Evaluation of the Role of Phosphatidylserine Translocase Activity in ABCA1-mediated Lipid Efflux*

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The following two theories for the mechanism of ABCA1 in lipid efflux to apolipoprotein acceptors have been proposed: 1) that ABCA1 directly binds the apolipoprotein ligand and then facilitates lipid efflux and 2) that ABCA1 acts as a phosphatidylserine (PS) translocase, increasing PS levels in the plasma membrane exofacial leaflet, and that this is sufficient to facilitate apolipoprotein binding and lipid assembly. Upon induction of ABCA1 in RAW264.7 cells by cAMP analogues there was a moderate increase in cell surface PS as detected by annexin V binding, whereas apoAI binding was increased more robustly. Apoptosis induced large increases in annexin V and apoAI binding; however, apoptotic cells did not efflux lipids to apoAI. Annexin V did not act as a cholesterol acceptor, and it did not compete for the cholesterol acceptor or cell binding activity of apoAI. ApoAI binds to ABCA1-expressing cells, and with incubation at 37 °C apoAI is co-localized within the cells in ABCA1-containing endosomes. Fluorescence recovery after photobleaching demonstrated that apoAI bound to ABCA1-expressing cells was relatively immobile, suggesting that it was bound either directly or indirectly to an integral membrane protein. Although ABCA1 induction was associated with a small increase in cell surface PS, these results argue against the notion that this cell surface PS is sufficient to mediate cellular apoAI binding and lipid efflux.

The ATP binding cassette (ABC) family of proteins serves to pump a diverse set of molecules out of cells. ABCA1, the Tangier disease gene, is required for cholesterol and phospholipid efflux to lipid-free apolipoproteins (for review see Ref. 1). The exact mechanism of this lipid efflux pump and the location of the lipid transfer and assembly onto apolipoprotein acceptors have yet to be determined definitively. It has been shown previously that ABCA1 expression leads to increased cell surface binding and uptake of apoAI (2). Protein cross-linking studies have shown that cell surface ABCA1 is closely associated with exogenously added apoAI (3, 4). Thus, one theory of ABCA1 action is that the apolipoprotein ligand is bound directly to the ABCA1 receptor, which can then mediate lipid assembly to form a nascent lipoprotein. An alternative theory of ABCA1 function has been put forth by Chimini and co-workers (5, 6). Phosphatidylserine (PS) is normally asymmetrically distributed on the plasma membrane such that most of it is sequestered on the inner leaflet. Increased levels of PS in the exofacial leaflet have been demonstrated in ABCA1 transiently transfected cells, thus providing evidence that ABCA1 may function as a PS translocase to pump PS from the cytoplasmic leaflet to the exofacial leaflet (5). This activity is referred to as “floppase” activity to distinguish it from the “flippase” activity that pumps PS from the outer to inner plasma membrane leaflets (7). These authors propose that PS translocase activity is sufficient to lead to apoAI binding to and lipid efflux from ABCA1-expressing cells and that the cross-linking of apoAI with ABCA1 might be circumstantial (6). They also used fluorescence correlation spectroscopy to demonstrate that apoAI had mobility on the cell surface, which indicated that it was not bound to an integral membrane protein on ABCA1 transfected cells and thus supported the hypothesis that apoAI was bound to cell surface PS (6).

In the current study, the role of ABCA1 as a PS translocase has been reexamined using non-transfected RAW264.7 cells in which ABCA1 is inducible by cAMP analogues (4, 9). We found that induction of ABCA1 in RAW cells led to a modest but significant increase in cell surface PS as measured by fluorescence annexin V binding and that cell surface redistribution of phospholipids induced by apoptosis was also associated with a dramatic increase in apoAI binding. However, increased cell surface PS induced by apoptosis was not sufficient to lead to lipid efflux. Furthermore, the PS-binding protein annexin V did not compete with apoAI for binding to ABCA1-expressing cells, and annexin V did not act as (or inhibit the ability of apoAI to act as) a cholesterol acceptor. Fluorescence recovery after photobleaching demonstrated that apoAI bound to ABCA1-expressing cells did not behave as if it were bound to phospholipids. Thus, the increase in cell surface PS found in ABCA1-expressing cells does not appear to be sufficient to account for the mechanism by which ABCA1 mediates apoAI binding and lipid efflux.

EXPERIMENTAL PROCEDURES

Fluorescent Annexin V and ApoAI Binding and Uptake Studies—RAW264.7 cells were trypsinized and plated in high glucose DMEM containing 10% fetal bovine serum on Permanox Lab-Tek 4-chamber tissue culture plastic slides (Nunc) at a density of 50,000 cells/chamber. One day after plating, cells were incubated for 24 h in serum-free DMEM containing 0.2% BSA in the presence or absence of 0.1–0.3 mM 8-Br-cAMP (Sigma), used to induce ABCA1. Staurosporine treatments took place during the last 4 h of this incubation period. ApoAI (BioDesign) was dialyzed against phosphate-buffered saline, adjusted to 0.5 mg/ml, labeled with Cy5 (a fluorescent dye that absorbs maximally at 649 nm and emits maximally at 670 nm; Amersham Biosciences, Mono-Reactive Dye Pack) as previously described (6), and passed through a 0.22-μm filter prior to use. For the initial cell binding studies 0.5 μg/ml Cy5-annexin V (Biovision) or 3 μg/ml Cy5-apoAI was incubated with the cells for 10 min at room temperature in phosphate-buffered saline.

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The abbreviations used are: ABC, ATP binding cassette; PS, phosphatidylserine; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; GFP, green fluorescent protein; NBD, 7-nitrobenz-2-oxa-1,3-diazole; FRAP, fluorescence recovery after photobleaching; NBD-PC, NBD phosphatidylethanolamine; EGFP, enhanced GFP.
containing 1 mM calcium chloride. For the competitive binding studies, the Cy5-labeled ligands were used at 1 μg/ml, and the unlabeled competitors were used at 40 μg/ml. The cells were then washed, fixed with 10% phosphate-buffered formalin, and overslipped prior to analysis by confocal microscopy using a 633-nm He laser on an upright Zeiss LSM510 system. All gain and offset settings were kept constant within each experiment. For quantitative analysis of ligand binding, images were captured using a broad 5-μm focal plane and adjusting the focus for maximal illumination. A brightfield image was also captured to count the cell number in each field. 5–10 random fields were captured, the images were exported to Photoshop software (Adobe) for intensity analysis, and the average fluorescence per cell was calculated for each image. For Cy5-apoAI uptake studies, this ligand was incubated at 37 °C for 5 or 60 min with cells in serum-free medium containing 0.2% BSA. After fixation, 0.6-μm confocal sections were obtained throughout the cell.

Cholesterol Efflux Studies—On day 1, RAW264.7 cells were plated onto a 24-well plate at a density of 250,000 cells/well. All subsequent incubations were performed in serum-free medium containing 0.2% BSA. On day 2, the cells were cholesterol-labeled and loaded for 24 h with 0.33 μCi/ml [3H]cholesterol and 50 μg/ml acetylated low density lipoprotein. This labeling medium was prepared by directly adding [3H]cholesterol dissolved in ethanol to the acetylated low density lipoprotein, preincubating at 37 °C for 20 min, and diluting in serum-free DMEM containing 0.2% BSA. On day 3, the cells were washed and incubated for 24 h in the presence or absence of 0.1–0.3 mM 8-Br-cAMP. On day 4, the cells were chased with 5 μg/ml apoAI for 4 h. At the end of this chase period, the radioactivity in the medium and cells was determined by liquid scintillation counting, and the percent efflux was calculated as 100× the total medium dpm divided by the sum of the total medium and cellular dpm. All treatments were performed in triplicate. Other variations on this protocol are described in the individual figure legends.

Generation of Stably Transfected mABCA1-GFP Cell Line—The mouse ABCA1 cDNA was cloned by reverse transcriptase-PCR using RNA extracted from 8-Br-cAMP-treated RAW cells. The entire clone was sequenced and found to contain only minor differences compared with the GenBank™ sequence AF287263. An in-frame green fluorescent protein (GFP) fused to the C terminus of ABCA1 was engineered in pCR expression vector (Invitrogen). This expression vector was transfected into HEK293 cells using LipofectAMINE (Invitrogen). Two days later the cells were trypanospined and plated at low density in p100 dishes for selection of neo+ colonies in medium containing 1 mg/ml Geneticin (Invitrogen). Colonies were examined by epifluorescence to confirm GFP expression and were subsequently picked and expanded.

Fluorescence Recovery after Photobleaching (FRAP) Studies—RAW cells were plated on collagen-coated coverslip bottom p35 dishes (MatTek). On the following day the cells were treated for 24 h in DMEM, 0.2% BSA with 50 μg/ml acetylated low density lipoprotein, 0.3 mM 8-Br-cAMP, and (for some dishes) 0.05–0.2 mM NBD-PC (diluted directly into this medium from a 10 mM stock in ethanol; Molecular Probes, catalog no. N-3786). Prior to FRAP, the NBD-PC-containing medium was aspirated, and the cells were washed once and preincubated at 37 °C for 10 min in serum-free DMEM. For the apoAI FRAP study, 1–3 μg/ml Cy5-apoAI was incubated for 10 min to 1 h at 21 °C with either 8-Br-cAMP or staurosporine-pretreated RAW cells. The cells were washed prior to FRAP. For the annexin V FRAP, RAW cells were treated with staurosporine for 4 h and then incubated with 1 μg/ml Cy5-annexin V for 10 min at 21 °C. FRAP was performed at 21 °C with a ×63 W lens using an inverted LSM510 system (Zeiss). NBD-PC photobleaching used three to five iterations of the Ar/Kr 488 laser set to 30% intensity, which did not lead to additional bleaching during the capture period. The luminosity of the bleach area was determined by the LSM510 software, and the data were exported for non-linear regression analysis.

Statistical Analyses—Two-tailed Student’s t tests, analysis of variance with Newman-Keuls post-test, and non-linear regression analysis were performed using Prism software (GraphPad).

RESULTS

ABCA1 Expression or Apoptosis Increases Cell Surface PS and ApoAI Binding—We have demonstrated previously that 8-Br-cAMP treatment of RAW267.4 cells leads to a 50-fold induction of ABCA1 mRNA, which in turn mediates cholesterol and phospholipid efflux to extracellular lipid-free apoAI or apoE (10). To determine whether 8-Br-cAMP-mediated ABCA1 induction in RAW cells was associated with PS floppase activity, cell surface PS was assessed in adherent cells by the binding of Cy5-labeled annexin V, a calcium ion-dependent PS-binding protein. RAW cells were grown on plastic slides and treated with or without 0.1 mM 8-Br-cAMP for 24 h or (for a positive control) for 4 h with 5 μM staurosporine, an apoptosis-inducing reagent. Treatment of RAW cells with the cAMP analogue induced a 64% mean increase in annexin V binding per cell as determined by confocal microscopy and quantitative image analysis (Fig. 1, A and B). This finding is qualitatively similar to the 40% increase in Cy5-annexin V binding previously reported using fluorescence-activated cell sorter analysis of trypsized ABCA1-transfected cells (5). Staurosporine treatment led to a much larger increase in annexin V binding, thus validating the annexin V binding assay (Fig. 1A). Binding of Cy5-labeled apoAI was also examined under similar conditions. Pretreatment of RAW cells with 8-Br-cAMP led to a 173% increase in Cy5-apoAI binding (Fig. 1, C and D), a 2.7-fold larger increase than was observed for binding of annexin V to the cAMP-treated cells. When the cells were treated with staurosporine, there was also a dramatic increase in Cy5-apoAI binding (Fig. 1C). Thus, apoptotic cells with increased levels of exofacial PS are competent to bind large amounts of Cy5-apoAI; however, whether the apoAI is actually binding to the cell surface PS cannot be determined from this experiment and will be addressed below.

Because staurosporine treatment was effective in inducing Cy5-apoAI binding to cells, the ability of staurosporine to induce cholesterol efflux to apoAI was also assessed. In the absence of staurosporine, a 16-h pretreatment with 8-Br-cAMP led to a 7.7-fold increase in [3H]cholesterol efflux to 3 μg/ml apoAI during a 4-h chase period at 37 °C (Fig. 2). 1.25 μM staurosporine was added during the entire 4-h chase period or during the last 1 or 2 h of this chase. In the absence of 8-Br-cAMP pretreatment, staurosporine treatment for 1–4 h failed to elicit cholesterol efflux to apoAI (Fig. 2). In the presence of 8-Br-cAMP, staurosporine treatment partially inhibited the cAMP-mediated cholesterol efflux to apoAI by ~30%. Thus, cells treated for varying times with staurosporine still retained most of the ability to release cholesterol to apoAI after induction by 8-Br-cAMP, and the failure of staurosporine by itself to induce efflux to apoAI cannot be totally attributed to the death of the cells. Staurosporine doses between 1.25 and 5.0 μM yields similar results, and all of these doses were capable of leading to increased levels of plasma membrane exofacial PS in a 4-h treatment as determined qualitatively by Cy5-annexin V binding (data not shown).

Annexin V Does Not Compete with ApoAI—An experiment was then performed to look at the ability of annexin V to act as a cholesterol acceptor or to alter the cholesterol acceptor activity of apoAI. RAW cells were cholesterol-loaded, pretreated with 8-Br-cAMP, and chased for 4 h in the presence or absence of apoAI with the addition of 0, 0.3, 3, or 30 μg/ml annexin V. Annexin V in the absence of apoAI was not capable of acting as a cholesterol acceptor, and in the presence of apoAI annexin V did not inhibit or stimulate cholesterol efflux (Fig. 3). Thus, annexin V does not compete for or cooperate with apoAI as a lipid acceptor.

The ability of apoAI and annexin V to compete with each
other for binding to RAW cells was examined next. Cells pre-
treated with 8-Br-cAMP were incubated for 10 min with 1
μg/ml Cy5-apoAI or Cy5-annexin V. Binding of Cy5 apoAI was
inhibited 76% by co-incubation with 40 μg/ml apoAI, but it was
not inhibited by co-incubation with 40 μg/ml annexin V (Fig.
4A). This competition by unlabeled apoAI brought Cy5-apoAI
binding down to the levels observed in cells that were not
exposed to 8-Br-cAMP pretreatment (data not shown). Con-
versely, the binding of 1 μg/ml Cy5-annexin V to the 8-Br-
cAMP-pretreated cells was not inhibited by co-incubation with
40 μg/ml apoAI but was inhibited 79% by co-incubation with 40
μg/ml annexin V (Fig. 4B). Thus, annexin V and apoAI do not
compete with each other for binding to ABCA1-expressing cells,
which implies that apoAI binding is not mediated by interac-
tion with cell surface PS. A similar study was undertaken with
apoptotic RAW cells that had been pretreated with staurospo-
rine for 4 h. Cy5-apoAI and Cy5-annexin V bound efficiently to
the apoptotic cells as observed above; however, when co-incu-
bated with excess unlabeled annexin V only the Cy5-annexin V
binding was competed off (Fig. 5). This implies that apoAI does
not bind to cell surface PS on apoptotic cells.

**ApoAI Bound to ABCA1-expressing Cells Does Not Have
Rapid Lateral Diffusion**—To determine the site of interaction
of apoAI with ABCA1, fluorescently labeled apoAI was incu-
bated with cells that either did or did not express ABCA1.
8-Br-cAMP pretreatment of RAW cells to induce ABCA1 led to
a large increase in the cellular uptake of Cy5-apoAI during a
1-h incubation at 37 °C (Fig. 6, A and B). Confocal slices
through ABCA1-expressing cells demonstrated that Cy5-apoAI

![Fig. 1. Induction of annexin V and apoAI binding to RAW264 cells by treatment with 8-Br-cAMP or staurosporine. RAW264 cells were treated for 24 h with 0.1 mM 8-Br-cAMP to induce ABCA1 or for 4 h with 5 μM staurosporine to induce apoptosis. A, representative confocal micrographs of Cy5-annexin V bound to RAW cells for 10 min at 24 °C. B, quantitative effect of 8-Br-cAMP treatment on Cy5-annexin V binding normalized to untreated cells (mean ± S.D.; *, p < 0.001; n = 5 fields of cells, each). C, representative confocal micrographs of Cy5-apoAI bound to RAW cells for 10 min at 24 °C. D, quantitative effect of 8-Br-cAMP treatment on Cy5-apoAI binding normalized to untreated cells (mean ± S.D.; ***, p < 0.0001; n = 10 fields of cells each).](image1)

![Fig. 2. Effect of staurosporine treatments on cholesterol efflux to apoAI. Cholesterol efflux to 3 μg/ml apoAI for 4 h was examined in cells with (filled bars) or without (open bars) 0.1 mM 8-Br-cAMP pretreatment to induce endogenous ABCA1. 1.25 μM staurosporine was added for the last 1 or 2 h of this chase or during the entire 4-h chase period (mean ± S.D., n = 3).](image2)

![Fig. 3. Effect of annexin V on cholesterol efflux. All cells were treated with 0.3 mM 8-Br-cAMP to induce ABCA1. Cholesterol efflux for 4 h was measured in the absence (squares) or presence (circles) of 3 μg/ml apoAI as an exogenous cholesterol acceptor and with the indicated concentration of annexin V (mean ± S.D., n = 3).](image3)
was on the cell surface as well as within the cell in what appear to be endocytic vesicles (Fig. 6B). Similar findings were obtained with apoAl labeled with either rhodamine or NBD (data not shown). To co-localize apoAl with ABCA1, stably transfected HEK293 cells expressing a murine ABCA1-EGFP fusion protein were prepared. Compared with non-transfected cells, these ABCA1-GFP-expressing cells have robust cholesterol efflux to apoAl (Fig. 7A). Confirming previous reports of transfections with human ABCA1-GFP fusion expression vectors (5, 11), the murine ABCA1-GFP fusion protein was expressed on the plasma membrane and in intracellular vesicles (Fig. 7, B and C). Cy5-apoAI was incubated with these cells at 37 °C for 5 or 60 min. After 5 min Cy5-apoAI was bound to the plasma membrane, often in large aggregates (Fig. 7B). After 60 min Cy5-apoAI was found on both the plasma membrane and in punctate ABCA1-GFP-containing vesicles within the cell, as demonstrated by confocal sectioning (Fig. 7C).

To examine the mobility of cellular phospholipids and proteins bound to cells, FRAP was performed at 21 °C. To assess lipid diffusion in the plasma membrane, cells were labeled with NBD-PC, a fluorescent phospholipid. The NBD-PC was highly mobile in the plasma membrane at 21 °C as indicated by the rapid diffusion during the photobleaching period leading to incomplete bleaching in the bleached area and partial bleaching beyond the perimeter of the bleach area. Thus, fluorescence recovery in the bleach area was not complete, because the total fluorescence in the adjacent donor membrane was also decreased (Fig. 8A). The partial fluorescence recovery occurred quite rapidly (Fig. 8, A and B), with an average t½ of 4.2 ± 1.4 s (n = 18, combined from two independent experiments). Cy5-apoAI was preincubated with ABCA1-expressing, 8-Br-cAMP-treated RAW cells for periods ranging from 10 min to 1 h at 21 °C, which led to binding to the cell surface in both large patches and punctate aggregates (the latter of which are not shown). In contrast to NDB-PC, bleaching of Cy5-apoAI in large patches on the cell surface led to a distinct area of bleach-

**Fig. 4.** Competition for annexin V and apoAI binding to ABCA1-expressing cells pretreated for 16 h with 0.3 mM 8-Br-cAMP. A, 1 μg/ml Cy5-apoAI was incubated for 10 min at 37 °C in the absence or presence of 40 μg/ml unlabeled apoAI or annexin V (AnnV) as indicated in DMEM-containing 0.2% BSA. Binding was measured on fixed cells by confocal microscopy as described under “Experimental Procedures” and normalized to that obtained in the absence of competitor (mean ± S.E., n = 6 fields of cells for each condition; *, p < 0.001 versus no competitor or annexin V competitor by analysis of variance). B, 1 μg/ml Cy5-annexin V was incubated for 10 min at 21 °C in the absence or presence of 40 μg/ml unlabeled apoAI or annexin V as indicated in phosphate-buffered saline containing 1 mM calcium chloride (mean ± S.E., n = 5 fields of cells for each condition; *, p < 0.001 versus no competitor or apoAI competitor by analysis of variance).

**Fig. 5.** Annexin V competition for Cy5-apoAI and Cy5-annexin V binding to apoptotic cells pretreated for 4 h with 5 μM staurosporine. Cy5-apoAI (5 μg/ml) or Cy5-annexin V (AnnV) (0.5 μg/ml) was incubated with cells in the absence (open bars) or presence (filled bars) of 40 μg/ml annexin V for 10 min at 21 °C. For each fluorescent ligand binding was normalized to that in the absence of competitor (mean ± S.E.; n = 5 fields of cells each; *, p = 0.001 compared with Cy5-annexin V binding in the absence of competitor).

**Fig. 6.** Increased intracellular uptake of Cy5-apoAI after ABCA1 induction by 8-Br-cAMP treatment. A, orthogonal projection of confocal sections of a representative cell in the absence of 8-Br-cAMP treatment shows minimal uptake of Cy5-apoAI during the 1-h incubation at 37 °C. B, orthogonal projection of confocal sections of a representative cell after pretreatment with 0.1 mM 8-Br-cAMP shows extensive intracellular accumulation of Cy5-apoAI during the 1-h incubation at 37 °C. For both panels the main image shows a section of a cell in the xy plane, near but not on the bottom of the cell at the z position indicated by the blue lines in the xy plane (small top panel) and the yz plane (small right side panel). In B it is apparent that Cy5-apoAI was taken into intracellular vesicles distributed asymmetrically in the xy plane of this cell, but in this region apoAI is distributed throughout the thickness of the cell in the z axis.
no fluorescence recovery after the bleaching of these structures (not shown). Thus, apoAI bound to ABCA1-expressing cells did not behave as if it were floating freely in the lipid phase of the plasma membrane. To determine the FRAP behavior of a comparably sized protein that is bound directly to plasma membrane lipids, staurosporine-pretreated RAW cells were incubated for 10 min with Cy5-annexin V (Fig. 8, E and F). After bleaching, the Cy5-annexin V fluorescence recovered with an average $t_{1/2}$ of $3.0 \pm 0.9$ s ($n = 9$). Thus, the annexin V bound to apoptotic cells did behave as if it were floating freely in the lipid phase of the plasma membrane. We also noted that Cy5-apoAI bound for 10 min at 21°C to staurosporine-pretreated cells did not exhibit fluorescence recovery after photobleaching (not shown), and in contrast to annexin V, which was bound exclusively to the cell exterior (Fig. 8E), apoAI was found throughout the cell despite this short incubation period at room temperature (Fig. 9). Thus, Cy5-apoAI could quickly penetrate the plasma membrane of apoptotic cells and adhere to intracellular structures that were relatively immobile during the first minute after photobleaching.

**DISCUSSION**

The plasma membrane has an asymmetrical distribution of phospholipids, with the external leaflet enriched with choline-containing phospholipids and the cytoplasmic leaflet enriched with aminophospholipids (12). This distribution is mediated by proteins that can translocate phospholipids between the inner and outer leaflets. The inward-directed flippase activity that normally keeps aminophospholipids in the inner leaflet is reportedly mediated by a P-type ATPase aminophospholipid translocase (12). In addition cells have outward-directed floppase activity that can be mediated by various members of the ABC gene family. The ABCB4 gene (also known as human MDR3, P-glycoprotein 3, or mouse mdr2) product is a PC-specific floppase with expression largely confined to the liver (13, 14). The ABCB1 (also known as MDR1 or P-glycoprotein 1) gene product can translocate a broad variety of short chain phospholipids and ceramides and can export platelet-activating factor (15, 16). Phospholipid floppase activity has also been claimed for the ABCG1 gene (MRP-1) product known to be needed for leukotriene C4 export, although the floppase activity might be an artifact caused by the analogues used in these studies (17, 18).

The *Caenorhabditis elegans* gene CED-7 codes for an ABC transporter that is implicated in the engulfment of apoptotic cells (19). Luciani and Chimini (20) have performed a series of studies indicating that ABCA1 has PS floppase activity, which is implicated in both apoptosis and lipid efflux to apoAI, and they propose that ABCA1 may function as the mammalian CED-7 homologue. A polyclonal antibody to the ABCA1 first ATP binding cassette stains phagocytes with ingested apoptotic bodies in the developing limb of mouse embryos, and when introduced into cultured macrophages by osmotic shock this antibody inhibited engulfment of apoptotic thymocytes (20). Glyburide or oligomycin treatments of macrophages inhibited engulfment of apoptotic cells as well as the exposure of PS on irradiated thymocytes (21). We speculate that these results might be caused by inhibition of a different mammalian ABC transporter because neither the polyclonal antibody nor the drugs have absolute specificity for ABCA1. In addition, our bleach analysis revealed that the human or mouse ABCA2 protein has greater identity to the *C. elegans* CED-7 protein than does the human or mouse ABCA1 protein, thus doubting the identity of ABCA1 as the mammalian homologue of CED-7.

Chimini’s laboratory reported less engulfment of apoptotic cells in *situ* and *ex vivo* in ABCA1-deficient mice compared with wild-type mice (5). However, ABCA1-deficient mice did not
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Display any gross morphological defects that can be attributed to defective apoptosis, implying that normal developmental apoptosis can proceed without an intact ABCA1 gene. Transfection of active versus mutant ABCA1 into HeLa cells was found to increase the fraction of cells competent to engulf apoptotic prey by approximately 2-fold (5). Although this effect may be caused by PS translocase activity, it may also be due to other effects on phagocytosis or actin- or microtubule-based membrane trafficking because ABCA1 recently has been noted to effect both receptor-mediated endocytosis and membrane trafficking from the Golgi (22, 23).

Chambenoit et al. (6) reported that the level of cell surface PS detected by Cy5-annexin V binding was related to the expression of ABCA1-EGFP in transfected HeLa cells that had been trypsinized and suspended prior to annexin V binding and analysis by fluorescence-activated cell sorter. In the current study using adherent cells in which endogenous ABCA1 was induced by 8-Br-cAMP, we confirm this prior result. We observed a 64% increase in fluorescent annexin V binding to adherent RAW264.7 cells treated with cAMP analogues, which induces ABCA1 mRNA levels by ~50-fold (10). Similarly treated cells had a 173% increase in fluorescent apoAI binding. In addition, we found that staurosporine-induced apoptosis of RAW cells, which results in increased cell surface PS, also led to a dramatic increase in cell-associated apoAI. These results together seem to support the hypothesis from Chimini's lab.
Chambenoit et al. (6) determined the mobility of apoAI by fluorescence correlation spectroscopy using ABCA1-GFP-expressing cells incubated for 5 min with Cy5-apoAI. In this procedure (designed for solution studies) confocal optics are used to scan the fluctuation of fluorescent emissions in a very small volume (~1 femtoliter). The diffusion of individual fluorescently labeled molecules into and out of this small area gives rise to this fluctuation, and the diffusion rate can be calculated. These authors determined that Cy5-apoAI diffused at room temperature on the cell surface more rapidly than would be expected if it were bound to an integral membrane protein; however, they did not display any micrographs of Cy5-apoAI bound to the ABCA1-GFP-expressing cells (6). Chambenoit et al. (6) speculate based on the fluorescence correlation spectroscopy study that ABCA1 induction of cell surface PS is sufficient to allow for binding of apoAI to the lipid phase of the plasma membrane without a direct ligand-receptor interaction with ABCA1. In the current study, we found that most of the cell surface apoAI was in large aggregates after a 5-min incubation of Cy5-apoAI with HEK293 cells expressing an ABCA1-GFP fusion protein. Thus, it is possible that the rapid diffusion of Cy5-apoAI measured by Chambenoit et al. (6) may be caused by diffusion within the aggregates and not the lateral diffusion of single apoAI molecules on the cell surface. If this is the case then the fluorescence correlation spectroscopy study could not definitively assess the characteristics of apoAI bound to the cell surface. In the current study, FRAP was used to directly visualize Cy5-apoAI mobility on the cell surface; we conclude that apoAI does not diffuse rapidly, and we speculate that it is either directly or indirectly tethered to an integral membrane protein, possibly ABCA1.

By analogy with other ABC transporters (18), it is possible that ABCA1 pumps lipids from within the cell to either the outer leaflet of the plasma membrane or perhaps into a recycling endosome. However, the lipids that may be the substrate for ABCA1 transport have not been clearly defined. Our data demonstrate that apoAI binding to ABCA1-expressing or apoptotic cells does not appear to be mediated by cell surface PS because we did not observe any competition between annexin V and apoAI for binding to these cells. Another lipid may be the substrate for ABCA1 floppase activity, and this lipid might initially interact with apoAI, although our data suggest that apoAI is then directly (or perhaps indirectly through a lipid bridge) bound to an integral membrane protein. An alternate hypothesis is that ABCA1 is a regulatory protein that directs membrane trafficking. This activity may also influence the plasma membrane lipid bilayer asymmetry by altering both the lipid and protein composition of the plasma membrane (for example, by altering the ratio of phospholipid flipases and floppases that are trafficked to the plasma membrane). Recently, the notion that ABCA1 functions as a regulator rather than a pump has been supported by the finding of very low ATPase activity of ABCA1 in isolated membrane preparations (8). It is possible that ABCA1 has two distinct activities: 1) functioning as a PS translocase, which appears not to play a direct role in apoAI binding or lipid efflux and 2) functioning as a protein that can directly or indirectly bind apoAI and facilitate the assembly of lipids on to the apoAI.

Our finding of ABCA1 and Cy5-apoI co-localized within cells is consistent with our prior hypothesis that endocytosis and resecretion is part of the ABCA1-mediated lipid efflux pathway (2). However, we cannot distinguish whether the observed co-localization is part of the lipid efflux pathway or part of a regular endosome to lysosome degradative pathway. We speculate that endocytosis might be a required step in ABCA1-mediated lipid efflux to allow for conditions in which a nascent lipoprotein can dissociate from cellular membranes and/or integral membrane proteins in a loose analogy to the release of iron from bound transferrin in acidic endosomes.

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