Quantitative Analysis of ABCA1-dependent Compartmentalization and Trafficking of Apolipoprotein A-I

IMPLICATIONS FOR DETERMINING CELLULAR KINETICS OF NASCENT HIGH DENSITY LIPOPROTEIN BIOGENESIS*

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The molecular mechanisms underlying the apoA-I/ABCA1 endocytic trafficking pathway in relation to high density lipoprotein (HDL) formation remain poorly understood. We have developed a quantitative cell surface biotinylation assay to determine the compartmentalization and trafficking of apoA-I between the plasma membrane (PM) and intracellular compartments (ICCs). Here we report that 125I-apoA-I exhibited saturable association with the PM and ICCs in baby hamster kidney cells stably overexpressing ABCA1 and in fibroblasts. The PM was found to have a 2-fold higher capacity to accommodate apoA-I as compared with ICCs. Overexpressing various levels of ABCA1 in baby hamster kidney cells promoted the association of apoA-I with PM and ICCs compartments. The C-terminal deletion of apoA-I Δ(187–243) and reconstituted HDL particles exhibited reduced association of apoA-I with both the PM and ICCs. Interestingly, cell surface biotinylation with a cleavable biotin revealed that apoA-I induces ABCA1 endocytosis. Such endocytosis was impaired by naturally occurring mutations of ABCA1 (Q597R and C1477R). To better understand the role of the endocytotic pathway in the dynamics of the lipidation of apoA-I, a pulse-chase experiment was performed, and the dissociation (re-secretion) of 125I-apoA-I from both PM and ICCs was monitored over a 6-h period. Unexpectedly, we found that the time required for 50% dissociation of 125I-apoA-I from the PM was 4-fold slower than that from ICCs at 37 °C. Finally, treatment of the cells with phosphatidylinositol-specific phospholipase C increased the dissociation of apoA-I from the PM. This study provides evidence that the lipidation of apoA-I occurs in two kinetically distinguishable compartments. The finding that apoA-I specifically mediates the continuous endocytic recycling of ABCA1, together with the kinetic data showing that apoA-I associated with ICCs is rapidly re-secreted, suggests that the endocytic pathway plays a central role in the genesis of nascent HDL.

*This work was supported by Grant MOP 15042 from the Canadian Institutes of Health Research and by the Heart and Stroke Foundation of Québec. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The molecular interaction of apoA-I with the cell membrane ABCA1 transporter has important implications in reverse cholesterol transport. It provides a mechanism whereby excess cholesterol is removed from peripheral cells that are unable to catabolize cholesterol, including macrophages in the vessel wall. This process plays a crucial role in both the formation and maintenance of HDL.3 levels in plasma and is believed to be one of the major mechanisms by which HDL protects against atherosclerotic cardiovascular disease (1, 2). Despite a large body of information identifying HDL as a potent physiological protector against atherosclerotic cardiovascular disease, the fundamental mechanisms underlying the genesis of HDL at the cellular level remain complex and poorly understood.

It is generally thought that the lipidation of apoA-I occurs inside the cell as a part of a retroendocytosis pathway. Of particular interest is the concept of Smith and co-workers (3) that cellular cholesterol efflux involves endocytosis and resecretion of apoA-I. This concept falls in line with the idea that the ABCA1 transporter may play an important role in the apoA-I retroendocytosis pathway and is supported by previous studies documenting the following: 1) apoA-I colocalizes with ABCA1-containing endosomes (4); and 2) apoA-I-mediated lipid efflux is defective in the lysosomal storage diseases Niemann-Pick type C disease (5) and Niemann-Pick type B disease (6).

The pioneering biophysical and biochemical studies by Neufeld et al. (7, 8) carried out with a functional fluorescent chimeric human ABCA1-GFP protein expressed in living cells have led to important new insights into the structural mechanisms involved in the lipidation of apoA-I. Indeed, ABCA1 was found to be present in late endosomes and lysosomes and to traffic between late endosomes and the plasma membrane (PM). The presence of both internalized apoA-I and ABCA1 in late endosomes is thought to be functionally important in
mediating lipid efflux from this intracellular location. It is not clear, however, whether apoA-I solubilizes a membrane domain that is created by ABCA1 (9–11) or if apoA-I/ABCA1 must interact directly and be internalized together for subsequent nascent HDL formation. This information is critical to the ongoing assessment of whether PM or intracellular compartments (ICCs) represent active cellular sites for the lipida
tion of apoA-I.

Although it is accepted that a retroendocytosis pathway plays an important role in the formation of nascent HDL particles, the structural determinants governing the dynamics of apoA-I lipidation at different cellular sites have not yet been elucidated. In this study, we have used a quantitative assay based on cell surface biotinylation to investigate the cellular compartmentalization and trafficking of apoA-I/ABCA1 in relation to the biogenesis of nascent HDL particles.

**EXPERIMENTAL PROCEDURES**

**Patient Selection**—For this study, we selected fibroblasts from three normal control subjects and two patients with TD (homozygous for Q597R at the ABCA1 gene and compound heterozygous for C1477R as described previously (12)). The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Centre. Separate consent forms for blood sampling, DNA isolation, and skin biopsy were provided.

**Cell Culture**—BHK cells stably transfected with an ABCA1 expression vector that is inducible by treating the cells with mifepristone and cells transfected with the same vector lacking the ABCA1 cDNA insert (mock-transfected) were generously provided by Dr. John F. Oram from the Department of Medi
cine, University of Washington, and were characterized and cultured as described previously (13, 14). These BHK cells do not normally express ABCA1.

Human skin fibroblasts were obtained from 3.0-mm punch biopsies of the forearm of patients and healthy control subjects and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.1% nonessential amino acids, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum.

**Human Plasma ApoA-I**—Purified plasma apoA-I (Biodes
design) was resolubilized in 4 M guanidine-HCl and dialyzed extensively against PBS buffer. Freshly resolubilized apoA-I was iodinated with $^{125}$I by IODO-GEN® (Pierce) to a specific activity of 3000–3500 cpm/ng apoA-I and used within 48 h. Expression of wild type (WT) apoA-I and its mutants in a bacterial system and the isolation and purification of these proteins have been described previously by Marcel et al. (15). Deletion mutant apoA-I $\Delta$(187–234) was provided by Dr. Yves L. Marcel.

**Cell Surface Biotinylation Assay**—BHK cells stably expressing ABCA1 or mock were treated or not with mifepristone as described by Oram and co-workers (13, 14). Confluent fibro
blasts were stimulated or not with 2.5 µg/ml 22(R)-hydroxycho
elsterol and 10 µM 9-cis-retinoic acid for 20 h. Cells were incubated in the presence of 10 µg/ml of apoA-I for 45 min at 37 °C or with increasing concentrations of apoA-I. Cells were washed three times with PBS, and surface proteins were biotin
ylated with 500 µg/ml sulfosuccinimidobiotin (sulfo-NHS-bio
tin; Pierce) for 30 min at 4 °C. The biotinylation reaction was quenched for 10 min at 4 °C by removal of the biotin solution and addition of 20 mM Tris-HCl (pH 7.5). Cells were washed twice with ice-cold PBS, lyzed, and homogenized. 200 µg of protein was added to 50 µl of streptavidin-Sepharose beads and incubated overnight on a platform mixer at 4 °C. The pellet (PM) or supernatant (ICCs) was washed with lysis buffer and counted for radioactivity. To test whether the biotinylation of cell surface proteins was complete, BHK cells induced with mifepristone and incubated with 10 µg/ml of $^{125}$I-apoA-I for 45 min at 4 °C were washed, and biotinylation was performed as described above. After streptavidin pull-down, the recovery of $^{125}$I-apoA-I associated with PM was estimated from total $^{125}$I-apoA-I (counts/min) added to the cells. We found that more than 85% of apoA-I at the cell surface was accessible to biotin.

**Preparation of Reconstituted HDL Particles**—Complexes comprising apoA-I and palmitoyloleoylphosphatidylcholine were prepared using the sodium cholate dialysis method as described by Jonas et al. (16). An apoA-I/palmitoyloleoylphosphatidylcholine molar ratio of 1:100 was used. rHDL particles were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, molecular weight cutoff 50,000, Amicon) to discard any lipid-free apoA-I or proteolytic peptides. apoA-I-lipid complex formation was verified by analysis with two-dimen
sional PAGGE as we have described previously (17).

**ABCA1 Endocytosis Assay**—Endocytosis assays were performed as described previously (18). Briefly, mifepristone-in
duced BHK-ABCA1 or 22OH/9CRA-stimulated normal fibro
blasts or ABCA1 mutant fibroblasts were biotinylated with a cleavable form of biotin (sulfosuccinimidyl 2-(bixinamido)-ethyl-1, 3-dithiopropionate (sulfo-NHS-SS-biotin); Pierce) at 4 °C for 30 min. Excess biotin was removed, and cells were incubated with warm DMEM containing 10 µg/ml apoA-I or not. Cells were maintained at 37 °C for various times to permit cellular trafficking. Subsequently, cells were washed with ice-cold PBS, and biotin cleavage was performed using a non-cell-per
meeble reducing reagent (50 mM glutathione in 75 mM NaCl, 10 mM EDTA containing 1% bovine serum albumin (pH 7.4)), which was applied to the cells twice for 15 min. Reducing reagent was quenched by two 5-min incubations with iodoacet
amide (5 mg/ml) at 4 °C. Biotinylated cells incubated with or without apoA-I at 4 °C throughout each assay and subjected to reducing agent were used as controls for the efficacy of biotin cleavage. Under these conditions, more than 95% of biotiny
lated ABCA1 was cleaved by glutathione. Integrin α4 and activin receptor type II, which localize to the plasma membrane in BHK cells and human fibroblasts, respectively, were used as controls for protein loading. The ratios of endocytosed ABCA1 over integrin α4 and ABCA1 over activin receptor type II were determined.

To ensure that application of cleavable biotin did not alter the function of ABCA1, cholesterol efflux assays were carried out on biotinylated cells. We found that labeling cell surface protein with cleavable biotin did not affect significantly apoA-I-mediated cholesterol efflux via ABCA1 pathway.
Dissociation of $^{125}$I-ApoA-I from Intact Cells—BHK-ABCA1, normal human fibroblasts, or fibroblasts with ABCA1 mutations (Q597R and C1477R) from Tangier disease subjects were used. Fibroblasts were grown to confluence in 100-mm diameter dishes and stimulated with 2.5 μg/ml 22(R)-hydroxycholesterol and 10 μM 9-cis-retinoic acid for 20 h in DMEM/bovine serum albumin. BHK cells stably expressing ABCA1 or mock were treated or not with mifepristone as described above. Cells were incubated in the presence of 10 μg/ml $^{125}$I-apoA-I for 45 min at 37 °C. After washing to remove unbound $^{125}$I-apoA-I, DMEM was added, and the plates were immediately incubated at 37 °C for increasing times. Biotinylation was performed, and $^{125}$I-apoA-I dissociation from both plasma membrane and intracellular compartments was quantitated as described above.

Analysis of Nascent ApoA-I-containing Particles—$^{125}$I-ApoA-I released to the medium at the time point (6 h) from mifepristone-induced BHK-ABCA1 cells was analyzed by two-dimensional PAGGE, and the number of apoA-I molecules per particle was assessed by cross-linking with dithiobis(succinimidyl propionate), as described previously (20).

Treatment with Phospholipases—Confluent BHK-ABCA1 cells were incubated for 45 min in DMEM containing 10 μg/ml $^{125}$I-apoA-I. After washing to remove unbound $^{125}$I-apoA-I, cells were subjected to treatment with 2.5 units/ml phosphatidylycholine-specific phospholipase C (PC-PLC) or 0.4 units/ml sphingomyelinase (SMase) (Sigma) for 30 min at 37 °C. Following washes, cell surface biotinylation was performed as described above.

Statistical Analysis—Results were compared statistically by Student’s t test. Two-tailed p values <0.05 were considered as significantly different.

RESULTS

Development of a Quantitative Biotinylation Assay—To investigate the cellular compartmentalization and trafficking pattern of apoA-I in a cell culture model, we developed a quantitative assay based on cell surface biotinylation. This permitted us to quantify the amount of apoA-I associated with the PM and ICCs as described under “Experimental Procedures.” However, both the incomplete biotinylation of cell surface proteins or the possible contamination of PM with ICCs fractions could result in an inaccurate quantification. To test whether the biotinylation of cell surface proteins was complete, 22OH/9CRA-stimulated fibroblasts were incubated with 10 μg/ml $^{125}$I-apoA-I for 45 min at 37 °C, washed, and incubated with increasing amounts of biotin (0.25–3 mg/ml). After streptavidin pulldown, $^{125}$I-apoA-I associated with PM (pellets) or ICCs (supernatants) was determined by γ-counting. Increasing the amount of biotin beyond 0.5 mg/ml did not result in any significant increase in $^{125}$I-apoA-I association with either the PM or ICCs (Fig. 1A).

To examine the specificity of the biotinylation reaction, a 30-fold excess of unlabeled apoA-I, absence of ABCA1 stimulation with 22OH/9CRA, and ABCA1 mutant fibroblasts (Q597R) were used as controls. As shown in Fig. 1B, the presence of excess unlabeled apoA-I, the absence of stimulation with 22OH/9CRA, or utilization of an ABCA1 mutant (Q597R)
ABCA1 expression promoted the association of apoA-I with the PM and ICCs. A, BHK cells stably overexpressing ABCA1 (BHK-ABCA1) were treated or not (B) with 10 nM mifepristone for 20 h. Cells were incubated with increasing concentrations of 125I-apoA-I for 45 min at 37 °C. Surface proteins were biotinylated and separated by streptavidin pulldown. A fraction of cell lysate was directly counted for the determination of total 125I-apoA-I associated with the cells. Recovered supernatants (ICCs) and pellets (PM) washed with lysis buffer were directly counted for radioactivity. Values represent the means ± S.D. from triplicate wells. Affinity parameters of 125I-apoA-I association with the PM and ICCs (B<sub>max</sub> and K<sub>d</sub>) were obtained using Graph Pad Prism 4.00 software and are reported in Table 1. C, BHK-ABCA1 cells were treated or not with 0.1 or 10 nM mifepristone for 20 h. Cells were incubated with 10 µg/ml 125I-apoA-I for 45 min at 37 °C. Surface proteins were biotinylated and separated by streptavidin pulldown. A fraction of cell lysate was directly counted for the determination of total 125I-apoA-I association to the cells. The inset shows the separation by SDS-PAGE of equal amounts of cell protein (40 µg) of ABCA1-transfected cells treated or not with 0.1 or 10 nM mifepristone for 20 h. ABCA1 was detected with an ABCA1 antibody. Cytosolic protein Hsp 70 serves as a loading control. D, 125I-apoA-I associated with recovered supernatant (ICCs) and pellet (PM) samples was determined by γ-counting. Values represent the mean ± S.D. from triplicate wells. Results shown are representative of two independent experiments. * and **, p < 0.001 by Student’s t test.

which is considered a marker for the PM. Similarly, Hsp-70 and tubulin were found exclusively associated with ICCs (Fig. 1C). These results indicate that the biotinylation is complete and limited to the PM.

In light of these findings, a concentration of 0.5 mg/ml biotin and an incubation period of 30 min at 4 °C were used for biotinylation throughout this study. Importantly, a period of 45 min of incubation of 125I-apoA-I with cells at 37 °C was chosen to permit sufficient time for equilibration of apoA-I with different cellular compartments. Although several groups used binding at 4 °C to determine the association of apoA-I with the PM, we obtained evidence that incubation at 4 °C alters the association of apoA-I with ABCA1 at the PM (data not shown), in agreement with previous studies (20, 21). These results underscore the importance of using physiological temperatures to study apoA-I/ABCA1 interactions.

Association of ApoA-I with the PM and ICCs—To determine the association of apoA-I with the PM and ICCs, either BHK cells treated or not with mifepristone or fibroblasts treated or not with 22OH/9CRA were incubated with increasing concentrations of 125I-apoA-I for 45 min at 37 °C. After washing to remove unbound 125I-apoA-I, biotinylation was performed and 125I-apoA-I associated with the PM and ICCs was determined by γ-counting. As shown in Fig. 2A, 125I-apoA-I exhibited saturable, concentration-dependent association with the PM and ICCs in BHK cells induced with mifepristone. Conversely, 125I-apoA-I showed no significant association with either fraction in noninduced BHK cells (Fig. 2B). Similarly, 125I-apoA-I exhibited saturable association with both PM and ICCs in 22OH/9CRA-stimulated fibroblasts (data not shown). Importantly, analysis of affinity parameters for the association of apoA-I with different cellular compartments revealed that the PM possessed nearly a 2-fold higher capacity (B<sub>max</sub>) to accommodate apoA-I as compared with ICCs in BHK expressing ABCA1 and stimulated fibroblasts. In contrast, apoA-I exhibited similar affinity for both the PM and ICCs (Table 1). This result indicates that under conditions of continuous exposure.

FIGURE 2.
to an excess of apoA-I, two-thirds of apoA-I is found associated with the PM and the remaining one-third with the ICCs.

Transferrin (Tf), a ligand that is known to specifically interact with the Tf receptor and recycle to the cell surface (22) was used as a control. We found that 77% of $^{125}$I-Tf was found associated with the PM and 23% with ICCs in HepG2 (0.76 ± 0.02 versus 0.22 ± 0.01 ng of Tf/µg of cell protein).

**Effect of the C-terminal Deletion of apoA-I and the Lipidation of WT apoA-I on the Association with the PM and ICCs**—It is well documented that the C-terminal region of apoA-I is important in the ABCA1-mediated lipid efflux pathway. Indeed, previous studies have documented that apoA-I deletion mutants lacking residues 187–243 of the C-terminal domain (Δ(187–243)) exhibit both reduced cell surface binding and the ability to promote lipid efflux (9, 11, 15). Furthermore, we and others have previously shown that lipid association with apoA-I or apoE3 reduced their ability to interact with ABCA1 (20, 25–27). We further examined the role of the C-terminal domain of apoA-I and the lipidation of WT apoA-I on the association with other cellular compartments. As shown in Fig. 3, A and B, apoA-I Δ(187–243) and rLpA-I exhibited drastically reduced cell association and association with both the PM and ICCs as compared with lipid-free WT apoA-I. These results indicate that the C-terminal domain of apoA-I is important for the compartmentalization of apoA-I between the PM and ICCs. Similarly, they indicate a requirement of lipid-free apoA-I for efficient compartmentalization.

**Overexpressing ABCA1 Promotes the Compartmentalization of apoA-I between the PM and ICCs**—It is well documented that ABCA1 in late endocytic vesicles plays a role in cellular lipid efflux (4, 8, 23, 24). To further examine the role of ABCA1 in the cellular compartmentalization of apoA-I, different levels of ABCA1 were expressed in BHK cells under a mifepristone-inducible ABCA1 gene. As shown in Fig. 2C (inset), without induction, BHK-ABCA1 cells had no detectable ABCA1 protein, whereas cells induced with 0.1 and 10 nM mifepristone for 20 h expressed increasing levels of ABCA1. Concomitantly, cellular cholesterol efflux was significantly increased (2 ± 0.35, 8 ± 0.22, and 13 ± 0.68%; 0.1, 0.1, and 10 nM mifepristone, respectively). Furthermore, ABCA1 induction by mifepristone was paralleled by elevated $^{125}$I-apoA-I association to both the PM and ICCs (Fig. 2D). Similarly, stimulation of normal fibrinogen with 22OH/9CRA increased the association of $^{125}$I-apoA-I with both the PM and ICCs (Fig. 1B). These results indicate that expression of ABCA1 is required for the association of apoA-I with both the PM and ICCs.

**The Endocytotic Pathway Is Required for the Association of apoA-I with ICCs**—Having demonstrated that ABCA1 expression is required for the compartmentalization of apoA-I, we examined the impact of ABCA1 trafficking on apoA-I association with the PM and ICCs. Cells were treated with either cyclosporin A (CsA) or probucol, which are known to specifically inhibit ABCA1-mediated cholesterol efflux to apoA-I (28, 29). CsA is a potent inhibitor of ABCA1 shown to block recycling of ABCA1 from the PM to endosomes, resulting in the inhibition of HDL biogenesis and of ABCA1 degradation by calpain proteases (28). Alternatively, Rothblat and co-workers (42) suggest that probucol inhibits cholesterol efflux by preventing trafficking of ABCA1 to the PM. However, Yokoyama and co-workers (29) did not observe any effect of probucol on the trafficking of ABCA1. Probucol is suggested to prevent the structural interaction of ABCA1 with apoA-I and to decrease calpain-mediated degradation of ABCA1 (29). As expected, treatment of BHK-ABCA1 cells with either CsA or probucol induced a marked increase in PM-ABCA1, we did not observe a significant increase in

### TABLE 1

| Kinetic parameters | Association | Dissociation |
|-------------------|-------------|-------------|
|                    | Apparent $K_{max}$ | Apparent $K_d$ | $K_{cat}$ | $t_{1/2}$ |
| BHK-ABCA1          |             |             |          |          |
| PM                 | 0.58 ± 0.05 | 2.52 ± 0.20 | 30 ± 5   | 82 ± 7   |
| ICCs               | 0.31 ± 0.04 | 2.20 ± 0.15 | 127 ± 11 | 20 ± 3   |
| Fibroblasts +     |             |             |          |          |
| 22OH/9CRA          |             |             |          |          |
| PM                 | 0.21 ± 0.01 | 1.52 ± 0.14 | 18 ± 6   | 126 ± 24 |
| ICCs               | 0.10 ± 0.02 | 1.68 ± 0.25 | 84 ± 18  | 29 ± 2   |

For apparent $K_{max}$, $p < 0.001$ for PM is compared with ICCs in BHK cells.

For apparent $K_d$, $p < 0.001$ for PM is compared with ICCs in fibroblasts.

For $t_{1/2}$, $p < 0.001$ for PM is compared with ICCs in BHK cells.

For $t_{1/2}$, $p < 0.001$ for PM is compared with ICCs in fibroblasts.

**FIGURE 3.** Effect of a C-terminal deletion of apoA-I and the lipidation of WT apoA-I on the compartmentalization of apoA-I between the PM and ICCs. A, iodinated apoA-I isolated from human plasma, WT apoA-I, apoA-I Δ(187–243), and rLpA-I (10 µg/ml, specific activity = 3800–4000 cpm/ng) were incubated for 45 min at 37°C with induced BHK-ABCA1. After washing to remove unbound ligands, surface proteins were biotinylated and separated by streptavidin pulldown. A fraction of cell lysate was directly counted for the determination of total cell-associated iodinated ligands, $B$, recovered supernatants (ICCs) and pellets (PM) washed with lysis buffer were directly counted for radioactivity, and the association of different ligands with the PM and ICCs was determined. Values represent the mean ± S.D. from triplicate wells. Results shown are representative of two independent experiments. * $p < 0.01$ by Student's t test.
PM-associated apoA-I. Although currently uninvestigated, this may be due to a decreased affinity of ABCA1 for apoA-I by CsA and probucol treatment. Taken together, these results indicate that alteration of apoA-I and ABCA1 trafficking has a profound effect on the apoA-I endocytotic pathway and the subsequent apoA-I lipidation process.

ApoA-I Induces ABCA1 Endocytosis—Having determined that alteration of ABCA1 endocytic trafficking affects apoA-I association with ICCs and its subsequent lipidation, the question was raised as to whether ABCA1 localized at the cell surface is internalized together with apoA-I during the lipidation reaction. To quantify the amount of internalized PM-ABCA1, we applied a cleavable biotinylation assay. Under this technique, PM proteins are labeled with a cleavable biotin (sNH₂SS-biotin) at 4 °C and are subsequently transferred to 37 °C to permit cellular trafficking. After incubating with or without 125I-apoA-I for various periods of time, cells were treated with glutathione, a non-cell permeable reducing agent, which cleaves all PM-biotin. Under these conditions, only PM proteins that have been internalized retain the biotin signal, thus giving an indication of the rate and level of internalization. Biotinylated cells incubated at 4 °C and subjected to a reducing agent were used as controls for the efficacy of biotin cleavage by glutathione as described under “Experimental Procedures.” As shown in Fig. 5A, a significant accumulation of glutathione-resistant ABCA1 was observed 5 min after the beginning of the internalization assay in the presence of apoA-I in BHK cells. Within 20 min, the amount of endocytosed ABCA1 reached a plateau in the presence of apoA-I, whereas ABCA1 exhibited no significant internalization in the absence of apoA-I (basal state) in BHK cells (Fig. 5B). This was in contrast to integrin α4, a known PM marker, whose level of internalization did not change throughout the assay. The ratio of endocytosed ABCA1 over integrin α4 is shown in Fig. 5C. The observed increase in ABCA1 internalization was not attributed to an overall increase in ABCA1 production because the total amount of ABCA1 protein remained constant (Fig. 5B). It is likely that the plateau reflects the steady state of the intracellular ABCA1 fraction, which represent the balance of ABCA1 endocytosis and recycling to the PM.

Similar results were obtained with normal fibroblasts but not TD mutants (Q597R and C1477R). The ratio of ABCA1 over activin receptor type II in 22OH/9CRA-stimu-
the PM was 4-fold slower than that of ICCs at 37 °C in both cell lines. At the same time, practically all radioactivity that disappeared from the cell appeared as intact lipidated $^{125}$I-apoA-I in the medium over a 6-h period (Fig. 6B). Dissociation of $^{125}$I-apoA-I from the PM and ICCs over the 6-h chase was almost completely inhibited at 4 °C (data not shown).

Over 95% of the radioactivity released to the medium and in cell lysates was precipitated by 10% trichloroacetic acid, indicating that endocytosis did not cause significant degradation of apoA-I, consistent with our previous findings (20) (data not shown). These results were verified visually by SDS-PAGE analysis in which a time-dependent loss of apoA-I from both the PM and ICCs was observed, without the appearance of lower molecular weight bands (Fig. 6C).

Analysis of the dissociated $^{125}$I-apoA-I product by two-dimensional PAGGE revealed the presence of nascent apoA-I-containing particles having $\alpha$-electrophoretic mobility with diameters of 9–20 nm (Fig. 6D) as we have previously documented (17, 20). Noninduced BHK cells were unable to form such particles (data not shown). The structural properties of these released particles were further examined by dithio-bis(succinimidyl propionate) cross-linking. These nascent particles contain one, two, three, or four molecules of apoA-I as we and others have previously documented (20, 44) (Fig. 6E).

These results indicate that cell-associated apoA-I was re-secreted more rapidly from the ICCs versus its dissociation from the PM, and apoA-I was released from both compartments as nascent apoA-I-containing particles.

**Modulation of ApoA-I Dissociation from the PM by Phospholipids**—Recently, we and others have described a phosphatidylcholine containing non-ABCA1 PM site with a high binding capacity for apoA-I (31–33). In light of this, we hypothesized that PM-associated apoA-I could be released by treatment with phosphatidylcholine-specific phospholipase C (PC-
apoA-I associated with ICCs. To assess how effectively phospholipids were removed by phospholipases, the cells were labeled with \(^{3}H\)choline, and the lipids were separated by TLC and counted. PC-PLC and SMase treatment digested greater than 65% of phosphatidyl\(^{3}H\)choline and 80% of \(^{3}H\)sphingomyelin. Cell membrane integrity and cellular toxicity following phospholipase treatment were monitored by \(^{3}H\)adenine leakage. No significant increase in \(^{3}H\)adenine release was observed by either PC-PLC or SMase treatment compared with untreated cells (data not shown). These results demonstrate that the association of apoA-I with the PM can be modulated by phospholipids and that a large fraction of PM-associated apoA-I is dependent on phosphatidylcholine (31–33).

**DISCUSSION**

Defining the structural characteristics of cellular apoA-I compartmentalization, trafficking, and re-secretion is key for understanding how nascent HDL genesis occurs at the cellular level. Although the HDL retroendocytosis hypothesis was proposed by Assmann and co-workers (30) 2 decades ago, its fate, route, and physiological relevance still remain enigmatic. Previous studies by Takahashi and Smith (3) and Neufeld et al. (7, 8) have proposed a model in which the endocytic pathway plays an important role in the formation of HDL particles. This is consistent with the work of Tall and co-workers (24) showing that deletion of the PEST sequence occurs at the cellular level. Although the HDL retroendocytosis hypothesis was proposed by Assmann and co-workers (30) 2 decades ago, its fate, route, and physiological relevance still remain enigmatic. Previous studies by Takahashi and Smith (3) and Neufeld et al. (7, 8) have proposed a model in which the endocytic pathway plays an important role in the formation of HDL particles. This is consistent with the work of Tall and co-workers (24) showing that deletion of the PEST sequence occurs at the cellular level.
Cellular Compartmentalization and Trafficking of ApoA-I/ABCA1

**A**

![Graph A](image1.png)

**B**

![Graph B](image2.png)

**FIGURE 7. Effect of phospholipases on the dissociation of apoA-I from the PM.**

A, BHK cells were treated with 10 nM mifepristone for 20 h and incubated with 10 µg/ml 125I-apoA-I for 45 min at 37°C. After washing to remove unbound 125I-apoA-I, cells were treated or not with either 2.5 units/ml PC-PLC or 0.4 units/ml SMase for 30 min 37°C, and the radioactivity appearing in the medium after treatment or not with phospholipases was determined. B, cells were incubated with 10 µg/ml of 125I-apoA-I for 45 min at 37°C and treated with phospholipases or not as described in A, and then surface proteins were biotinylated. Recovered supernatants (ICCs) and pellets (PM) were directly counted for radioactivity. Values represent the mean ± S.D. from triplicate wells. *p < 0.001 by Student’s t test.

We obtained evidence that under conditions of continuous exposure to an excess of apoA-I, the PM exhibited a 2-fold higher capacity to accommodate apoA-I as compared with ICCs (Table 1). We propose that the PM is able to accommodate more apoA-I because of the presence of a phosphatidylcholine-rich high-capacity binding site as identified recently by our group (31–32) as well as Phillips and co-workers (33). Despite the fact that only one-third of cell-associated apoA-I was found within ICCs, 125I-apoA-I was re-secreted four times faster from ICCs versus its dissociation from the PM (Fig. 6 and Table 1). In both cases, apoA-I re-secreted from ICCs and/or dissociated from the PM was released as nascent HDL (Fig. 7C).

Although the kinetic findings indicate that ICCs represent a metabolically active compartment for the lipidation of apoA-I, in agreement with previous work (3, 8), the quantitative assessment suggests an important role of the PM to associate apoA-I and supports the idea that the PM represents an independent lipidation compartment. This is consistent with our previous findings that disruption of the HCBS inhibited the formation of nascent HDL (31). It is possible, however, that the PM could act as an initial tether point or apoA-I reservoir, allowing apoA-I to be brought into close proximity for interaction with the endocytotic pathway.

The cause of the disparity between the kinetics of apoA-I dissociation from the PM and its re-secretion from ICCs is unknown, but it may be attributed to the retention of apoA-I by PM structures (lipids, rafts, caveolin, or HCBS), consistent with the data showing that PC-PLC treatment increased the dissociation of apoA-I from the PM (Fig. 7A). Although it cannot be ruled out that apoA-I re-secreted from ICCs reassociates with the PM, our results showing that apoA-I dissociated and/or re-secreted from both the PM and ICCs are lipidated (Fig. 6C), together with the finding that lipidated rLpA-I exhibited decreased association with the PM and ICCs (Fig. 3B), do not support such a mechanism.

The structural requirements for apoA-I to associate with the PM and ICCs are as yet unknown. In this study we obtained evidence that deletion of the C-terminal region of apoA-I, residues 187–243, drastically reduced the compartmentalization of apoA-I between the PM and ICCs (Fig. 3B), consistent with our previous report documenting that apoA-I Δ(187–243) exhibited reduced binding to both ABCA1 and the HCBS (31). This is in agreement with a previous study by Zannis and co-workers (9) showing that direct cross-linking of apoA-I Δ(185–243) and Δ(220–243) to ABCA1 produced 3-fold higher Kd values for these mutants compared with WT apoA-I. It is well established that the C-terminal α-helices are important for effective cellular lipid efflux (9, 11). Previously, we have reported that apoA-I Δ(185–243) incubated with HepG2 failed to form larger nascent LpA-I particles compared with WT apoA-I (32). These results indicate that the C-terminal region of apoA-I is required for the formation of a productive complex with ABCA1 that leads to the compartmentalization of apoA-I between the PM and ICCs. Loss of compartmentalization by the C-terminal deletion mutant could be due to an inability of apoA-I Δ(185–243) to bind directly to specialized phospholipid domains, such as the PC-containing HCBS within the PM (31–33) and/or to an impairment of the apoA-I endocytotic pathway.

This study supports the concept that the association of apoA-I with the PM and ICCs is dependent on the formation of a high affinity complex between apoA-I and ABCA1, which allows the compartmentalization and trafficking of apoA-I. We demonstrated that increasing apoA-I binding to ABCA1 by 22OH/9CRA stimulation or overexpression by mifepristone induction promoted the association of apoA-I to both the PM and ICCs (Fig. 1B and Fig. 2D). Conversely, disruption of the high affinity complex formation by deletion of the C-terminal or by lipidation of apoA-I impaired the association of apoA-I with both the PM and ICCs. Taken together, our findings indicate that the initial interaction between apoA-I and ABCA1 plays a pivotal role in determining the subsequent cellular compartmentalization and trafficking of apoA-I, thereby allowing the formation of nascent HDL particles at different subcellular compartments. However, it is possible that this low capacity site involving direct apoA-I/ABCA1 interaction serves a regulatory function and stabilizes ABCA1, as reported recently by Phillips and co-workers (33).

Although the mechanisms governing ABCA1 turnover and its relationship to the compartmentalization and trafficking of apoA-I remain unknown, previous studies by Tall and co-workers (35) have shown that apoA-I increased cell surface ABCA1 by decreasing its degradation by calpain proteolysis. Additionally, we hypothesized that apoA-I induces continuous rapid
Internalization of PM-ABCA1. We proposed that, when the ABCA1 transporter system functions at steady state, the internalized apoA-I-ABCA1 complex must dissociate rapidly to allow replenishment of lost cell surface ABCA1 and completion of a large number of apoA-I lipidation cycles. This scenario is strongly supported by the following findings. 1) ApoA-I specifically mediates the continuous endocytic recycling of ABCA1, and this process is impaired in TD cells (Fig. 5). 2) The re-secretion of $^{125}$I-apoA-I from ICCs was faster than its dissociation from the PM (Table 1). 3) Alteration in apoA-I-ABCA1 trafficking impaired the lipidation process (Fig. 4). Our recent results showing that disruption of the high capacity binding site impaired HDL formation (31) indicate that a complex regulation mechanism exists for the lipidation of apoA-I within different subcellular compartments. It is likely that under conditions of continuous exposure to an excess of apoA-I the accumulation of apoA-I within different compartments reflects the balance of two opposing processes of endocytosis from the PM and re-secretion from ICCs. This is consistent with the concept that the bi-directional vesicular trafficking of ABCA1 is essential not only for the lipidation of apoA-I during its intracellular trafficking, but also for its lipidation within the high capacity binding site at the PM.

It is well documented that ligand binding can induce the phosphorylation of cellular receptors, triggering their oligomerization and endocytosis and, in turn, modulating their function. In light of this, apoA-I may have important implications in the activation of ABCA1, consistent with our previous study (19) which showed that brief treatment of 22OH/9CRA-stimulated fibroblasts or Chinese hamster ovary cells overexpressing ABCA1 with apoA-I triggered cAMP production and consequently induced ABCA1 phosphorylation via a cAMP-dependent protein kinase-dependent mechanism. This is in agreement with the finding of Oram and Heinecke (34) that ABCA1 activity could be regulated by different signaling processes, including the JAK2 pathway. Although apoA-I binds to both the dimeric and tetrameric forms of ABCA1 found in both the PM and ICC compartments as we have documented previously, the presence or absence of apoA-I does not affect the oligomerization of ABCA1 (41).

Although cellular factors affecting the degradation of apoA-I are poorly understood, a recent study by Zha and co-workers (43) reported that after incubation of BHK cells overexpressing ABCA1 with apoA-I for 2 h at 37 °C, internalized apoA-I is largely targeted for lysosomal degradation. However, we obtained evidence that under these conditions degradation of apoA-I is increased 2-fold as compared with incubation with apoA-I for 45 min at 37 °C (9.52 ± 0.53% versus 4.13 ± 0.20%, respectively) as assessed by trichloroacetic acid precipitation. Freeman and co-workers (10) demonstrated that in ABCA1-expressing 293 cells, the apoA-I-ABCA1 complex has a half-life of less than 30 min and that 50% of the cell-associated apoA-I is lipidated within 40 min. Similarly, we demonstrated previously (17) that in HepG2 cells and fibroblasts, formation of nascent particles occurs within 1 h at 37 °C and presently (Table 1) that the kinetics of dissociation from the ICCs is rapid, with a half-life of 20 min. As such, a large number of lipidation cycles will have occurred within 2 h of incubation and, in doing so, will have reduced the lipid burden of the cell. In the absence of excess cellular cholesterol and/or phospholipids, it is likely that apoA-I could be targeted for degradation. This is consistent with our finding that depletion of PM-cholesterol by treatment of BHK cells with β-cyclodextrin (50 mM for 45 min at 37 °C), followed by incubation with $^{125}$I-apoA-I for 45 min at 37 °C, causes a 3-fold increase of $^{125}$I-apoA-I degradation compared with untreated cells (16.04 ± 1.16% versus 4.13 ± 0.20%, respectively). More thorough investigations are required to examine cellular factors regulating the degradation of apoA-I.

A major paradigm shift in the current concept of reverse cholesterol transport comes from the proposition of Brewer and co-workers (36), suggesting that the liver is a major source of plasma HDL-C. This concept is strongly supported by a recent study by Parks and co-workers (37) showing that tar-
targeted disruption of hepatic ABCA1 in mice dramatically reduced the level of circulating HDL. It would be of great interest to determine whether similar mechanisms that we propose here for the lipidation of exogenously added apoA-I could operate in hepatocytes and enterocytes. Indeed, Marcel and co-workers (38) have documented that newly synthesized apoA-I in hepatocytes undergoes an early ABCA1-independent phospholipidation in the endoplasmic reticulum that is followed by significant phospholipidation in Golgi. Furthermore, it has been shown that apoA-I acquires some cholesterol in the endoplasmic reticulum and Golgi but that the major transfer occurs at the cell surface of hepatocytes, consistent with our results showing the greater capacity of the PM to associate with apoA-I (Table I). At the same time, our kinetic data suggest that the endocytotic pathway plays a central role in the formation of nascent HDL, in agreement with current models of nascent HDL biogenesis. Thus, targeting apoA-I to ICCs rich in cholesterol may have important implications in preventing atherosclerotic cardiovascular disease. This is supported by the finding of Tabas and co-workers (39, 40) that the endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages.

This study has allowed for a direct quantitative comparison to be made between the kinetics of apoA-I compartmentalization, dissociation, and/or re-secrection from the PM and ICCs. Furthermore, we obtained evidence that the specific interrelationship between apoA-I and ABCA1 endocytic trafficking plays a key physiologic role in the lipidation process. Our findings support a two-compartment lipidation model for nascent HDL genesis, as illustrated in Fig. 8. The interaction of apoA-I with ABCA1 at the cell surface creates a new apoA-I high capacity binding site at the PM (31–33) where apoA-I acquires lipids locally and is slowly released directly from the PM as nascent HDL particles (slow lipidation compartment). A second parallel compartment involves endocytosis of both apoA-I and ABCA1 followed by rapid dissociation and targeting of apoA-I to ICCs. ABCA1 is recycled to allow replenishment of lost cell surface ABCA1. ApoA-I extracts lipids locally from these intracellular sites and is rapidly released from the cell as nascent HDL particles (fast lipidation compartment). However, we wish to make clear that no attempt was made to use these models to give a definitive interpretation concerning the lipidation of apoA-I at different cellular compartments. Our model presented in Fig. 8 is a simple illustration of our current findings and previous investigations on the HCBS (31, 32) and reconciles many of the apparently discrepant findings in the literature.

The detailed mechanisms underlying HDL genesis at the cellular level require more extensive investigations, which are currently ongoing. Development of a kinetic model as reported here is a suitable approach for dissecting a complex intracellular trafficking pathway for apoA-I/ABCA1 that plays an important role in cellular cholesterol homeostasis and the genesis of nascent HDL particles.

Acknowledgments—We thank Drs. John F. Oram and Ashley M. Vaughan for generously providing BHK cells overexpressing ABCA1 and Dr. Yves L. Marcel for kindly providing apoA-I Δ(187–243).

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