ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL

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Abstract **Recent developments in lipid metabolism have shown the importance of ATP binding cassette transporters (ABCs) in controlling cellular and total body lipid homeostasis. ABCA1 mediates the transport of cholesterol and phospholipids from cells to lipid-poor apolipoprotein A-I (apoA-I), whereas ABCG1 and ABCG4 mediate the transport of cholesterol from cells to lipidated lipoproteins. ABCA1, ABCG1, and ABCG4 are all expressed in cholesterol-loaded macrophages, and macrophages from ABCA1 and ABCG1 knockout mice accumulate cholesteryl esters. Here, we show that the lipidated particles generated by incubating cells overexpressing ABCA1 with apoA-I are efficient acceptors for cholesterol released from cells overexpressing either ABCG1 or ABCG4. The cholesterol released to the particles was derived from a cholesterol oxidase-accessible plasma membrane pool in both ABCG1 and ABCG4 cells, which is the same pool of cholesterol shown previously to be removed by high density lipoproteins. ABCA1 cells incubated with apoA-I generated two major populations of cholesterol- and phospholipid-rich lipoprotein particles that were converted by ABCG1 or ABCG4 cells to one major particle population that was highly enriched in cholesterol. These results suggest that ABCG1 and ABCG4 act in concert with ABCA1 to maximize the removal of excess cholesterol from cells and to generate cholesterol-rich lipoprotein particles.**—Vaughan, A. M., and J. F. Oram. **ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL.** ***J. Lipid Res.*** **2006.** 47: **2433–2443.**

Supplementary key words ATP binding cassette transporter A1 • ATP binding cassette transporter G1 • ATP binding cassette transporter G4 • high density lipoprotein

Atherosclerosis is an inflammatory disease process (1), and monocyte-derived macrophages play a key role in both the initiation and progression of atherosclerosis. Among the earliest events in atherosclerosis are insults to the arterial endothelium and the deposition and retention of atherogenic apolipoprotein B (apoB)-containing particles in the subendothelial space (2). This deposition promotes a local inflammatory response, leading to the release of soluble signaling factors such as chemokines and to the expression of endothelial cell adhesion molecules (3). These proteins recruit monocytes to the artery wall (4), which then differentiate into macrophages, the major cellular components of both early and advanced atherosclerotic lesions (5). The uptake of modified lipoproteins by macrophages leads to the accumulation of cholesteryl esters and the formation of macrophage-derived foam cells, the trademark of the fatty streak. The accumulation of cholesteryl ester in the macrophage foam cell can be viewed as an imbalance between cholesterol influx and efflux.

The cholesterol loading of macrophages leads to the formation of oxysterols and the subsequent activation of the liver X receptor (LXR) (6, 7). A number of ATP binding cassette transporters (ABCs) involved in lipid transport are regulated through activation by LXR, including ABCA1 (8), ABCG1, and ABCG4 (9–11). However, a recent report using ABCG4$^{-/-}$ mice has suggested that ABCG4 is not expressed in macrophages and does not contribute to HDL-mediated cholesterol efflux (12). Until an ABCG4-specific antibody becomes available, the presence of ABCG4 in macrophages will remain elusive. The incubation of apoA-I with J774 mouse macrophages expressing ABCA1 (13) leads to the efflux of cholesterol and phospholipid and the subsequent formation of nascent HDL particles (14). *ABCG1* transcription is highly upregulated in cholesterol-loaded macrophages (11), and ABCG1 facilitates the efflux of cholesterol from cells to HDL, rather than to free apoA-I (15–17). The peroxisome proliferator-activated receptor γ activators also increase *ABCG1* transcription in macrophages and stimulate cholesterol efflux to HDL independent of their induction of LXR (18). Like ABCG1, ABCG4 also facilitates the efflux of cholesterol from cells to HDL (16, 17), although there is evidence that ABCG1 and ABCG4 can form a heterodimer (19). ABCA1 and ABCG1 have been shown to synergize to mediate the efflux of cholesterol to apoA-I (20), and in the brains of mice, the expression of ABCG1 but not ABCA1

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correlates with cholesterol release from cerebellar astroglia (21). These findings suggest a significant role for ABCG1 and possibly ABCG4 in the release of cholesterol from cells into the reverse cholesterol transport pathway.

The importance of macrophage ABCA1 expression in atherosclerosis has been demonstrated in mice, in which the bone marrow transplantation of ABCA1 null macrophages was shown to lead to increased atherosclerosis in hyperlipidemic animals (22). Conversely, the overexpression of macrophage ABCA1 in mice reduces atherosclerotic lesion development in low density lipoprotein receptor-deficient mice (23). The targeted disruption of *ABCG1* in mice on a high-fat and high-cholesterol diet causes the massive accumulation of both neutral lipids and phospholipids in hepatocytes and macrophages of multiple tissues (24). Conversely, tissues in *ABCG1* transgenic mice are protected from dietary fat-induced lipid accumulation (24).

The groundwork for this study was laid by Gelissen et al. (20), who showed that particles released from ABCA1-expressing cells incubated with apoA-I acted as acceptors for cholesterol from ABCG1-expressing cells. In this study, we have analyzed the ability of both ABCG1 and ABCG4 to mediate the efflux of cholesterol to the nascent HDL particles formed by the incubation of apoA-I with ABCA1-expressing cells. The efflux of cholesterol to these particles is dependent on both the apoA-I concentration used to efflux lipid from ABCA1-expression cells and the time the particles are incubated with ABCG1- and ABCG4-expressing cells. The cholesterol released from the ABCG1- and ABCG4-expressing cells by HDL comes mainly from a cell surface cholesterol domain that is formed upon expression of the transporters (16). Gel filtration chromatography demonstrated that small, phospholipid-rich nascent HDL particles formed by the incubation of apoA-I with ABCA1-expressing cells became a larger, more cholesterol-rich particle population upon incubation with ABCG1- and ABCG4-expressing cells.

EXPERIMENTAL PROCEDURES

Cultured cells

All cell culture incubations were performed at 37°C in a humidified 5% CO_2 incubator. BHK cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). BHK cells expressing human ABCA1 (ABCA1 cells), ABCG1 (ABCG1 cells), and ABCG4 (ABCG4 cells) were generated using the mifepristone-inducible GeneSwitch system (Invitrogen, Carlsbad, CA) as described previously (16, 25). A clonal line, which expressed an N-terminal FLAG-tagged cDNA clone containing the reported full-length 678 amino acids of ABCG1, was used in the current studies and had a high expression level of ABCG1. A human IMAGE clone (No. 5763981; ATCC) encoding ABCG4 was used to amplify the predicted full-length 646 amino acid open reading frame, which was then ligated into pGene/V5 HisA in-frame with the C-terminal V5-His tag. A clonal line of this construct was used in the current studies and had a high expression level of ABCG4. Mock-transfected BHK (Mock) cells were derived from the same pSwitch clonal line transfected with linearized pGene/V5-HisA. Cells were grown and maintained in DMEM containing 10% fetal bovine serum until experimental treatments. Unless indicated otherwise, ABCA1, ABCG1, and ABCG4 were induced by incubating cells for 18–20 h in DMEM with 1 mg/ml fatty acid-free BSA (DMEM/BSA) and 10 nM mifepristone. Studies were carried out on standard 24-well plates unless stated otherwise.

ApoA-I

HDL was prepared by sequential ultracentrifugation in the density range 1.125–1.21 g/ml. HDL was subsequently depleted of apoE and apoB by heparin-agarose chromatography. ApoA-I was purified from HDL and subsequently delipidated (26). ApoA-I was radiolabeled with ^{14}C using the method of Krebs, Ibdah, and Phillips (27).

Lipid efflux and cellular cholesterol distribution

To radiolabel cellular cholesterol to equilibrium, [^{3}H]cholesterol (2.0 μCi/ml, 40–60 Ci/mmol; Amersham Biosciences) was added to the 10% serum medium 1–2 days before mifepristone treatment. To radiolabel cellular phospholipids to equilibrium, [^{3}H]choline (2.0 μCi/ml, 40–60 Ci/mmol; Amersham Biosciences) was added to the 10% serum medium 1–2 days before mifepristone treatment.

To measure lipid efflux, cells were incubated with DMEM-BSA with or without 10 μg/ml apoA-I or lipidated apoA-I particles (see below) for various times at 37°C and chilled on ice, and the medium was collected and centrifuged to remove detached cells. For cholesterol efflux, the medium was counted for ^{3}H, and the cells were assayed for ^{3}H after hexane-isopropanol extraction. For phospholipid efflux, media and cells were extracted with chloroform-methanol-water, and the organic phase was counted for ^{3}H.

To determine the cholesterol and phospholipid mass in the media of treated cells, cells were plated in 150 mm diameter dishes. The cholesterol mass of effluxed particles was measured by adapting the methodology of Heider and Boyett (28). Briefly, the cellular lipids were extracted and hydrolyzed with alcoholic potassium hydroxide. The total cholesterol was then oxidized with cholesterol oxidase, which resulted in the release of peroxide. The peroxide reacts with a fluorogen (*p*-hydroxyphenylacetic acid) to form a fluorescent product in the presence of peroxidase that is quantified and compared with standards of known cholesterol content. The precision of the method is greater than that obtained from gas-liquid chromatography. The phospholipid mass of effluxed particles was measured by adapting the methodology Bartlett (29). Briefly, the phospholipids were extracted in chloroform-methanol and the phosphate head groups were converted to inorganic phosphate in the presence of perchloric acid. The addition of a molybdate solution and Fiske-Subbarow (30) reducing reagent resulted in the production of a stable blue solution. Standards of know amounts of inorganic phosphate were treated similarly, the wavelengths of samples and standards were measured at 820 nm, and the amount of inorganic phosphate (and thus phospholipid) in the samples was determined.

To measure cell surface cholesterol redistribution, cells were washed once with PBS and then incubated with 1 U/ml cholesterol oxidase (Calbiochem) in DMEM at 37°C for 10 min (25). Cells were then washed twice with PBS, cellular lipids were extracted, and [^{3}H]cholesterol and [^{3}H]cholestenone were measured after isolation by thin-layer chromatography.

Immunoblotting

Cells were solubilized in 50 mM Tris buffer containing 1% SDS, 0.1 M mercaptoethanol, and 0.5 mM EDTA, and proteins were resolved by 8% polyacrylamide gel electrophoresis. Proteins were

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transblotted onto nitrocellulose. Transporters were identified with an ABCA1 antibody (Novus Biologicals); ABCG1 was identified with an anti-FLAG antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO), and ABCG4 was identified using an anti-V5 antibody conjugated to horseradish peroxidase (Invitrogen).

Formation of lipidated apoA-I particles

ABCA1 cells were plated in 150 mm dishes and radiolabeled or not with either [^{3}H]cholesterol or [^{3}H]choline. On the day that cells reached confluence, they were incubated with DMEM/BSA for 6 h and then incubated with 15 ml of DMEM/BSA containing 10 nM mifepristone and 10 μg/ml apoA-I for 18 h so that apoA-I would be lipidated by its interaction with ABCA1. A subset of dishes that had not been radiolabeled were treated with [^{14}C]apoA-I. The medium was centrifuged to remove cell debris and stored at 4°C. Mock cells were treated in a similar manner as a control. Lipidated apoA-I particles were subsequently used for gel filtration chromatography analysis (see below).

Gel filtration chromatography

When the medium was analyzed by gel filtration chromatography, cells were set up on standard six-well plates at 550,000 cells per well. Cells were treated on day 4, and the final incubation was in a volume of 1.5 ml. Medium was collected after incubating ABCA1 cells with apoA-I and Mock, ABCG1, and ABCG4 cells with ABCA1-lipidated apoA-I particles. The medium was centrifuged to remove cell debris, loaded (1.0 ml) onto a Superdex 200 column (60 × 1.6 cm) using the Pharmacia FPLC system, and eluted with 10 mM Tris buffer at a flow rate of 1 ml/min (14). Fractions (1.5 ml) were collected between 30 and 100 ml (initial experimentation found no substantial recovery of radioactivity before 30 ml or after 100 ml). Fractions were counted to determine either [^{3}H]cholesterol or [^{14}C]apoA-I radioactivity from [^{3}H]cholesterol-labeled cells or cells treated with [^{14}C]apoA-I, respectively. Phospholipids from 1.5 ml fractions of medium from [^{3}H]choline-labeled cells were extracted with chloroform-methanol and counted for [^{3}H]choline. This enabled a cholesterol, phospholipid, and apoA-I profile for each experiment. The total run time was 180 min, and each experimental condition was repeated at least three times.

In using gel filtration, the elution of a solute is characterized by a distribution coefficient (K_d) and represents the fraction of the stationary phase that is available for the solute. In practice, K_d is difficult to determine and is replaced by K_{av} since there is a constant relationship between K_d and K_{av}. The particle sizes of the various fractions were determined by comparing their K_{av} values with those of proteins of known diameter (particle diameter range, 6.1–17 nm; and protein mass range, 29–669 kDa). K_{av} was calculated using the following equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_0 is the void volume, V_t is the total column volume, and V_e is the elution volume. For this purpose, a plot of log particle size or log molecular mass against K_{av} was constructed, and the points were fitted using a linear regression analysis. The apparent molecular mass (in kilo Daltons) and particle size (hydrodynamic diameter in nanometers) were calculated using the following equations: $\log_{10}$ mass $= -2.57\ K_{av} + 5.93$ and $\log_{10}$ diameter $= -1.00\ K_{av} + 1.94$.

Statistics

Data were analyzed by paired Student's *t*-test to determine significance. Each experiment shown is representative of at least three similar experiments. The apparent K_d for apoA-I-mediated cholesterol efflux specifically to ABCA1 was calculated using the one-site saturation model of nonlinear regression.

RESULTS

ABCG1 and ABCG4 promote the efflux of cholesterol to nascent HDL particles formed by incubating apoA-I with ABCA1 cells

We and others have shown that ABCG1 and ABCG4 are able to promote the efflux of cholesterol to HDL (11, 16, 17, 24). Incubation of J774 macrophages with apoA-I, treated with cAMP to induce ABCA1 expression, generates nascent HDL particles (14), and treatment of macrophages with LXR and RXR agonists simultaneously increases the transcription of *ABCA1*, *ABCG1*, and *ABCG4* (9, 31). However, it has been documented, based on studies on ABCG4$^{-/-}$ mice, that ABCG4 does not play a role in the efflux of cholesterol to HDL in macrophages (12). Thus, it is not clear whether ABCG4 plays a role in cholesterol efflux in the macrophage. Nonetheless, we surmised that treatment of ABCA1-expressing cells with apoA-I would generate lipidated particles that would promote ABCG1- and ABCG4-dependent cholesterol efflux.

ABCA1 and Mock cells were incubated with medium alone and with 10 μg/ml apoA-I for 18 h. The medium from these cells was then removed, centrifuged to remove cell debris, and added to [^{3}H]cholesterol-labeled Mock, ABCG1, or ABCG4 cells. Efflux of [^{3}H]cholesterol was measured after a 6 h incubation. Conditioned medium from apoA-I-treated ABCA1 cells promoted significant efflux of [^{3}H]cholesterol from either ABCG1 or ABCG4 cells but not from Mock cells (**Fig. 1A**). This demonstrates that the nascent HDL particles formed by incubation of apoA-I with ABCA1 then promote further cholesterol efflux from cells through both ABCG1 and ABCG4.

We then investigated the possibility that ABCG1 or ABCG4 could enhance ABCA1-dependent cholesterol efflux in a mixture of cells expressing these different transporters. We plated an equal number of cells from two different transfected BHK cell lines, grew them to confluence, and measured apoA-I-mediated [^{3}H]cholesterol efflux. Although we did not determine the relative fraction of each cell line when confluent, the individual lines proliferate at similar rates and thus should have represented approximately half of the final cell population. As expected, apoA-I stimulated [^{3}H]cholesterol efflux from a mixture of Mock and ABCA1 cells (Fig. 1B). Mixing ABCA1 cells with either ABCG1 or ABCG4 cells enhanced apoA-I-mediated [^{3}H]cholesterol efflux >2-fold compared with the Mock/ABCA1 cell mixture (Fig. 1B). In addition, as expected, apoA-I had no effect on cholesterol efflux from a mixture of ABCG1 and ABCG4 cells. These results indicate that ABCA1, ABCG1, and ABCG4 cooperate to promote cholesterol efflux when highly expressed in the same cell population.

ABCG1- and ABCG4-mediated cholesterol efflux to nascent HDL particles is time- and concentration-dependent

To determine whether the degree of lipidation of apoA-I by ABCA1 influenced the subsequent ABCG1- and ABCG4-mediated cholesterol efflux, ABCA1 or Mock cells were treated with apoA-I for increasing times, and the medium

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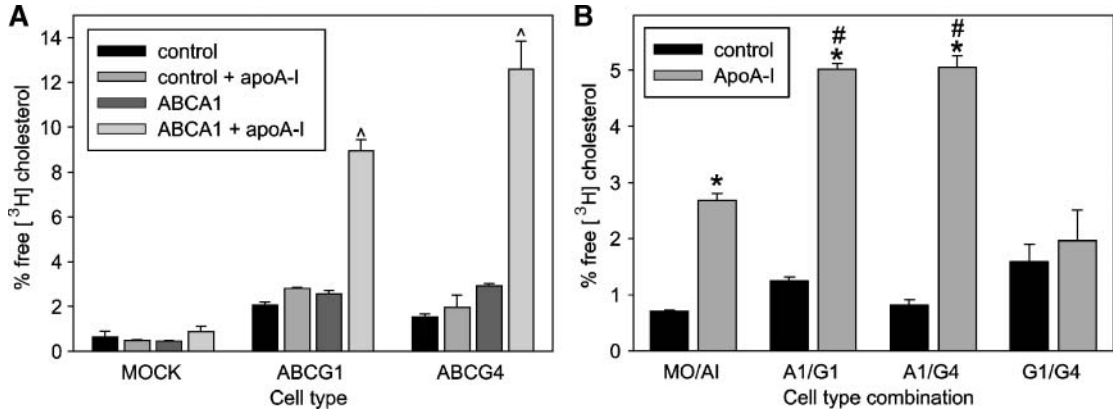

Fig. 1. Nascent HDL particles formed by the incubation of apolipoprotein A-I (apoA-I) with ABCA1 cells promote the efflux of cholesterol to ABCG1 and ABCG4 cells. A: Mock cells (control) and ABCA1 cells were treated with medium alone or medium containing 10 μg/ml apoA-I for 18 h. The medium was centrifuged to remove cellular debris and subsequently added to [^{3}H]cholesterol-labeled Mock cells, ABCG1 cells, or ABCG4 cells; efflux of [^{3}H]cholesterol was measured after 6 h. Results are means ± SD of triplicate experiments expressed as the percentage of total radiolabeled cholesterol (medium plus cell) released into the medium. B: Equal numbers of Mock (MO), ABCA1 (A1), ABCG1 (G1), or ABCG4 (G4) cells were plated in combination and labeled with [^{3}H]cholesterol. Cells were then treated with medium or medium containing 10 μg/ml apoA-I for 6 h, and efflux of [^{3}H]cholesterol was measured. The results are means ± SD of triplicate experiments expressed as the percentage of total radiolabeled cholesterol (medium plus cell) released into the medium. ^ $P < 0.01$ versus incubation of apoA-I medium from ABCA1-expressing cells added to Mock cells; * $P < 0.005$ versus control incubation; # $P < 0.01$ versus apoA-I incubated with the Mock and ABCA1 cell combination.

was then transferred to ABCG1 and ABCG4 cells. ApoA-I-mediated [^{3}H]cholesterol efflux from ABCA1 cells was linear over 7 h (**Fig. 2A**). When conditioned medium from a parallel experiment with unlabeled ABCA1 cells was transferred to [^{3}H]cholesterol-labeled Mock, ABCG1, or ABCG4 cells for 6 h, [^{3}H]cholesterol efflux was much higher with ABCG1 or ABCG4 cells than with Mock cells (Fig. 2B). Although this efflux continued to increase with time of preincubation with ABCA1 cells, the largest incremental increase occurred between 0 and 1 h (Fig. 2B). These results show that only partial lipidation of apoA-I is sufficient to generate efficient acceptors for ABCG1- and ABCG4-transported cholesterol and suggest that this lipidation occurs within 1 h of the addition of apoA-I to ABCA1-expressing cells.

When apoA-I conditioned medium from an 18 h incubation with ABCA1 cells was transferred to radiolabeled ABCG1, ABCG4, or Mock cells, [^{3}H]cholesterol efflux was linear over an 8 h incubation and markedly higher when comparing ABCG1 or ABCG4 cells with Mock cells (Fig. 2C). Thus, the nascent apoA-I-containing apolipoproteins produced by ABCA1 are poor acceptors for cellular cholesterol unless cells are expressing these ABCG transporters.

Concentration curves showed that apoA-I-mediated [^{3}H]cholesterol efflux from ABCA1 cells was saturable, with an apparent K_d of 1.6 ± 0.3 μg/ml apoA-I (**Fig. 3A**). When conditioned medium from a parallel experiment with unlabeled ABCA1 cells was then transferred to [^{3}H]cholesterol-labeled Mock, ABCG1, or ABCG4 cells for 6 h, [^{3}H]cholesterol efflux from ABCG1 or ABCG4 cells was also saturable but had a slightly lower K_d than for ABCA1 (0.56 ± 0.13 μg/ml for ABCG1 and 0.58 ± 0.18 μg/ml for ABCG4). Efflux was significantly higher compared with the [^{3}H]cholesterol efflux from Mock cells (Fig. 3B). These results suggest that the nascent particle-mediated cholesterol efflux from ABCG1 or ABCG4 cells was dependent on the degree of lipidation by ABCA1 but that other factors may play a role.

Cholesterol removed from ABCG1 and ABCG4 cells by ABCA1-generated nascent HDL particles is from cholesterol oxidase-accessible pools

We previously showed that both ABCG1 and ABCG4 are robustly expressed in transfected BHK cells and mediate the cellular redistribution of cholesterol to a pool in the plasma membrane that is accessible to added cholesterol oxidase, and that cholesterol efflux to HDL is primarily from this pool (16). To ascertain that the nascent HDL particles also remove cholesterol from this pool, the conditioned medium from ABCA1 cells treated with increasing concentrations of apoA-I was added to [^{3}H]cholesterol-labeled ABCG1, ABCG4, or Mock cells for 6 h, and the fraction of cellular [^{3}H]cholesterol accessible to cholesterol oxidase was measured. As shown previously, overexpressing either ABCG1 or ABCG4 markedly increased the fraction of cellular [^{3}H]cholesterol accessible to cholesterol oxidase (Fig. 3C). The majority of the cholesterol removed from the ABCG1 or ABCG4 cells by the ABCA1-conditoned medium was from this oxidase-accessible pool (Fig. 3C). This removal had an apoA-I concentration profile that was saturable and paralleled that of cholesterol efflux. The cholesterol oxidase-accessible pool in the Mock cells, which was much lower, was unaffected by the ABCA1-generated particles (Fig. 3C). If ABCG1- or ABCG4-expressing cells were not treated with mifepristone, the results obtained were similar to those with Mock cells, although mock-transfected cells were routinely used as they provide a more relevant control. These results suggest that depletion of this oxidase-accessible pool of cholesterol contributes to the saturability of cholesterol efflux from ABCG1 and ABCG4 cells.

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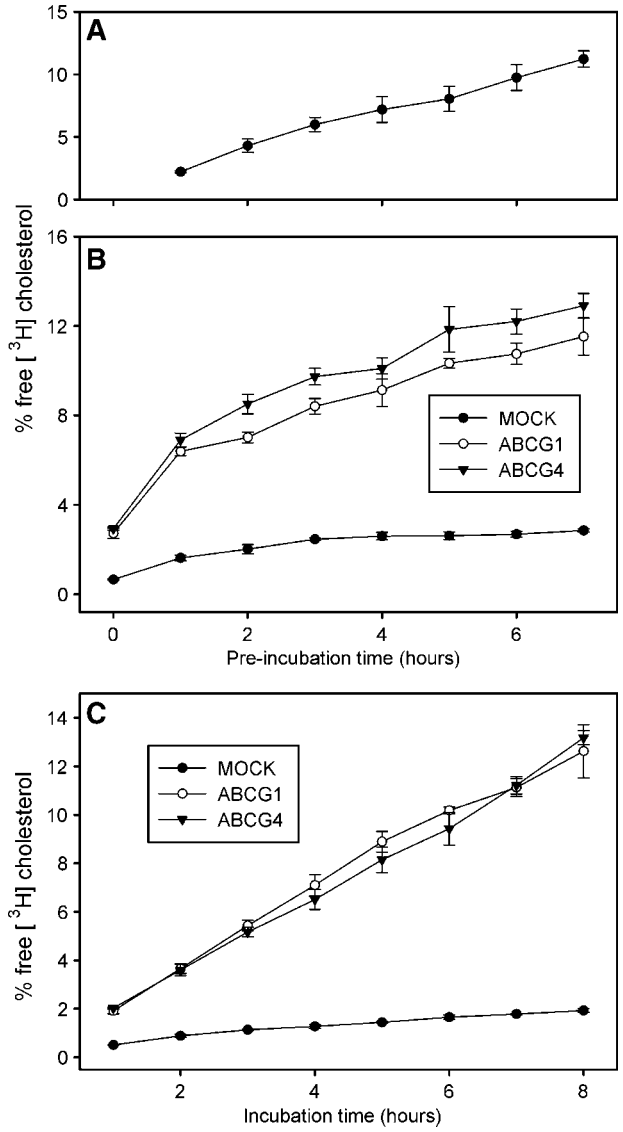

Fig. 2. ABCG1- and ABCG4-mediated cholesterol efflux to nascent HDL particles is dependent on apoA-I lipidation and time of incubation with the nascent HDL particle. A: [^{3}H]cholesterol-labeled ABCA1 cells were treated with 10 μg/ml apoA-I, and medium was collected hourly for 7 h for cholesterol efflux measurements. B: The medium from nonlabeled cells treated as described for A was collected, centrifuged to remove cellular debris, added to [^{3}H]cholesterol-labeled Mock, ABCG1, or ABCG4 cells for 6 h, and assayed for cholesterol efflux. C: ABCA1 cells were treated with 10 μg/ml apoA-I for 18 h. The medium was spun to remove cellular debris and added to [^{3}H]cholesterol-labeled Mock, ABCG1, or ABCG4 cells hourly for 1–8 h and assayed for efflux. In all cases, efflux of [^{3}H]cholesterol is shown as means ± SD of triplicate experiments expressed as the percentage of total radiolabeled cholesterol (medium plus cell) released into the medium.

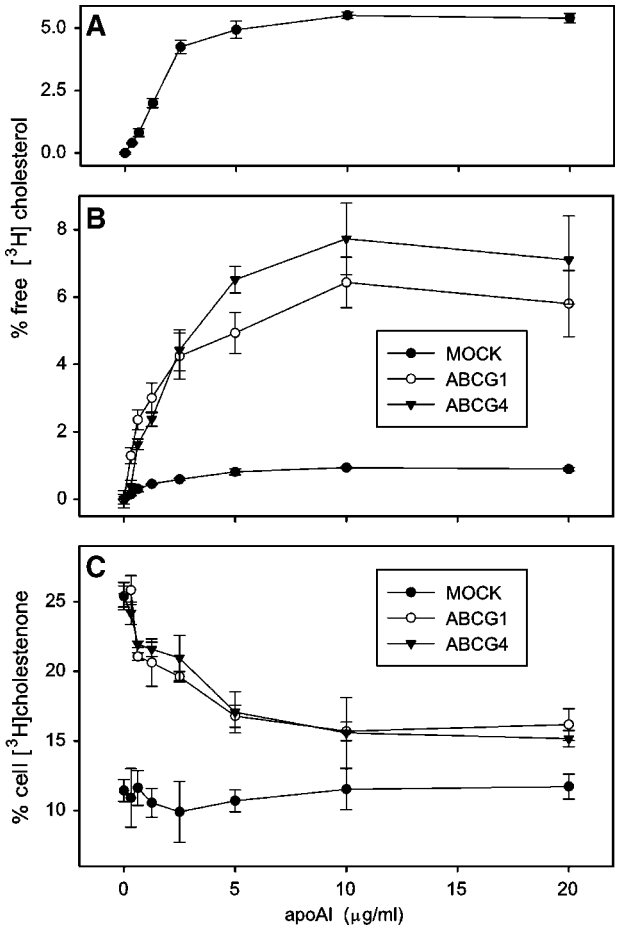

Fig. 3. Saturability of ABCA1-mediated cholesterol efflux to apoA-I and subsequent ABCG1- and ABCG4-mediated cholesterol efflux to these ABCA1-generated particles. A: [^{3}H]cholesterol-labeled ABCA1 cells were treated with increasing concentrations of apoA-I for 18 h and assayed for cholesterol efflux. B: The medium from unlabeled ABCA1 cells treated as described for A was collected, centrifuged to remove cellular debris, added to [^{3}H]cholesterol-labeled Mock, ABCG1, or ABCG4 cells for 6 h, and assayed for efflux. For A and B, efflux of [^{3}H]cholesterol is shown as means ± SD of triplicate experiments expressed as the percentage of total radiolabeled cholesterol (medium plus cell) released into the medium. C: The cells described for B were treated with 1 U/ml cholesterol oxidase for 10 min at 37°C and assayed for [^{3}H]cholestenone. Results are means ± SD of triplicate experiments expressed as the percentage of total (medium plus cell) radiolabeled free cholesterol plus cholestenone.

ABCG1 and ABCG4 promote the maturation of ABCA1-generated nascent HDL particles to larger, more cholesterol-rich lipoproteins

We used gel filtration chromatography to analyze the lipid composition and size of the lipidated particles being formed by the incubation of apoA-I with ABCA1 cells and their subsequent maturation when incubated with ABCG1 or ABCG4 cells. Incubating ABCA1 cells for 18 h with 10 μg/ml apoA-I removed ~10% and 6% of the total cellular [^{3}H]cholesterol and [^{3}H]choline-labeled phospholipid, respectively (**Fig. 4**). We used Superdex 200 column gel filtration to analyze the conditioned medium, because it is able to separate free apoA-I from HDL-like particles and has been used previously to study the nascent HDL particles that are formed by J774 macrophages incubated with apoA-I (14). The gel filtration profile after the 18 h incubations of 10 μg/ml apoA-I with ABCA1 cells shows multiple peaks (**Fig. 5**, **Table 1**). Peak I is present in the void volume, does not contain appreciable apoA-I, and probably represents membrane vesicles shed by the cells

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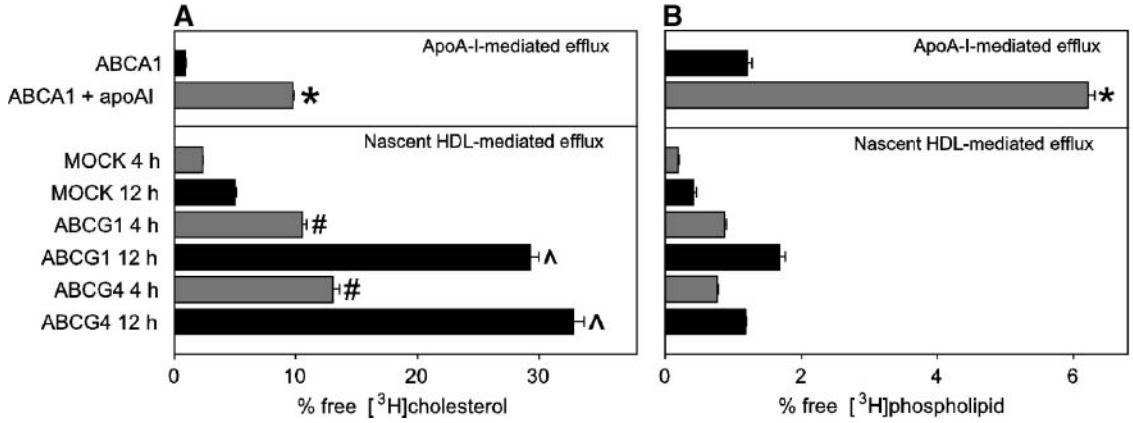

Fig. 4. ApoA-I-mediated [^{3}H]cholesterol and [^{3}H]phospholipid efflux from ABCA1 cells and nascent HDL-mediated [^{3}H]cholesterol and [^{3}H]phospholipid efflux from Mock, ABCG1, or ABCG4 cells. [^{3}H]cholesterol-labeled (A) and [^{3}H]phospholipid-labeled (B) ABCA1 cells were incubated with medium alone (ABCA1) or with medium and 10 μg/ml apoA-I (ABCA1 + apoA-I) for 18 h. In a parallel experiment, unlabeled cells were treated with 10 μg/ml apoA-I, and this medium was added to [^{3}H]cholesterol-labeled (A) and [^{3}H]phospholipid-labeled (B) Mock, ABCG1, or ABCG4 cells for 4 and 12 h. Efflux of [^{3}H]cholesterol (A) and [^{3}H]phospholipid (B) was measured and expressed as means ± SD of the percentage of total radiolabeled cholesterol (medium plus cell) released into the medium for triplicate incubations. * $P < 0.01$ versus incubation with medium alone; # $P < 0.01$ versus 4 h incubation with Mock cells; ^ $P < 0.005$ versus 12 h incubation with Mock cells.

(32, 33). A similar peak is generated from J774 cells treated with cAMP and is thought to contain membrane rafts (34). Peak IV corresponds to the elution position of lipid-free apoA-I added directly to the Superdex 200 column (results not shown) and contains no appreciable cholesterol. Most of the phospholipid present in this peak appears to be from the right shoulder of peak III and is probably attributable to the phospholipidation of apoA-I. Peaks II and III contain the majority of the cholesterol, phospholipid, and apoA-I and thus are nascent HDL particles. Peak III is phospholipid-rich, peak II is more cholesterol-rich, and the two peaks have similar amounts of apoA-I. The profile is very similar to that generated by cAMP-treated J774 macrophages exposed to apoA-I (14), although the amount of peak I in this study is a lot smaller in comparison (Fig. 5). These profiles suggest that the incubation of apoA-I with ABCA1 cells is sufficient to generate nascent HDL particles.

We then evaluated how ABCG1 and ABCG4 modified these ABCA1-generated nascent particles with time of incubation. ApoA-I mediated the release of both [^{3}H]cholesterol and [^{3}H]phospholipid from ABCA1 cells to pro-

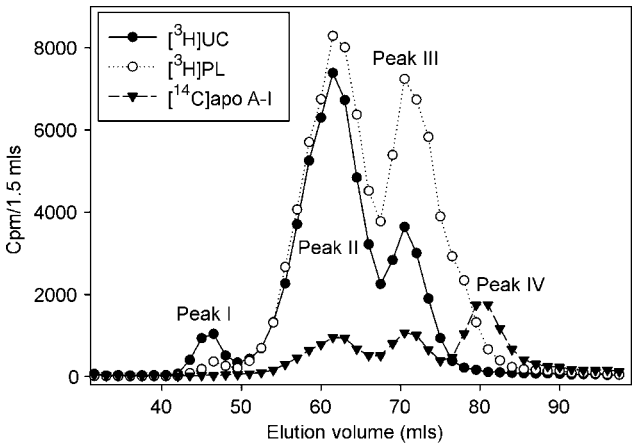

Fig. 5. Gel filtration of nascent HDL particles. ABCA1 cells were labeled with [^{3}H]cholesterol or [^{3}H]choline and treated with 10 μg/ml apoA-I for 18 h. Alternatively, nonlabeled cells were treated with [^{14}C]apoA-I. The nascent HDL particles formed were centrifuged to remove cellular debris and subjected to gel filtration chromatography on a calibrated Superdex 200 column. Fractions of 1.5 ml were collected, and radioactivity for [^{3}H]cholesterol (UC) and [^{14}C]apoA-I was determined by direct liquid scintillation counting. [^{3}H]choline-containing phospholipids (PL) were separated from aqueous [^{3}H]choline by solvent extraction as described in Experimental Procedures. One representative profile is shown out of multiple independent experiments.

TABLE 1. Characteristics of nascent HDL particles

Peak	K_{av}[a]	Molecular Mass[b]	Diameter[b]
		kDa	*nm*
I	0	>1,200	>20
II	0.16–0.20	265–337	11.1–12.2
III	0.32–0.34	115–129	8.0–8.4
IV	0.45–0.46	56–59	6.1–6.2

ABCA1 cells were labeled with [^{3}H]cholesterol or [^{3}H]choline and treated with 10 μg/ml apolipoprotein A-I (apoA-I) for 18 h. Alternatively, nonlabeled cells were treated with [^{14}C]apoA-I. The medium was centrifuged to remove cellular debris and subjected to gel filtration chromatography on a calibrated Superdex 200 column. Fractions of 1.5 ml were collected, and radioactivity for [^{3}H]cholesterol and [^{14}C]apoA-I was determined by direct liquid scintillation counting. [^{3}H]choline-containing phospholipids were separated from aqueous [^{3}H]choline by solvent extraction as described in Experimental Procedures. The particle sizes of the various peaks (see Fig. 4) were determined by comparing their K_{av} values with those of proteins of known diameter (particle diameter range, 6.1–17 nm; protein mass range, 29–669 kDa).

[a] K_{av} was calculated as described in Experimental Procedures and relates to the distribution coefficient of the filtered particle.

[b] The molecular mass and particle diameter of an equivalent globular protein were derived using the calibration equations described in Experimental Procedures.

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duce nascent HDL particles (Fig. 5). We then transferred this conditioned medium from unlabeled ABCA1 cells to ABCG1, ABCG4, or Mock cells and measured the efflux of radiolabeled cholesterol and phospholipids after 4 and 12 h incubations (Fig. 4). Mock cells released a small amount of [^{3}H]cholesterol (Fig. 4A) and [^{3}H]choline-labeled phospholipid (Fig. 4B) into the ABCA1 conditioned medium that increased slightly from 4 to 12 h. As also shown in Fig. 1, ABCG1 and ABCG4 cells released significant amounts of radiolabeled cholesterol into the ABCA1 conditioned medium by 4 h, and this increased >2-fold by 12 h (Fig. 4A). There was also an increased efflux of [^{3}H]phospholipid from ABCG1 and ABCG4 cells compared with Mock cells at both time points (Fig. 4B), although this was far less than the apoA-I-mediated [^{3}H]phospholipid efflux from ABCA1-expressing cells (Fig. 4B) and the [^{3}H]cholesterol efflux from ABCG1 and ABCG4 cells (compare Fig. 4A, B). These results confirm the previous data showing that ABCG1 and ABCG4 selectively promote cholesterol efflux from cells.

We subjected the 4 and 12 h chase media to gel filtration to monitor changes in the distribution of lipids between particles (**Fig. 6**). In parallel, we first incubated ABCA1 cells with [^{14}C]apoA-I and then transferred the medium to unlabeled cells so that we could track the changes in apoA-I distribution after the chase incubations.

For the Mock cells, there was very little [^{3}H]cholesterol and [^{3}H]phospholipid efflux, and the lipids that did efflux were distributed mainly to peaks II and III (Fig. 6A, B). This suggests that the Mock cells are not able to efflux cholesterol or phospholipid to the nascent HDL particles and are unlikely to have any ABCG1 or ABCG4 activity.

For the ABCG1 cells (Fig. 6D), most of the ABCA1-generated particles were remodeled into a single particle population slightly larger than peak II, and these particles became slightly larger with incubation time. The shift in

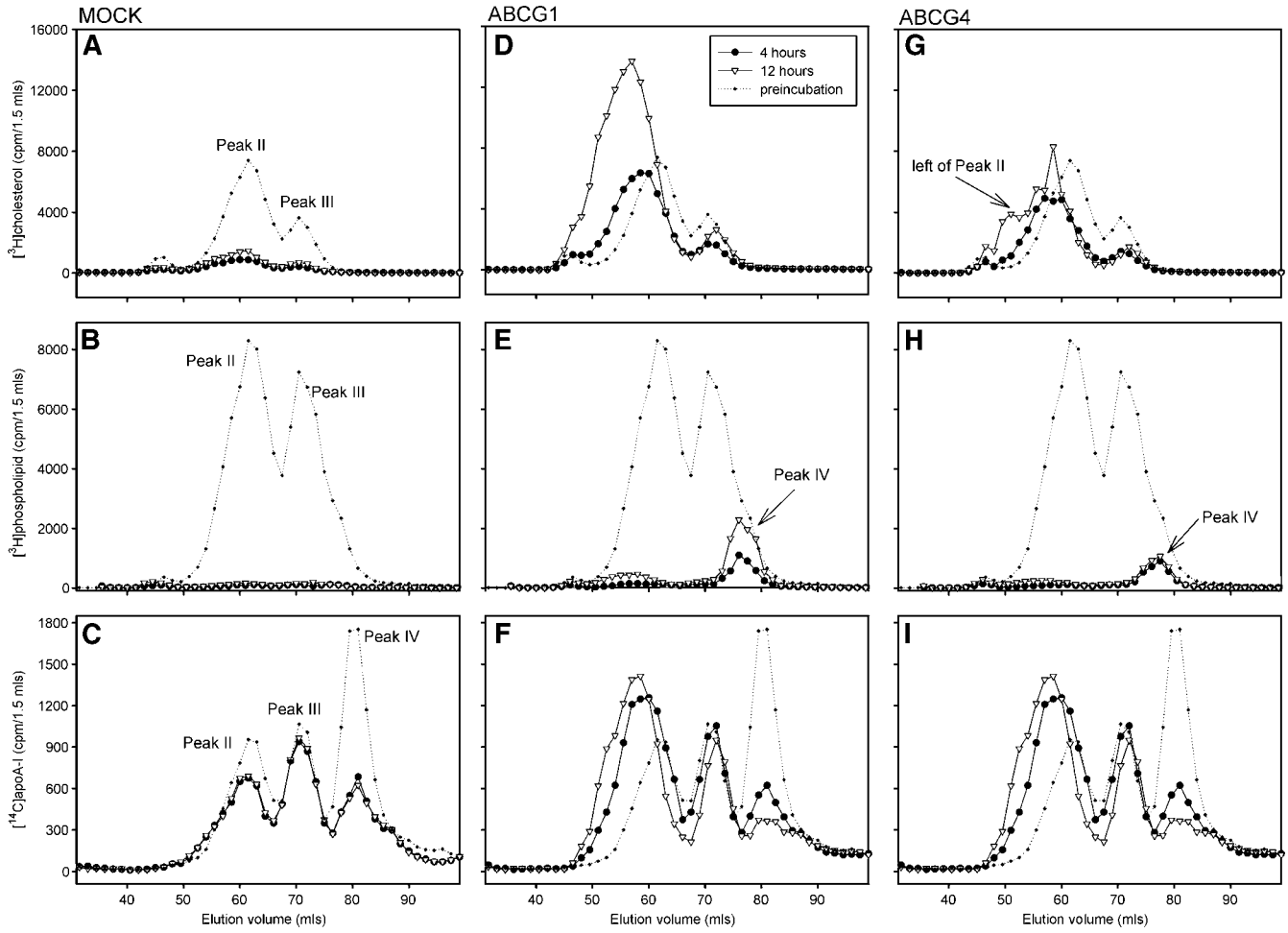

Fig. 6. Gel filtration of HDL particles generated by the incubation of Mock cells (A–C), ABCG1 cells (D–F), or ABCG4 cells (G–I) incubated with ABCA1-generated particles. The nascent HDL particles formed by the incubation of apoA-I with ABCA1 cells (see Fig. 5) were incubated with [^{3}H]cholesterol-labeled or [^{3}H]choline-labeled Mock, ABCG1, or ABCG4 cells for 4 h (closed circles) or 12 h (open triangles). In parallel, [^{14}C]apoA-I-labeled nascent HDL particles were incubated with nonlabeled cells. The HDL particles were subjected to gel filtration chromatography on a calibrated Superdex 200 column. Fractions of 1.5 ml were collected, and radioactivity for [^{3}H]cholesterol (A, D, G) and [^{14}C]apoA-I (B, E, H) was determined by direct liquid scintillation counting. [^{3}H]choline-containing phospholipids (C, F, I) were separated from aqueous [^{3}H]choline by solvent extraction as described in Experimental Procedures. One representative profile is shown out of multiple independent experiments. As a comparison, the results obtained from the incubation of apoA-I with ABCA1-expressing cells are shown as dotted lines.

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particle size appeared to be attributable to an enrichment in cholesterol, as there was a marked time-dependent increase in [^{3}H]cholesterol content (Fig. 6D). There was appreciable phospholipid efflux to peak IV and a slight increase in size of these particles (Fig. 6E), which is probably attributable to further lipidation of lipid-poor apoA-I. The [^{14}C]apoA-I was redistributed from both peak IV and peak III into peak II, and this redistribution and particle size increase became more pronounced with time (Fig. 6F). This suggests that ABCG1-mediated cholesterol efflux caused the smaller, phospholipid-rich peak III particles to be converted to larger, more cholesterol-rich peak II particles. The studies by Gelissen et al. (20) suggest that the phospholipid concentration of the conditioned medium formed by the incubation of apoA-I with ABCA1-expressing cells is important in promoting the further efflux of cholesterol from ABCG1-expressing cells. The data presented here are in agreement with this finding and suggest that the phospholipid-rich peak III particle (Fig. 5) is an ideal acceptor of further cholesterol from ABCG1-expressing cells and probably then becomes the larger peak II particle (Figs. 5, 6D).

The results of gel filtration chromatography for the ABCG4 cells were similar to those for the ABCG1 cells (Fig. 6G–I), with the possible exception of some heterogeneity in the [^{3}H]cholesterol distribution in the major particle population at the 12 h time point (Fig. 6G). Figure 6G shows that there is an additional, larger, cholesterol-rich peak to the left of peak II. These findings raise the possibility that the ABCG1 and ABCG4 cholesterol export pathways remodel nascent HDL particles in slightly different ways. However, the slight increase in efflux of cholesterol from ABCG4 cells, compared with ABCG1 cells (Fig. 4A), to the nascent HDL particles could lead to the formation of the additional large peak to the left of peak II. In addition to ABCG1 and ABCG4, scavenger receptor class B type I (SR-BI) has also been shown to be involved in the efflux of cholesterol to HDL in macrophages (35), and it is thus possible that SR-BI also plays a role in the formation of nascent HDL. However, in our experiments, there was a negligible efflux of cholesterol from Mock cells to the nascent HDL particles formed by the incubation of apoA-I with ABCA1-expressing cells, and the incubation of ABCG1- and ABCG4-expressing cells with an SR-BI blocking antibody did not alter the efflux of cholesterol from these cells to HDL (results not shown). It is possible, however, that SR-BI expressed in the macrophage could play a role in nascent HDL formation.

Gel filtration shows that the incubation of the nascent HDL particles with ABCG1- and ABCG4-expressing cells, but not with Mock cells, increases cholesterol and phospholipid efflux to a peak just to the left of peak IV (Figs. 6D, G and E, H, respectively). This suggests that ABCG1 and ABCG4 are involved in lipidation of the free apoA-I remaining in the nascent HDL mixture. Alternatively, the remodeling of the nascent HDL particles by the efflux of cholesterol through ABCG1 and ABCG4 could lead to the formation of this lipid-poor apoA-I-containing particle.

To determine the cholesterol and phospholipid composition of the apoA-I particles generated by ABCA1 and remodeled by ABCG1 or ABCG4, we measured cholesterol and phospholipid mass in apoA-I-containing medium preincubated for 18 h with Mock or ABCA1 cells. The cholesterol and phospholipid masses of the medium from the ABCA1-expressing cells subsequently incubated with Mock, ABCG1, or ABCG4 cells for 8 h were also determined. Induction of ABCA1 in the presence of apoA-I led to a nearly equal molar increase in the export of cellular cholesterol and phospholipids (cholesterol-to-phospholipid molar ratio of 0.7) (**Fig. 7A**, second set of bars). When this medium was transferred to Mock cells, the cholesterol content decreased by ~40%, presumably because of some surface binding or cellular uptake of the cholesterol (Fig. 7B, first set of bars). This is in contrast with the radiotracer study (Fig. 4A), in which a modest efflux of [^{3}H]cholesterol was seen. However, this could be attributable to an exchange of unlabeled cholesterol in the nascent HDL particle with [^{3}H]cholesterol in the Mock cells. The phospholipid content of the nascent HDL-containing medium did not change significantly when incubated with Mock, ABCG1-, or ABCG4-expressing cells (Fig. 4B, all bars). However, modest increases in phospholipid efflux were seen in the radiotracer studies with the ABCG1- and ABCG4-expressing cells. As before, these increases could be attributable to an exchange of [^{3}H]phospholipid in the cell with unlabeled phospholipid in the medium. Compared with the incubations with

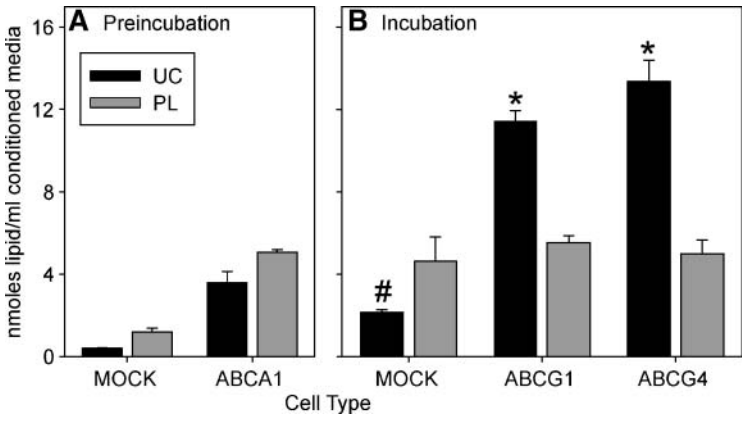

Fig. 7. ApoA-I-mediated cholesterol and phospholipid mass efflux from ABCA1 cells and nascent HDL-mediated cholesterol and phospholipid mass efflux from Mock, ABCG1, or ABCG4 cells. A: The mass of cholesterol (UC) and phospholipid (PL) released into the medium of Mock and ABCA1 cells treated with 10 μg/ml apoA-I for 18 h. B: The medium from ABCA1 cells treated as described for A was then incubated with Mock, ABCG1, or ABCG4 cells for 12 h, and the cholesterol and phospholipid mass of the medium was measured. # $P < 0.01$, * $P < 0.005$ versus the cholesterol content of the preincubation medium (A, second set of bars).

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Mock cells, incubating ABCA1-conditioned medium with ABCG1 or ABCG4 cells caused a significant increase in cholesterol content (Fig. 7B). The ABCG1- and ABCG4-modified particles had cholesterol-to-phospholipid mass ratios of 2.1 and 2.7, respectively. These results confirm that ABCA1 generates partially lipidated apoA-I particles that then become enriched with cholesterol when exposed to cells expressing either ABCG1 or ABCG4.

DISCUSSION

Previous studies have shown that ABCG1 and ABCG4 mediate the efflux of cellular cholesterol to HDL (16, 17, 24). This is achieved by the ability of ABCG1 and ABCG4 to redistribute cholesterol to plasma membrane domains that are accessible for removal by lipoprotein particles (16). It has been shown that ABCA1 and ABCG1 synergize to mediate cellular cholesterol efflux to apoA-I (20). That study was very important in laying the groundwork for this current report. The authors showed, using the same BHK cells that overexpress ABCA1 that we used in this study, that the phospholipid content of the nascent HDL particles was indicative of the further efflux of cholesterol from cells overexpressing ABCG1. However, the process by which this occurs is not fully understood. Here, we show that nascent HDL-like particles formed by the incubation of apoA-I with ABCA1-expressing cells act as acceptors of cholesterol exported from either ABCG1- or ABCG4-expressing cells. Gel filtration chromatography revealed that the incubation of apoA-I with ABCA1 cells generates two predominant nascent HDL-like particle populations: smaller phospholipid-rich particles and larger more cholesterol-rich particles. The cholesterol exported by ABCG1 or ABCG4 remodels most of the phospholipid-rich ABCA1-generated particles into one larger particle population that is markedly enriched in cholesterol. ABCG4 also generates an additional particle population that is larger than the ABCG1-modified particles. Thus, exposing lipid-free apoA-I to ABCA1-expressing cells forms a nascent HDL particle that is an efficient acceptor for cholesterol exported by either the ABCG1 or ABCG4 pathway. These findings indicate that ABCA1 and ABCG1 or ABCG4 act sequentially to optimize the secretion of cholesterol from cells and to increase the formation of cholesterol-rich HDL particles.

The nascent apoA-I particles generated by ABCA1 cells were efficient acceptors for cholesterol when incubated with ABCG1 or ABCG4 cells but not with control cells, indicating that further lipidation of the nascent particles depended on the activity of these ABCG transporters. These studies also showed that the enhanced cholesterol efflux does not require coexpression of ABCA1, ABCG1, or ABCG4 in the same cell, indicating that an ABCA1-lipidated apoA-I could remove additional cholesterol from distant sites containing cells expressing ABCG1 or ABCG4. This concept was supported by our results showing that mixing either ABCG1 or ABCG4 cells with ABCA1 cells markedly increased the amount of cholesterol released into the medium. With cholesterol-loaded macrophages, however, the transcription levels of these transporters, particularly ABCA1 and ABCG1, are induced to high levels, making it likely that they also coordinate the removal of excess cholesterol from the same cell (8–11).

We showed previously that all three ABC transporters mediate cholesterol export from cells by promoting the translocation of cellular cholesterol to plasma membrane domains that are accessible for removal by either lipid-poor apolipoproteins or lipidated lipoproteins (16, 25). Using a cholesterol oxidase treatment assay, we found that the nascent lipoprotein particles produced by ABCA1 cells also selectively removed cholesterol from cell surface domains formed by ABCG1 or ABCG4 expression, similar to what was observed previously with HDL particles (16). This cholesterol removal appears to require only partial lipidation of apoA-I by ABCA1. The largest incremental increase in the ability of apoA-I to remove cholesterol from ABCG1 or ABCG4 cells was observed after exposing apoA-I to ABCA1 cells for only 1 h, despite continual lipidation of apoA-I with time of exposure to ABCA1 cells. It is remarkable that these partially lipidated apoA-I particles have very little ability to promote cholesterol efflux from cells by processes independent of ABC transporters. The ability of ABCA1 to acutely generate efficient acceptor particles for ABCG1 and ABCG4 is consistent with the idea that these transporters can act in tandem and in close proximity to remove cellular cholesterol.

Gel filtration studies showed that incubation of apoA-I with ABCA1-expressing cells caused the formation of two predominant lipid particles containing apoA-I, phospholipid, and cholesterol (~8 and 12 nm in size). Similar particles were shown previously to be generated by ABCA1 in macrophages (14). The distinct sizes of the lipoprotein particle populations likely reflect different compositions of apoA-I, phospholipids, and cholesterol, as has been reported for the macrophage-produced particles (34). ABCA1-expressing macrophages exposed to apoA-I were reported to release a significant amount of what appeared to be large apoA-I-free membrane vesicles (14). We observed the formation of similar vesicles when BHK cells were incubated with apoA-I, although the amount of cholesterol and phospholipid in these vesicles was much smaller than that in apoA-I-containing particles. We found, however, that cells lacking ABC transporters also generated these vesicles in the presence of apoA-I, suggesting that they can be produced by an ABCA1-independent process.

When subsequently incubated with ABCG1 or ABCG4 cells, most of the ABCA1-generated nascent HDL particles were remodeled to one large particle population that was slightly larger than the 12 nm particles produced by ABCA1 alone. Although there was a modest increase in their phospholipid composition, these particles became markedly enriched in cholesterol. Compared with incubations with cells lacking ABC transporters, incubating ABCA1-conditioned medium with ABCG1 or ABCG4 cells for only 8 h led to a 4.4- or 5.8-fold increase, respectively, in the cholesterol-to-phospholipid mass ratio.

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Interestingly, the ABCA1-generated particles remodeled by ABCG4 appeared to be more heterogeneous than those remodeled by ABCG1. In particular, ABCG4 distributed cholesterol to a particle population that appeared as a 14 nm shoulder on the major peak. This observation raises the possibility that ABCG1 and ABCG4 remodel nascent lipoproteins by different mechanisms. Alternatively, the increased amount of cholesterol efflux from the ABCG4 cells, compared with the ABCG1 cells used in this study, could cause the formation of the larger particles attributable to a threshold of peak II particle concentration in the medium that then converted to the larger particle. Further studies with the ABCG1 and ABCG4 cells, using longer incubation times, should address this question. Based on studies using ABCG4$^{-/-}$ mice, it was recently shown that ABCG4 does not play a significant role in cholesterol mass efflux to HDL from macrophages (12), suggesting that it is ABCG1 that plays a major role in macrophage cholesterol efflux. It has been shown, however, that ABCG4 transcription levels are increased in the brain's neuronal cells on stimulation with 24(*S*)-hydroxycholesterol (36), consistent with the idea that ABCG4 could be involved in cholesterol homeostasis in the brain and other neuronal tissue.

In summary, this study shows that the lipidation of apoA-I by the ABCA1 pathway forms nascent HDL particles that then serve as acceptors for cholesterol exported by both ABCG1 and ABCG4 transporters. Thus, ABCA1 and either or both ABCG1 and ABCG4 can act in tandem to remove excess cholesterol from cells and generate cholesterol-rich HDL particles. The expression of these three transporters ensures that both lipid-free and lipidated apoA-I can act to remove excess cholesterol from cholesterol-loaded macrophages and aid in reverse cholesterol transport. Thus, this process sets up a sequence of actions that provide a defense against cholesterol accumulation in the artery wall.

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