ATP-binding cassette transporter A1 (ABCA1) is known to mediate cholesterol efflux to lipid-poor apolipoprotein A-I. In addition, ABCA1 has been shown to influence functions of the plasma membrane, such as endocytosis and phagocytosis. Here, we report that ABCA1 expression results in a significant redistribution of cholesterol and sphingomyelin from rafts to non-rafts. Caveolin, a raft/caveola marker also redistributes from punctate caveola-like structures to the general area of the plasma membrane upon ABCA1 expression. Furthermore, we observed significant reduction of Akt activation in ABCA1-expressing cells, consistent with raft disruption. Cholesterol content in the plasma membrane is, however, not altered. Moreover, we provide evidence that a non-functional ABCA1 with mutation in an ATP-binding domain, A937V, fails to redistribute cholesterol, sphingomyelin, or caveolin. A937V also fails to influence Akt activation. Finally, we show that apolipoprotein A-I preferentially associates with non-raft membrane fractions (non-rafts) (7). It is far from clear whether these domains do not seem to play a major role in efflux, because apoA-I was shown to preferentially acquire cholesterol from the loosely packed, “non-raft” microdomains (6, 7). ABCA1 itself also appears to be localized in Triton X-100 soluble fractions (non-rafs) (7). It is far from clear whether non-rafs are required for ABCA1-mediated cholesterol efflux. Indeed, recent work performed by Duong and colleagues (8) further revealed a need for a better understanding of the relative contribution of these microdomains to the that generates high density lipoprotein and transports excess cholesterol from the peripheral tissues, including the arterial wall, to the liver for biliary secretion. This process is absent in Tangier disease, due to mutations in ABCA1 (1). Without a functional ABCA1, apoA-I is rapidly catabolized, leading to cholesterol accumulation in peripheral tissues and low plasma high density lipoprotein. ABCA1 therefore plays a key role in cholesterol efflux to lipid-poor lipoproteins, such as apoA-I.

There has been considerable debate as to whether ABCA1 mediates apoA-I acquisition of phospholipids and cholesterol separately or simultaneously. In a “two-step model,” lipid-poor apoA-I firstly forms a high affinity complex with ABCA1 (2–4), which facilitates apoA-I association with phospholipid-rich domains. Second, this phospholipid-primed apoA-I acquires cholesterol from cholesterol-rich domains, a process thought to be independent of ABCA1. On the other hand, Smith et al. (5) reported that ABCA1 mediates concurrent efflux of phospholipid and cholesterol. These contrasting models demonstrate that the mechanism by which ABCA1 mediates lipid efflux to apoA-I has yet to be clarified.

Interestingly, the plasma membrane bilayer is thought to contain a mosaic of tightly packed lipid microdomains termed “lipid rafts.” Commonly defined based on their insolubility in non-ionic detergents, these microdomains contain high concentrations of cholesterol, sphingolipids, caveolin, and many proteins involved in cell signaling. In terms of apoA-I-mediated efflux, these microdomains represent a conceptually attractive “in situ” reservoir of cholesterol in the plasma membrane. Despite their enrichment in cholesterol, however, these domains do not seem to play a major role in efflux, because apoA-I was shown to preferentially acquire cholesterol from the loosely packed, “non-raft” microdomains (6, 7). ABCA1 itself also appears to be localized in Triton X-100 soluble fractions (non-rafs) (7). It is far from clear whether non-rafs are required for ABCA1-mediated cholesterol efflux. Indeed, recent work performed by Duong and colleagues (8) further revealed a need for a better understanding of the relative contribution of these microdomains to the

Apolipoprotein A-I (apoA-I)5-mediated lipid efflux is one of the earliest events in reverse cholesterol transport, a process

**ATP-binding Cassette Transporter A1 Expression Disrupts Raft Membrane Microdomains through Its ATPase-related Functions**

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5 The abbreviations used are: apoA-I, apolipoprotein A-I; ABCA1, ATP-binding cassette transporter A1; MCD, methyl-β-cyclodextrin; YFP, yellow fluorescent protein; GFP, green fluorescent protein; EGF, epidermal growth factor; DiIC18, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate; BHK, baby hamster kidney; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; wt, wild type.
ABCA1 Alters Membrane Microdomains

ABCA1-mediated formation of nascent high density lipoprotein particles.

Aside from cholesterol efflux, ABCA1 has been linked to several functions on the plasma membrane, such as phagocytosis, endocytosis, and microvesiculation (9–12). These findings collectively imply that ABCA1 exerts significant influence on the plasma membrane. It is however unclear whether ABCA1 influences raft/non-raft partition and, consequently, how this may affect apoA-I-mediated cholesterol efflux. An attractive concept would be that ABCA1 actively disrupts the raft microdomains and makes cholesterol more readily available, thus facilitating cholesterol-apoA-I interaction and efflux.

To test this possibility, we analyzed in detail the impact of ABCA1 expression on membrane micro-organizations. We observed that ABCA1 disrupts microdomains (rafts) and redistributes cholesterol/sphingomyelin to non-raft domains. ABCA1 also disrupts caveolea formation. Consequently, ABCA1 expression impairs EGF-induced Akt activation, a process known to be sensitive to raft integrity (13). We also provide evidence that this membrane reorganization is dependent on a functional nucleotide binding domain of ABCA1, suggesting involvement of ATPase-related functions in membrane reorganization. Furthermore, we observed preferential association of apoA-I with the non-raft fraction of the plasma membrane. Our results thus provide a potential mechanism by which ABCA1 facilitates apoA-I cell association and cholesterol efflux by disrupting the tightly packed lipid raft microdomains.

MATERIALS AND METHODS

Material and Reagents—Baby hamster kidney (BHK) cells stably expressing either an empty vector (mock) or ABCA1 under the control of a mifepristone-inducible Gene-switch promoter were prepared as described previously (14). To facilitate morphological analysis, we further subcloned ABCA1 or mutant cells and chose the colonies that express equal levels of ABCA1 among individual cells. Cell culture media and reagents were from Invitrogen. Mifepristone, methyl-β-cyclodextrin, and Triton X-100 were purchased from Sigma. A cholesterol kit was from VWR International (West Chester, PA). Polyclonal antibody against ABCA1 was from Novus Biological Inc. (Littleton, CO). Anti-caveolin antibodies were from BD Transduction Laboratories. Polyclonal anti-Akt antibody (C-20) was from Santa Cruz Biotechnology (Santa Cruz, CA), and polyclonal anti-phospho-Akt (Ser-473) was from Cell Signaling Technology (Beverly, MA). Fluorescent secondary antibodies and DiIC_{18} were purchased from Molecular Probes (Eugene, OR). YFP-caveolin is a C-terminally YFP-tagged caveolin 1 and was reported to traffic similarly to caveolin-GFP that transports to caveolea identically to untagged caveolin (15). YFP-caveolin was obtained originally from Dr. Robert G. Parton (University of Queensland, Brisbane, Australia).

Cell Culture—BHK cells were maintained in DMEM plus 10% fetal calf serum at 37 °C in a 5% CO2 incubator. Incubating cells for 18–20 h in DMEM with 1 mg/ml BSA and 10 nm mifepristone induced ABCA1 expression. Mock or mutant cells were treated identically as controls.

Endogenous Caveolin-1 Distribution—ABCA1- and mock-expressing BHK cells were grown to near confluence in glass bottom dishes and incubated for 18–20 h in DMEM containing 1 mg/ml BSA and 10 nm mifepristone. Endogenous caveolin-1 was visualized using a plasma membrane-specific mouse monoclonal antibody against caveolin-1 followed by incubation with an Alexa-488 goat anti-mouse secondary antibody. Confocal fluorescent images of the basal membrane were taken using a Nikon TE300 inverted fluorescent microscope with a 60× objective and the 488 nm line of an argon ion laser.

Cholesterol Mass Determination—Cellular lipids were extracted by organic solvent and dried under N2. The dry samples were directly resuspended by vigorous vortexing in 500 μl of the Free Cholesterol E reagent supplied with the cholesterol mass determination kit (Wako Chemicals, Richmond, VA), incubated for 10 min at 37 °C, and the absorbance was determined at 600 nm. The Triton X-100-extractable cholesterol fraction was calculated as % cholesterol as follows: (Triton X-100-soluble fraction)/(Triton X-100-insoluble fraction).

Triton X-100 Extraction—Mifepristone-induced 75% confluent BHK cells grown in 6-well plates were washed twice with ice-cold PBS and chilled on ice for 30 min in DMEM containing 10 nM Hepes, pH 7.4. The medium was then replaced with 1 ml/well DMEM/Hepes in the presence or absence 1% Triton X-100 and further incubated on ice for 30 min. The medium was then collected, and the first wash with 500 μl of ice-cold PBS was combined with the medium. Lipids were then extracted overnight at 4 °C by adding 4 volumes of Folch solution (chloroform/methanol (2:1)). The organic phase was collected and evaporated under N2 atmosphere. Lipids were also extracted twice from cells in 1 ml/well of a 3:2 hexane:isopropanol solution, and the organic phase was evaporated under N2 atmosphere as described previously. For labeling free and esterified cholesterol, cells were labeled for 48 h with 1 μCi/ml [3H]cholesterol. After Triton X-100 treatment, tritiated samples were separated by high-performance thin layer chromatography (Whatman) using a 80:20:6.7:1 hexane:ether:methanol:acetic acid mixture as the elution system. For [3H]-labeled phospholipids, half-confluent cells were labeled for 24 h with 1 μCi/ml [3H]cholesterol and the elution system was composed of chloroform:methanol:acetic acid:water (35:15:6:2:1). Spots corresponding to cholesteryl ester, free cholesterol, phosphatidylcholine, and sphingomyelin were scraped and counted in a β-scintillation counter.

Cholesterol Efflux—BHK cells were incubated in normal growing media (DMEM plus 10% fetal calf serum) with 0.5 μCi/ml [3H]cholesterol for 2 days to label cellular cholesterol to equilibrium. Cells were then switched to DMEM with 1 mg/ml BSA and 10 nm mifepristone for 18–20 h. To measure cholesterol efflux, cells were incubated with 10 μg/ml apoA-I for 4 h at 37 °C. Medium was collected, centrifuged to remove detached cells, and counted for 3H. Cells were lysed with NaOH (0.5 n) overnight and counted for 3H. Cholesterol efflux was calculated as the percentage of total [3H]cholesterol released into the medium. For methyl-β-cyclodextrin (MCD)-induced cholesterol efflux, cells were also labeled with 0.5 μCi/ml [3H]cholesterol for 2 days, followed by 18–20 h of incubation with 1 mg/ml BSA and 10 nm mifepristone. Cells were then incubated...
with 5 mM MCD either at 37 °C for 1 min or on ice for the indicated time. Medium was collected, centrifuged, and counted for \[^{1}H\]cholesterol. Cell-associated \[^{1}H\]cholesterol was measured from NaOH lysates as mentioned above. Efflux was again calculated as the percentage of total \[^{1}H\]cholesterol released into the medium.

**Fluorescent Microscopy**—For immunofluorescent staining of ABCA1, mutant, and mock-expressing BHK cells were incubated for 18–20 h in DMEM containing BSA and 10 mM mifepristone. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1 mg/ml saponin. ABCA1 was visualized by a primary polyclonal antibody against ABCA1 followed by an Alexa-488 goat anti-rabbit secondary antibody. Confocal fluorescent images were taken using a Nikon TE2000 inverted fluorescence microscope with C1 confocal attachment and a 60× (numerical aperture, 1.4) objective. Images from ABCA1 and mock cells were taken under identical conditions.

For caveolin distribution, ABCA1, mutant, and mock-expressing BHK cells were transiently transfected with YFP-caveolin using Lipofectamine 2000. Cells were then incubated overnight in DMEM/BSA plus mifepristone prior to fluorescent microscopic observations. For caveolin and ABCA1 colocalization experiments, an Alexa-594 secondary antibody and the 594 nm line of a HeNe laser were used. Filipin staining of cellular free cholesterol was performed as described previously (16).

**DiIC\(_{18}\) Cold Triton X-100 Extractability**—Cold Triton X-100 extractability of surface-bound DiIC\(_{18}\) on the plasma membrane was performed as described previously (17). Cells were transferred from growth medium to Medium 1 (150 mM NaCl, 5 mM KCl, 1 mM Ca\(_{2+}\), 1 mM Mg\(_{2+}\), 2 g/liter glucose, and 20 mM HEPES, pH 7.4). Lipid analogs were transferred as monomers from fatty acid-free BSA carriers (18). In some of the experiments, DiIC\(_{18}\) in ethanol was directly added to labeling medium at 37 °C. In particular, cells were labeled with DiIC\(_{18}\) for 20 s at 37 °C, rinsed with ice-cold Medium 1, and then incubated on ice with cold Triton X-100 (1%) for 30 min. Triton X-100-resistant membranes were visualized by imaging DiIC\(_{18}\) using a standard rhodamine filter set. Images were quantified by manually outlining each cell using the Image software, and measuring fluorescent intensity from entire cell area. This is the fraction of DiIC\(_{18}\) remaining after Triton X-100 treatment (insoluble fraction, \(F_s\)). To extrapolate DiIC\(_{18}\) fluorescent intensity before Triton X-100 in the same cell, we randomly sampled several regions of interest from areas excluding any visible holes and determined average of fluorescent intensities from these regions of interest. We used this to calculate fluorescent intensity before Triton X-100 treatment from the entire cell area, or total DiIC\(_{18}\) (\(F_t\)). The percentage of DiIC\(_{18}\) extracted by Triton X-100 (\(F_e = (F_i - F_t) \times 100/F_t\)). For each data point, more than 50 individual cells were measured and pooled to produce an average and standard error of the mean (S.E.).

**Cy3-Transferrin Labeling of EYFP-Caveolin-1-transfected Cells**—Mock, ABCA1, and A937V mutant cells were grown to 80% confluence in 35-mm glass-bottom dishes and transfected with EYFP-caveolin-1 as described above. All cell lines were then incubated in DMEM with 1 mg/ml BSA and 10 mM mifepristone for 18 h. The cells were placed on ice and washed with ice-cold PBS. To stain the transferrin receptor on the cell surface, the cells were incubated with 80 ng/ml of Cy3-transferrin in Medium 1 for 30 min on ice. The cells were then washed 2× with ice-cold PBS and fixed with 4% paraformaldehyde for 10 min on ice. Cy3-transferrin and plasma membrane EYFP-caveolin-1 were visualized using a Nikon TE2000 inverted fluorescent microscope with a 60× (numerical aperture, 1.4) objective. Fluorescence images were taken using a cooled digital camera (Cascade 512B EM, Photometrics).

**Detergent-free Purification of Caveolae Membrane**—The procedure used was based on an established protocol by Smart et al. (19). Mock, ABCA1, and A937V mutant cells were grown to 80% confluence in 20-cm plates (five per cell line), and incubated in DMEM with 1 mg/ml BSA and 10 mM mifepristone for 18 h to induce ABCA1 expression. All of the following steps were carried out at 4 °C. Plates were washed twice with 3 ml of ice-cold buffer A (250 mM sucrose, 2.0 mM EDTA, 40 mM Tricine, pH 7.8). Cells were then scraped and collected in 3 ml of buffer A. Pelleting of cells was performed by centrifugation at 1000 \(g\), for 5 min, in a Beckman Coulter Allegra 6R centrifuge. The pellets were resuspended in 1 ml of buffer A and homogenized with 20 strokes of a Dounce homogenizer. Homogenates were transferred to 1.5-ml Eppendorf tubes and centrifuged at 1000 \(g\) for 10 min to remove nuclei. After collection of supernatants, the pellets were resuspended in 1 ml of buffer A, and homogenization and centrifugation steps were repeated as described. Postnuclear supernatants from both homogenizations were combined (2 ml total) and layered onto 23 ml of 30% Percoll, and then centrifuged in a Ti-70 rotor at 86,000 \(g_{max}\) for 30 min, using a Beckman Coulter Optima L-90K Ultracentrifuge. The Percoll gradient was then collected in 2-ml fractions, including the 2-ml plasma membrane band. The plasma membrane fractions were transferred to glass test tubes and sonicated, on ice, three times in succession for 10 s on setting 3 of a Branson Sonifier 450. The sonicated plasma membrane fractions were then combined with 1.84 ml of 50% Optiprep and 160 \(\mu\)l of buffer A, and placed in the bottom of SW41 tubes. After vortexing, a 6-ml continuous 20–100% Optiprep gradient was layered above the 4-ml plasma membrane preparations. Gradients were then centrifuged at 52,000 \(g\) for 90 min, in a Beckman SW41 rotor and Beckman Coulter Optima L-90K Ultracentrifuge. 10-ml Optiprep gradients were then collected in 1-ml fractions, and protein was trichloroacetic acid-precipitated. The pellets from all fractions were dissolved in sample buffer and loading buffer (60 \(\mu\)l of total volume) and analyzed by SDS-PAGE. All fractions were probed for caveolin-1 and clathrin by Western blot.

**Subcellular \[^{1}H\]Cholesterol Distribution**—Mock, ABCA1, and A937V mutant cells were grown to 80% confluence in 20-cm plates (two for each cell line). Radiolabeling of the cellular cholesterol pool was performed by incubation with 0.5 \(\muCi/ml\) \[^{1}H\]cholesterol for 24 h. Cells were then incubated in DMEM with 1 mg/ml BSA and 10 mM mifepristone for 18 h. All of the following steps were carried out at 4 °C. Plates were washed twice with 3 ml of ice-cold buffer A (250 mM sucrose, 2.0 mM EDTA, 40 mM Tricine, pH 7.8). Cells were then scraped.
ABCA1 Alters Membrane Microdomains

and collected in 3 ml of buffer A. Pelletting of cells was performed by centrifugation at 1,000 × g, for 5 min, in a Beckman Coulter Allegra 6R centrifuge. The pellets were resuspended in 1 ml of buffer A, and then homogenized with 20 strokes of a Dounce homogenizer. Homogenates were transferred to 1.5-ml Eppendorf tubes and centrifuged at 1000 × g, for 10 min, in a Fischer Scientific accuSpin MicroR centrifuge, to remove nuclei. Postnuclear supernatant fractions were collected and completed to 2 ml with buffer A. 45–10% continuous sucrose gradients were formed in SW41 tubes, and the gradients were subjected to centrifugation at 137,000 × g for 20 h at 4 °C. Each gradient was collected into 12 fractions (1 ml each). Equal volumes of each fraction were analyzed by SDS-PAGE, followed by Western blotting. Fractions were probed for the following cellular markers: caveolin-1 (plasma membrane), hsp-70 (cytosol), and calnexin (endoplasmic reticulum). [3H]Cholesterol levels in each fraction were analyzed by mixing 300 μl of sample with 2 ml of liquid scintillation mixture, and radioactivity was detected using a Beckman Coulter LS 6500 multipurpose scintillation counter.

**Determination of Akt Activation**—The procedure used was based on a protocol developed by Pike et al. (20). Briefly, mock, ABCA1, and A937V mutant cells were grown to 80% confluency in 20-cm plates. Cells were incubated in DMEM with 1 mg/ml BSA and 10 nM mifepristone for 18 h. The use of serum-free DMEM effectively starved the cells during the 18-h incubation. Cells were then stimulated with 50 ng/ml EGF. The cells were washed twice with 5 ml of ice-cold PBS. Once again, all of the following steps were carried out at 4 °C. Cells were washed twice with 3 ml of ice-cold radioimmuno precipitation assay buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA), scraped, and collected in 3 ml of radioimmuno precipitation assay buffer, supplemented with 100 μM orthovanadate, 20 mM p-nitrophenylmethylsulfonyl fluoride, 1 μg/ml leupeptin. Pelletting of cells was performed by centrifugation at 1000 × g, for 5 min, in a Beckman Coulter Allegra 6R centrifuge. The pellets were resuspended in 1 ml of supplemented radioimmuno precipitation assay buffer, and then homogenized with 20 strokes of a Dounce homogenizer. Homogenates were transferred to 1.5-ml Eppendorf tubes and centrifuged at 1000 × g for 10 min. The supernatants were collected, and aliquots were assayed for protein. Equivalent amounts of protein were mixed with SDS loading buffer and analyzed by SDS-PAGE and Western blotting. Phospho-Akt (activated) was probed using a goat polyclonal antibody recognizing phosphorylated Akt1 (Ser-473). Total Akt was probed using a goat polyclonal antibody against Akt1 (C-20). Akt activation was expressed as a ratio of phospho-Akt1/total Akt1.

**125I-ApoA-I Association**—Purified plasma apoA-I (Biodesign) solubilized in 4 mM guanidine-HCl was dialyzed extensively against PBS buffer. ApoA-I was iodinated with 125-iodine by IODO-GEN® (Pierce) to a specific activity of 2800 cpm/ng of apoA-I, dialyzed, and used within 48 h. Mifepristone-induced BHK cells were washed two times with DMEM and incubated for 30 min at 37 °C in DMEM containing 10 μg/ml 125I-apoA-I.

The medium was removed, cells were washed three times with ice-cold PBS, chilled on ice for 30 min in DMEM plus 10 mM Heps, pH 7.4, and cold Triton X-100 extraction was performed. Cells were lysed with 0.5 N NaOH, and the protein concentration was determined by using the Lowry method. Radioactivity found in the medium and in the cells was determined by gamma counting.

**RESULTS**

We hypothesized that ABCA1 modifies the plasma membrane raft/non-raft microdomains to facilitate apoA-I mediated efflux. To examine this, we used a BHK cell model stably transfected with a mifepristone-inducible plasmid containing an ABCA1 insert. A cell line containing identical mifepristone-inducible plasmid but without insert serves as control (Mock) (14). These BHK cells do not normally express ABCA1. An 18- to 20-h induction led to ABCA1 expression (Fig. 1, A). The expression level of ABCA1 in these cells is comparable to that of human THP-1 macrophages induced with 22(R)-hydroxysterol and 9-cis-retinoic acid, as determined by immunoblot (data not shown). Accordingly, ABCA1-express-
ing BHK cells had a high efficiency to efflux cholesterol to apoA-I (Fig. 1B), comparable to RAW264 cells, which typically efflux ~15% cholesterol in 6 h (21) or THP cells (1.7% cholesterol in 2 h (22)). These results indicate that wt ABCA1 expressed in BHK cells is fully functional.

We then examined ABCA1 localization in BHK cells by immunofluorescent staining. We found that ABCA1 was mainly on the plasma membrane. Using confocal microscopy we took either a single slice (Fig. 2A, first and third columns) or serial images along the Z-axis that cover the whole cell volume and then projected onto a single image (second and fourth columns). We observed that ABCA1 predominantly decorated the plasma membrane and projections (Fig. 2A), in agreement with previous reports using GFP-ABCA1 (10, 23). Details of Z-stacks are shown in the supplement data. At steady state, ABCA1 can also be found intracellularly (first and second columns). These intracellular structures have the characteristics of the endoplasmic reticulum and the Golgi. To examine whether these ABCA1-containing intracellular structures represent the biosynthesis pathway that delivers ABCA1 to the plasma membrane, we treated cells with a general protein synthesis inhibitor, cycloheximide. We found that, when the biosynthesis pathway is blocked, ABCA1 can only be found on the plasma membrane (Fig. 2A, third and fourth columns, also see supplement data). This indicates that the intracellular portion at steady state most likely represents newly synthesized ABCA1 that transiently passes through these organelles.

Furthermore, we examined free cholesterol in these cells and found that total free cholesterol mass was identical between Mock- and ABCA1-expressing cells (4.87 ± 0.88 μg/mg of protein versus 4.56 ± 0.77 μg/mg of protein, respectively). To rule out the possibility that cholesterol might be abnormally sequestered to some intracellular compartments in ABCA1 cells, which could significantly diminish cholesterol in the plasma membrane without apparent alteration in total cholesterol, we stained cells with filipin. We found that filipin staining patterns were comparable in all cell types (Fig. 2B), ensuring that ABCA1 expression did not alter either cholesterol content or subcellular distribution. Furthermore, we performed cell fractionation in these cells to characterize the cholesterol distribution biochemically. Results, shown in Fig. 2C, demonstrate that the plasma membrane is peaked at fraction 7 as marked by caveolin and so is the cellular cholesterol. There is no major difference in the cell types tested, largely consistent with our microscopy observations. ABCA1-expressing cells, however, seemed to have slightly less cholesterol in the endoplasmic reticulum (fractions 9 and 10) than Mock or A937V cells, which may reflect higher cholesterol mobility from internal membranes to the plasma membrane upon ABCA1 expression. Collectively, these results demonstrate that most of ABCA1 is localized on the plasma membrane and its expression does not cause significant cholesterol redistribution.

We also included a BHK cell line that expresses a mutant form of ABCA1 (A937V) upon mifepristone induction. These cells express a similar level of ABCA1 protein (Fig. 1A) and exhibit identical cellular distribution as wt ABCA1 (Fig. 2A). Cholesterol distribution in A937V-expressing cells was also identical to that of wt ABCA1-expressing or Mock cells (Fig. 2, 

**FIGURE 2.** **ABCA1 and cholesterol intracellular distributions.** A, ABCA1 distribution in Mock, ABCA1, and A937V cells. Cells were immunostained using an anti-ABCA1 antibody. Single slices of images (first and third columns) or a series of Z-sections (second and fourth columns) were taken throughout the cell volume using confocal fluorescent microscopy and then projected to a single plane. Some of the cells were also treated with cycloheximide for 2 h before immunostaining (third and fourth columns). B, Mock, ABCA1, and A937V cells were stained with filipin, and fluorescent images were taken under identical conditions. C, Cholesterol intracellular distribution by cell fractionation. [3H]Cholesterol-labeled cells were fractionated as described under "Materials and Methods." Each fraction was analyzed by 3H counts and protein markers: Hsp70 for the cytosol (peaked at fractions 3 and 4), caveolin for the plasma membrane (peaked at fraction 7) and calnexin for the endoplasmic reticulum (peaked at fraction 9). Error bars are the standard errors of the mean from two independent experiments.
ABCA1 Alters Membrane Microdomains

**Results** are expressed as mean ± S.D. of triplicate wells. *p < 0.05; #, p < 0.025.

To determine whether ABCA1 expression affects the raft/non-raft partitioning of the plasma membrane, we first analyzed free cholesterol mass in terms of tightly packed membrane fractions (rafts) versus loosely packed fractions (non-rafts). We first employed a widely used criterion, the solubility in cold Triton X-100 extraction for 30 min on ice. ABCA1 has been suggested to generate phospholipid-rich microdomains through its postulated lipid-flipping activity. This could affect the general lipid packing of the plasma membrane. Indeed, we found that Triton X-100-soluble free cholesterol of total free cholesterol (Triton X-100-soluble plus insoluble) in ABCA1-expressing cells was extracted with cold Triton X-100 on ice for 30 min. Cellular lipids in Triton X-100-insoluble fraction or in Triton X-100-soluble fraction were extracted and separated by TLC and radioactivity associated with free cholesterol was quantified by a colorimetric kit as described under "Materials and Methods." The results are presented as percentage Triton X-100-soluble free cholesterol of total free cholesterol (Triton X-100-soluble plus insoluble). A, cells labeled with [3H]cholesterol overnight were treated with 1% cold Triton X-100 on ice for 30 min. Cellular lipids in Triton X-100-insoluble fraction or in Triton X-100-soluble fraction were extracted and separated by TLC and radioactivity associated with free cholesterol was counted. B, results are presented as percentage Triton X-100-soluble [3H]cholesterol for 24 h, chilled on ice for 30 min prior to 1% Triton X-100 extraction at 4 °C. Lipids were extracted from media and cells and separated by thin-layer chromatography. Radioactivity associated with sphingomyelin (C) and phosphatidylcholine (D) was determined by β-scintillation counting. Results are expressed as mean ± S.D. of triplicate wells. *p < 0.05; #, p < 0.025.

**FIGURE 3. Distribution of free cholesterol and phospholipids.** BHK cells induced to express either empty vector (Mock), ABCA1 (ABCA1), or ABCA1 mutant A937V (A937V) cells were incubated with mifepristone overnight and subjected to 1% cold Triton X-100 extraction for 30 min on ice. A, free cholesterol contents in the media (Triton X-100-soluble) and in the cell (Triton X-100-insoluble) were analyzed by a colorimetric kit as described under "Materials and Methods." The results are presented as percentage Triton X-100-soluble free cholesterol of total free cholesterol (Triton X-100-soluble plus insoluble). B, cells labeled with [3H]cholesterol overnight were treated with 1% cold Triton X-100 on ice for 30 min. Cellular lipids in Triton X-100-insoluble fraction or in Triton X-100-soluble fraction were extracted and separated by TLC and radioactivity associated with free cholesterol was counted. Results are presented as percentage Triton X-100-soluble [3H]cholesterol for 24 h, chilled on ice for 30 min prior to 1% Triton X-100 extraction at 4 °C. Lipids were extracted from media and cells and separated by thin-layer chromatography. Radioactivity associated with sphingomyelin (C) and phosphatidylcholine (D) was determined by β-scintillation counting. Results are expressed as mean ± S.D. of triplicate wells. *p < 0.05; #, p < 0.025.

Mock cells (Fig. 3B). ABCA1 expression did not influence [3H]cholesteryl ester solubility in Triton X-100 (data not shown). This suggests that ABCA1 cells expanded the non-raft cholesterol pool without any change in total cholesterol mass or microscopic subcellular distribution as shown in Fig. 2 (B and C).

This cholesterol redistribution to Triton X-100-soluble pools in ABCA1-expressing cells could be driven by intrinsic functionalities of ABCA1. Alternatively, overexpression per se could create disturbances in the plasma membrane, regardless of the functionality of the protein. To address this, we used BHK cells expressing A937V. The cold Triton X-100-extractable cholesterol fraction in A937V-expressing cells was identical to that in Mock cells (Fig. 3A and B), indicating that cholesterol redistribution from raft to non-rafts requires functional ABCA1 protein.

ABCA1 has been suggested to generate phospholipid-rich microdomains through its postulated lipid-flipping activity. This could affect the general lipid packing of the plasma membrane. Indeed, we found that Triton X-100 solubility of [3H]sphingomyelin, another major component of rafts, also significantly increased (~50%) in comparison with Mock cells (Fig. 3C).

Interestingly, ABCA1 expression had no effect on the distribution of [3H]phosphatidylcholine, a phospholipid mainly in non-raft domains (Fig. 3D). Once again, the A937V mutant failed to redistribute [3H]sphingomyelin, consistent with its incapability to redistribute cholesterol in these cells.

We then evaluated the general effect of ABCA1 expression on the packing of the plasma membrane. To specifically visualize the plasma membrane, cells were pulse-labeled with [3H]choline for 24 h, chilled on ice for 30 min prior to 1% Triton X-100 extraction at 4 °C. Lipids were extracted from media and cells and separated by thin-layer chromatography. Radioactivity associated with sphingomyelin (C) and phosphatidylcholine (D) was determined by β-scintillation counting. Results are expressed as mean ± S.D. of triplicate wells. *p < 0.05; #, p < 0.025.

We found that YFP-caveolin had no significant influence on this cholesterol redistribution to Triton X-100-soluble pools in ABCA1-expressing cells. To determine whether ABCA1 expression affects the raft/non-raft partitioning of the plasma membrane, we first analyzed free cholesterol mass in terms of tightly packed membrane fractions (rafts) versus loosely packed fractions (non-rafts). We first employed a widely used criterion, the solubility in cold Triton X-100 extraction for 30 min on ice. A, free cholesterol contents in the media (Triton X-100-soluble) and in the cell (Triton X-100-insoluble) were analyzed by a colorimetric kit as described under "Materials and Methods." The results are presented as percentage Triton X-100-soluble free cholesterol of total free cholesterol (Triton X-100-soluble plus insoluble). B, cells labeled with [3H]cholesterol overnight were treated with 1% cold Triton X-100 on ice for 30 min. Cellular lipids in Triton X-100-insoluble fraction or in Triton X-100-soluble fraction were extracted and separated by TLC and radioactivity associated with free cholesterol was counted. Results are presented as percentage Triton X-100-soluble [3H]cholesterol for 24 h, chilled on ice for 30 min prior to 1% Triton X-100 extraction at 4 °C. Lipids were extracted from media and cells and separated by thin-layer chromatography. Radioactivity associated with sphingomyelin (C) and phosphatidylcholine (D) was determined by β-scintillation counting. Results are expressed as mean ± S.D. of triplicate wells. *p < 0.05; #, p < 0.025.

So far, we have used detergent insolubility, a commonly used operational definition of rafts, to characterize the effect of ABCA1 expression. Although informative, this method is not entirely without limitations. We therefore sought next to use a detergent-free approach to verify our observations. Caveolin is a well known raft/caveolae marker, and its intracellular localization is highly sensitive to cholesterol content and the lipid organization of the membrane (26, 27). We transiently transfected Mock, ABCA1, and A937V cells with YFP-caveolin. Because the role of caveolin in cholesterol efflux has been highly inconsistent among the cell models tested to date (28), we first examined cholesterol efflux in YFP-caveolin-expressing cells. We found that YFP-caveolin had no significant influence on
cholesterol efflux in BHK cells (ABCA1, 4.35 ± 0.2%; ABCA1/YFP-caveolin, 4.12 ± 0.02%). This ensured us that YFP-caveolin expression itself would not likely alter cholesterol movement in these cells. We then morphologically analyzed the caveolin distribution in live cells. By confocal microscopy, we found that YFP-caveolin distribution in Mock cells was punctated and clustered, especially on the edges of the cells (Fig. 5A), characteristic of the reported caveolae distribution on the plasma membrane (29). At higher magnification, YFP-caveolin was seen to decorate cable-like structures in these Mock cells (Fig. 5C), presumably along the actin filaments (30). In ABCA1-expressing cells, strikingly, most of YFP-caveolin was diffusely distributed on the general area of the plasma membrane, largely lacking clustered caveolae-like structures (Fig. 5B and D). Furthermore, when we scored the cell population according to their YFP-caveolin appearance, we found that ~80% of ABCA1 cells had a diffuse YFP-caveolin distribution on the plasma membrane, whereas nearly all Mock cells showed punctated YFP-caveolin distribution (Fig. 5E). The examination of endogenous caveolin distribution using an anti-caveolin-1 antibody in untransfected cells by immunofluorescent staining gave similar results (not shown). Moreover, caveolin distribution in ABCA1 cells was identical to that of Mock cells before induction (not shown), indicating a direct impact by ABCA1 expression.

To further ensure that caveolin redistribution indeed resulted from ABCA1 function, we analyzed YFP-caveolin-expressing cells co-stained with ABCA1. We took advantage of the fact that, even within the same clone, there were variations among individual cells in terms of ABCA1 expression. We found that caveolin becomes diffusely distributed at the plasma...
membrane only in cells expressing ABCA1 (Fig. 6, A and B, cells a and b). In two neighboring cells (c and d) that expressed no detectable ABCA1, YFP-caveolin was strictly observed in punctated and clustered structures, similar to Mock cells (Fig. 5C). Furthermore, when we tested ABCA1 mutant A937V, we found that A937V was mainly localized on the plasma membrane and expressed at levels similar to wild-type ABCA1 (Fig. 6C). Yet, this mutant failed to redistribute YFP-caveolin (Fig. 6D). YFP-caveolin in A937V cells was mostly clustered, identical to that in Mock cells. This is consistent with our observations presented above that A937V is defective in redistributing rafts (Fig. 3). Caveolin distribution is known to be sensitive to cholesterol in the plasma membrane, but we could not detect any differences in terms of free cholesterol mass or subcellular cholesterol distribution among these cell lines. We therefore had to conclude that a shift in lipid packing conditions in ABCA1 cells, such as significant expansion of non-raft membrane fractions, hindered caveolin from forming caveolae clusters, because caveolae assembly requires tightly packed lipid rafts. ABCA1 likely depletes cholesterol from caveolae, therefore redistributing caveolin from clusters to the general area of the plasma membrane (31). Caveolin redistribution in ABCA1 cells, therefore, is consistent with our biochemical analysis (Fig. 3).

Interestingly, when we treated cells with apoA-I for 4 h, we did not observe any visible effect on YFP-caveolin distribution in either mock or ABCA1 cells (not shown). However, more drastic changes such as cholesterol depletion by 30-min incubation with MCD, a condition known to deplete >60% cholesterol and disrupt rafts, could disperse YFP-caveolin from the clusters to the general area of the plasma membrane in Mock cells. The same treatment had little effect on YFP-caveolin in ABCA1 cells. When we exogenously loaded cholesterol into these cells by using a cholesterol-MCD complex to enforce rafts formation, we could shift YFP-caveolin into clusters in ABCA1 cells without much effect in Mock cells (data not shown). Taken together, our caveolin distribution results from live cells support the idea that ABCA1 re-organizes the raft/non-raft partition of the plasma membrane.

If altered caveolin distribution in ABCA1 is indeed due to membrane lipid reorganization, this should also alter caveolin floatation properties, another method commonly used to define rafts. We therefore performed experiments using a non-detergent-based method, Optiprep gradient floatation, on Mock, ABCA1, and A937V cells (Fig. 7A). We found that the majority of caveolin was in fractions 1–6, typical of rafts. A minor portion of caveolin (~5%) was found in fractions 7–9, co-localizing with clathrin, a non-raft maker, in Mock and A937V cells. However, there was much more caveolin in the non-raft fractions (20%) in ABCA1 cells than either Mock or A937V cells (Fig. 7B). There was no significant difference between Mock and A937V cells. The caveolin floatation in non-detergent-based gradient, therefore, is in agreement with our observations in live cells.

Rafts are generally thought to be particularly important in signal transduction. To test whether such shift from raft to non-raft in ABCA1-expressing cells influences cell signaling, we...
characterized EGF-induced Akt phosphorylation, a process known to be sensitive to microdomain organizations (13). As shown in Fig. 8, a brief treatment with EGF triggered Akt phosphorylation in Mock cells as detected by anti-phospho-Akt antibody (Ser-473) (Fig. 8A). By contrast, we detected little Akt phosphorylation in ABCA1 cells (Fig. 8A). Expression of A937V once again does not alter Akt activation (Fig. 8A). Total amounts of Akt were similar in all three cell lines (Fig. 8A). The ratio of Ser-473-phosphorylated Akt versus total Akt is shown in Fig. 8B. This demonstrates a specific impairment of Akt activation upon ABCA1 expression. ABCA1 therefore negatively impacted on Akt activation, perhaps due to its function in redistributing raft to non-raft microdomains.

Another likely consequence of loosely packed membranes is that extracellular acceptors could now have better access to cholesterol. To examine this, we performed MCD extraction at 37 and 0 °C. A short pulse (1 min) with 5 mM MCD at 37 °C can extract cholesterol specifically from the plasma membrane without any significant involvement of intracellular transport (32). We found that MCD was extremely potent, and 1-min treatment extracted >30% [3H]cholesterol from cells, ABCA1, and Mock cells alike (Fig. 9A). This assured us that there is no obvious difference in terms of plasma membrane total cholesterol content in ABCA1 and Mock cells. It is also consistent with previous reports that at 37 °C, MCD-mediated cholesterol efflux is independent of ABCA1 function (33–35). Under these conditions, however, the high potency of MCD could easily override any native mechanisms, such as ABCA1-induced microdomain organization. We therefore resorted to a low temperature condition. We reasoned that lowering the temperature could, first of all, enforce microdomain organizations in the plasma membrane (Triton X-100 can only distinguish rafts from non-rafts at 0 °C). Additionally, lowering the temperature could potentially decrease the potency by which MCD extracts cholesterol, thus allowing the native mechanism to participate.
When we performed experiments on ice, we indeed found that MCD was much less potent even after prolonged incubations. Interestingly, MCD (5 mM) can now extract 40–50% more cholesterol from ABCA1 cells than from Mock cells at all the time points examined (Fig. 9B). A higher concentration of MCD (10 mM) gave similar results (not shown). These observations thus support the idea that cholesterol in the plasma membrane of ABCA1-expressing cells is more accessible to extraction, once again consistent with its increased non-raft localization.

Increased non-raft fractions in ABCA1-expressing cells could have functional consequences in terms of cholesterol efflux. ApoA-I has been suggested to acquire lipids primarily from non-raft membrane fractions (7), and an enlarged non-raft membrane pool could favor apoA-I action. To explore this possibility, we analyzed membrane domains with which apoA-I primarily associates. We incubated cells with 125I-apoA-I for 30 min at 37 °C and performed cold Triton X-100 extraction. We found in ABCA1 cells that apoA-I is indeed preferentially associated with non-raft membrane fractions (Fig. 9C), whereas no such preference could be observed in Mock cells. This argues for a role of ABCA1 in facilitating the association of apoA-I with non-raft microdomains. Altogether, our results suggest that ABCA1 favors the functional proximity of phospholipids, cholesterol, and apoA-I in loosely packed lipid microdomains of the plasma membrane. This is likely achieved by generating more non-raft microdomains.

**DISCUSSION**

In the present study, we obtained several lines of evidence indicating that ABCA1 expression alters the general packing of the plasma membrane by generating more loosely packed microdomains (non-rafts). First, ABCA1 expression increased the Triton X-100 solubility of cholesterol and sphingomyelin. Second, fluorescence microscopy using DiIC18 revealed an increased proportion of the plasma membrane area solubilized by Triton X-100 in ABCA1-expressing cells. Third, upon expression of ABCA1, caveolin was found more diffusely localized, characteristic of caveolae disassembly. This is also consistent with our observation that, in ABCA1-expressing cells, more caveolin was found in non-raft fractions obtained by non-detergent-based Optiprep gradient ultracentrifugation. Fourth, cholesterol was ~50% more available to cold MCD extraction from ABCA1-expressing fractions. Interestingly, ABCA1 expression also impaired Akt activation, a known raft-dependent process. Most importantly, we found that a functional nucleotide-binding domain in ABCA1 is required for non-raft expansion, because the A937V mutant failed to induce any cholesterol, sphingomyelin, or caveolin redistribution. A937V mutant also failed to impair Akt phosphorylation. These observations collectively demonstrate that, through its ATPase-related functions, ABCA1 re-organizes the plasma membrane and generates more loosely packed domains. These loosely packed domains likely facilitate apoA-I association with cells and, consequently, lipid acquisition by apoA-I to form nascent high density lipoprotein particles.

The results presented here potentially shed new light on previous studies (7, 14). In both primary human fibroblast and BHK cells, ABCA1 expression was found to enhance cholesterol oxidase accessibility. Cholesterol oxidase was able to convert more cholesterol into cholestenone in ABCA1-expressing cells. Mock BHK cells had significantly lower conversion rates. The action of cholesterol oxidase on membrane cholesterol is thought to highly depend on the environment of the substrate, and loosely packed cholesterol is likely more accessible to oxidase (36, 37). Our results thus suggest that a higher percentage of cholesterol susceptible to oxidase in ABCA1-expressing cells may reflect an increased availability of free cholesterol in what we may now call non-raft microdomains. Interestingly, cholesterol removed by apoA-I in ABCA1 cells was found to be exclusively derived from the oxidase-sensitive membrane domains (7, 14). Those results therefore support the concept presented here that ABCA1-mediated expansion of non-raft microdomains facilitates lipid efflux to apoA-I.

It has been proposed that the plasma membrane is essentially a quilt containing many heterogeneous patches, namely microdomains (38). We are just beginning to understand this complex lateral heterogeneity within the plasma membrane. Microdomains have been operatively defined by detergent solubility (39). ABCA1 appears to be localized in detergent-soluble fractions. How ABCA1 expands the non-raft fractions is not immediately clear. Because this event appeared to be independent of apoA-I, it likely represents some intrinsic functions of ABCA1 on the membrane. ABCA1 could influence membrane lateral organization simply by being an integral protein with multiple transmembrane domains and partitioning into non-raft fractions. Alternatively, ABCA1 may actively flip phospholipids and cholesterol from the inner to outer leaflet, leading to membrane destabilization. Our observations with the A937V mutant seem to support this latter notion.

The precise nature of ABCA1-generated non-raft microdomains remains to be elucidated. Here we show that Mock cells have a significant proportion of the plasma membrane that was Triton X-100-soluble. Yet, apoA-I-mediated cholesterol efflux is totally absent in these cells. An obvious explanation for this is that a protein–protein interaction between apoA-I and ABCA1 may be required for efficient lipid acquisition from non-rafts. However, it remains as a possibility that ABCA1 may generate non-raft domains compositionally distinct from those present in Mock cells. In Mock cells, ABCA1-independent mechanisms must maintain the raft/non-raft partition. Different packing and/or lipid composition of the non-rafts found in Mock cells might prevent them from participating in lipid efflux. Unfortunately, we are limited by techniques currently available that could reveal further details about differences in the nature and composition of these membrane micro-organizations (24).

The concept that ABCA1 alters lipid-packing conditions may have important implications on the understanding of the apoA-I-mediated lipid efflux. Fielding et al. (42) have reported that cholesterol efflux is still possible in cells that do not express ABCA1 when apoA-I was first lipided with phospholipids. This apparently argues against ABCA1 being required for cholesterol efflux. Evidence presented here suggests rather that ABCA1 plays a major role in increasing the accessibility of cholesterol to extraction by apoA-I, therefore also actively participating to the second step of the efflux process. This may also explain why Smith et al. (5) (and others) have reported difficulties in separating phospholipid from cholesterol efflux. By co-
localizing apoA-I and cholesterol in loosely packed lipid microdomains, ABCA1 may promote nearly simultaneous phospholipid and cholesterol efflux, i.e. too rapid to be separately detected at a physiological temperature.

Our observations may also have more general implications. Membrane microdomains are thought to be functionally significant in live cells by providing platforms to facilitate specific interactions among proteins (40). Our results support the notion that such micro-organizations in live cell membrane are actively maintained (41) and, most likely, far from equilibrium (24). In contrast to artificially created model membranes, which are mostly in equilibrium, membranes in live cells are intrinsically subjected to constant disturbance. For example, vesicles are continuously budding from and fusing to these membranes. Most lipid components in the membrane are also rapidly turning over. In addition, membranes with transmembrane asymmetry, such as the plasma membrane, have to be maintained by a large number of ATPases through consumption of metabolic energy. The lateral organization of the plasma membrane likely results collectively from all these actions. It is thus not surprising that the properties of membranes in live cells are frequently found to significantly depart from that of the model membranes. Such disturbances, intrinsic to live cells, may also explain why rafts are highly dynamic and invisible (below optical resolution) as we understand them now (24).

In summary, the current study demonstrates for the first time that ABCA1 is capable of re-organizing the microdomains on the plasma membrane, such that there are significantly more non-raft microdomains upon ABCA1 expression. Importantly, this reorganization depends on its nucleotide binding domain. Because apoA-I is primarily associated with non-raft membrane fractions, we speculate that this expansion of non-raft microdomains plays a critical role in apoA-I-mediated cholesterol efflux. ABCA1 likely pre-conditions cells and generates a favorable membrane environment for apoA-I to acquire lipids, including cholesterol. Further studies are needed to clarify the molecular requirements for ABCA1 to achieve this function. Such capacity to manipulate membrane microdomains may represent a general mechanism for cells to dynamically maintain functional membrane micro-organizations.

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