phospholipid efflux. ABC1 expression specifically increases binding of apoA-I, but not HDL₃, to the cell surface. The chemical cross-linking and immunoprecipitation analysis reveal that apoA-I directly interacts with ABC1. Compared with HDL₃, apoA-I is the preferred acceptor for ABC1-promoted cholesterol and phospholipid efflux. ABC1 is primarily localized on the cellular plasma membrane. It is likely that the direct interaction between apoA-I and ABC1 at the cell surface is required for the lipid efflux facilitated by these two proteins.

Increased apoA-I-mediated cholesterol and phospholipid ef-
flux by ABC1 expression has been reported by other groups, but in these studies the requirement for an additional 60 amino acids at the amino terminus was apparently not appreciated. One group used ABC1 cDNA equivalent to ABC1Δ in this study (29), whereas the 5'-end of the ABC1 cDNA used by another group was not clearly defined (30). ABC1Δ failed to increase apoA-I-mediated cholesterol efflux under the experimental conditions of this study. We cannot explain the apparent discrepancy of these findings. The necessity of this amino-terminal 60-amino acid sequence has also been suggested recently by DNA sequence analysis (31), although no functional studies were conducted.

The binding of apoA-I to fibroblasts from Tangier disease patients has been reported to be abnormal in some studies (8) but not in others (26). The great heterogeneity of ABC1 mutations and therefore the different biochemical bases for Tangier disease could potentially explain these different findings. In addition to apoA-I, cholesterol efflux mediated by other apolipoproteins such as apoA-II, apoA-IV, apoC-I, and apoC-III are all decreased in Tangier cells (26). Based on this, doubt has been raised as to whether a single receptor really binds apoA-I in the process of lipid efflux (26). Our findings unambiguously demonstrate that apoA-I directly binds to ABC1. In contrast, ABC1 overexpression fails to increase the cell surface binding of HDL₃ and minimally affect HDL₃-mediated cholesterol efflux. Together, these findings suggest that direct interaction of ligand (i.e., lipid-free apolipoproteins) with ABC1 is necessary for the stimulation of cholesterol efflux.

The inverse ability of ABC1 and SR-BI to bind apoA-I and HDL₃ has implications for their physiological roles in vivo.
Although this and other studies (28) have shown the binding of both apoA-I and HDL to SR-BI, recent studies demonstrate that lipid-free apoA-I or preβ-1 HDL binds to SR-BI less efficiently than native HDL (32). Indeed, the binding affinity of native HDL is inversely correlated with the density of the lipoprotein particles, suggesting that SR-BI binds most tightly to large, relatively low density, CE-rich HDL particles to maximize the efficiency of CE uptake (32). In contrast, the current study demonstrates that ABC1 binds lipid-free apoA-I with high affinity but binds HDL poorly. Furthermore, it is notable that, unlike HDL, lipid-free apoA-I fails to promote cholesterol efflux mediated by SR-BI even though it binds SR-BI (12). Therefore, it is conceivable that in vivo newly synthesized apoA-I from liver and intestine circulates and interacts with ABC1 on the cell surface, recruiting phospholipids and cholesterol from peripheral cells. Accumulation of lipids on apoA-I may reduce the affinity for ABC1 and promote dissociation from the transporter. These nascent lipoprotein particles are further converted into larger HDL particles by accepting more lipids from peripheral cells, by phospholipid transfer protein-mediated lipid transfer from triglyceride-rich lipoprotein particles (33) and by lecithin:cholesterol acyltransferase-mediated cholesterol esterification. The mature large, CE-rich HDL circulates back to liver and preferentially binds SR-BI. This process may be facilitated by apoE on such particles (34). Following selective uptake of HDL CE by SR-BI, the particles become smaller and have reduced affinity for SR-BI. In addition to secretion of apoA-I by the liver and small intestine, CETP and hepatic lipase also participate in the remodeling of HDL to generate free apoA-I. The anti-atherogenic effect of CETP in CETP-apoC-III double transgenic mice may be related to the increased production of small HDL particles and release of free apoA-I from HDL (35). In addition to apoA-I, other apolipoproteins also may function as acceptors for ABC1-mediated lipid efflux, because, in contrast to ABC1 knockout mice (29), apoA-I knockout mice do not accumulate cholesterol esters in macrophages (30). Besides apoA-I, apoA-IV appears to be a potential physiological acceptor. Indeed, lipoprotein-deficient serum from apoA-IV transgenic mice has markedly increased cholesterol efflux in J774 cells only after treatment with cAMP, a condition that increases expression of ABC1 (37). This could potentially explain the reduced atherosclerosis in apoA-IV transgenic mice (38). In contrast, the serum from apoA-IV transgenic mice does not increase cholesterol efflux from Fu5AH cells (37), a cell line with a high level of SR-BI expression (12). These observations imply that apoA-IV resembles apoA-I in ABC1- and SR-BI-mediated lipid efflux.

The low EC_{50} (~2 μg/ml) for apoA-IV-mediated cholesterol efflux in cells expressing ABC1 and the direct binding of apoA-I to ABC1 suggest a high affinity of ABC1 for apoA-I. In humans, the average plasma concentration of free apoA-I and lipid poor preβ HDL has been estimated to be 60–120 μg/ml (39, 40). This would suggest that the availability of apoA-I is not normally rate-limiting for ABC1-mediated cholesterol efflux. However, the real concentration of lipid-free apoA-I available to ABC1 in atherosclerotic lesions is likely to be much lower. ApoA-I transgenic mice have markedly increased apoA-I production and greatly reduced atherogenesis (41), suggesting that apoA-I synthesis is a rate-limiting factor in reverse cholesterol transport. The up-regulation of ABC1 expression upon cholesterol loading in macrophages (42) could be another physiological response in vivo to remove excess of cellular cholesterol. However, this response is mediated by liver X receptors and involves responses to specific oxiysterols, which are probably not abundant in atheroma foam cells (18). This suggests that macrophase ABC1 expression is also rate-limiting for reverse cholesterol transport from atheroma foam cells.

Cells overexpressing ABC1 assumed a striking morphology (Fig. 4), resembling echinocytes. Echinocytes are red blood cells containing spiky plasma membrane protrusions, which form upon addition of phospholipids or other amphipathic molecules to the outer leaflet of plasma membrane (43). Platelets also form similar plasma membrane projections upon activation or addition of phospholipids (44, 45). The distinctive morphological changes and inhibition of these changes by addition of apoA-I suggest that they arise from an increased quantity of lipid, probably phospholipid and cholesterol, in the outer leaflet of the plasma membranes. Together with an example of mr2, an ABC transporter that acts as a phospholipid translocase at the canalicular membranes (46, 47), these results suggest that ABC1 acts at the plasma membrane as a lipid translocase.

Like the multi-drug resistance P-glycoprotein, ABC1 is a full transporter containing two clusters of six transmembrane domains and internal loops with nucleotide binding motifs. A detailed electron microscopy study suggests that the overall shape of P-glycoprotein approximates a cylinder of about 10 nm in diameter with a large central chamber of about 5 nm in diameter (48). The aqueous pore is open at the extracellular face of the membrane but closed at the cytoplasmic face. In addition, an opening to the lipid phase, within the plane of the membrane, is also apparent. A working hypothesis is that lipids enter the protein channel by lateral diffusion in the plane of the inner leaflet hemimembrane and the polar headgroup is then flipped across the bilayer with the consumption of ATP (47). If ABC1 assumes a similar structure to the P-glycoprotein, lipid-free apoA-I could enter the chamber of the transporter and recruit flipped lipids. The limiting diameter of the pore could explain the poor binding and cholesterol efflux mediated by HDL.

In conclusion, our studies provide evidence for a direct interaction between apoA-I and ABC1 but not between HDL and ABC1, suggesting that lipid poor apoA-I or other lipid poor apolipoproteins such as apoA-IV is/are the physiological acceptor(s) for ABC1-mediated lipid efflux. The availability of free apoA-I, as well as the expression of ABC1 in atheroma foam cells, may both be rate-limiting for reverse cholesterol transport.

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