The mechanism of formation of high density lipoprotein (HDL) particles by the action of ATP-binding cassette transporter A1 (ABCA1) is not defined completely. To address this issue, we monitored efflux to apoA-I of phosphatidylcholine (PC), sphingomyelin (SM), and unesterified (free) cholesterol (FC) from J774 macrophages, in which ABCA1 is up-regulated, and investigated the nature of the particles formed. The various apoA-I-lipid particles appearing in the extracellular medium were separated by gel filtration chromatography. The presence of apoA-I in the extracellular medium led to the simultaneous formation of more than one type of poorly lipitated apoA-I-containing particle; there were 9- and 12-nm diameter particles containing ~3:1 and 1:1 phospholipid/FC (mol/mol), respectively, which were present together with 6-nm monomeric apoA-I. Removal of the C-terminal α-helix (residues 223–243) of apoA-I reduced phospholipid and FC efflux and prevented formation of the 9- and 12-nm HDL particles; the apoA-I variant formed larger particles that eluted in the void volume. FC loading of the J774 cells also led to the formation of larger apoA-I-containing particles that were highly enriched in FC. Besides creating HDL particles, ABCA1 mediated release of larger (20–450-nm diameter) FC-rich particles that were not involved in HDL formation and that are probably membrane vesicles. These particles contained 1:1 PC/SM in contrast to the HDL particles, which contained 2:1 PC/SM. This is consistent with lipid raft and non-raft plasma membrane domains being involved primarily in ABCA1-mediated vesicle release and nascent HDL formation, respectively.

Understanding both the factors that control plasma high density lipoprotein (HDL) levels and the functions of HDL in cholesterol transport is of great biomedical significance because there is an inverse relationship between plasma HDL cholesterol levels and the incidence of coronary heart disease (1). The anti-atherogenic properties of HDL have been demonstrated directly by increasing HDL levels in mice and observing reductions in the sizes of atherosclerotic lesions (2). The anti-oxidant (3) and anti-inflammatory (4) capabilities of HDL probably contribute to its cardioprotective effects, but the role of HDL in reverse cholesterol transport is generally thought to be critical (5, 6). According to this concept, HDL is anti-atherogenic because it transports cholesterol away from cells in the vessel wall (and from foam cells in atherosclerotic lesions, in particular) to the liver, where the cholesterol can be incorporated into the fecal sterol pool and removed from the body. Recognition of the beneficial effects of raising HDL levels has increased interest in understanding the mechanisms of HDL biogenesis. The discovery that the low HDL levels associated with Tangier disease and other familial HDL deficiencies are due to the mutations in ATP-binding cassette transporter A1 (ABCA1) (for a review, see Ref. 7) has revealed that this transporter plays a key role in HDL biogenesis (8). ABCA1-mediated efflux of phospholipid (PL) and free (unesterified) cholesterol (FC) from macrophages to apoA-I in the extracellular medium (9–11) reduces the accumulation of FC in foam cells and protects against atherosclerosis (12). The release of cellular lipids to lipid-free (poor) apoA-I via ABCA1 is an active process, which is in contrast to other mechanisms of passive efflux to HDL (9, 13, 14). The above observations have led to a surge of interest in ABCA1-mediated lipid efflux from cells and recognition of the need to understand how nascent HDL particles are created by the activity of this transporter.

It is well established that ABCA1 can promote the release of cellular PL and FC to various lipid-free (poor) apolipoproteins (for reviews, see Refs. 13 and 14). However, the molecular mechanisms involved in this process, which we termed membrane microsolvulation (15), have not been defined unambiguously. Reaction schemes involving either simultaneous or sequential release of PL and FC have been proposed. Similarly, there is uncertainty about involvement of various domains of the plasma membrane; the relative contributions of lipids in rafts and more fluid domains to overall lipid efflux are not known (10, 11, 14). In the case of apoA-I, binding to the cell surface is involved, but whether the interaction is directly with
ABC1 or with adjacent membrane PL is a matter of debate (11, 16). The presence of an amphipathic α-helix is required in the acceptor protein for efficient PL and FC efflux (17–19), and it is well established that the strongly lipid-binding C-terminal α-helix in apoA-I plays a key role in efflux to this protein (20–24). This domain of apoA-I could mediate the initial interaction with the plasma membrane and/or be required for retention of released PL molecules and stabilization of the nascent HDL particles. Elucidation of the structures of the apoA-I-containing particles created in the extracellular medium is an aspect of ABC1-mediated lipid efflux that has received relatively little attention.

Prior to the discovery of ABC1, it was established that apolipoprotein-mediated efflux of cellular PL and FC leads to the formation of HDL particles (25, 26). A thorough characterization of the HDL formed by Chinese hamster ovary cells showed that the first extracellular nascent apoA-I-containing particles are small and lipid-poor; these eventually transform to larger, lipid-enriched, discoidal complexes (27). A similar HDL particle size distribution was observed when apoA-I was incubated with THP-1 macrophages (28). The predominant PL incorporated into the nascent HDL particles are phosphatidylcholine (PC) and sphingomyelin (SM) (28, 29), although the incorporation of acidic PL can cause the particles to exhibit pre-α-electrophoretic mobility (30). When apoA-I is incubated with cholesterol-enriched cells, the FC/PL ratio in the extracellular medium is enhanced (15, 31). There is a need for more characterization of the different apoA-I-containing species formed by ABC1 in terms of their lipid/protein stoichiometries and PL/cholesterol contents. Also, the effects of changes in apoA-I structure on nascent HDL formation have not been examined. In this study, we address some of these issues by monitoring the lipoproteins of apoA-I induced by ABC1-driven efflux of cellular PL and FC from J774 macrophages. The influence of the C-terminal α-helix of apoA-I on the formation of nascent HDL particles is examined. Importantly, besides the formation of HDL particles, ABC1 induces release of FC/PL particles that do not contain apoA-I and that are apparently formed from a different domain of the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fetal bovine serum, gentamycin, and b-(4-chlorophenylthio) (CPT)-cAMP were purchased from Sigma. Bovine serum albumin (BSA; essentially fatty acid-free) was obtained from Intergen Co. (Purchase, NY). [1,2-3H]Cholesterol (51 Ci/mmol), [4-14C]cholesterol (45 Ci/mmol), and [methyll-3H]choline chloride (86 Ci/mmol) were obtained from PerkinElmer Life Sciences. Organic solvents were purchased from Fisher. Tissue culture dishes, flasks, and plates were from Corning Inc. (Corning, NY) and Falcon (Lincoln, NJ). RPMI 1640 medium and phosphate-buffered saline were purchased from CellGro (Herndon, VA). Minimal essential medium buffered with 25 mM Hepes (pH 7.4) (referred to below as Hepes-buffered minimal essential medium) was obtained from BioWhittaker, Inc. (Walkersville, MD). The acyl-CoA:cholesterol acyltransferase inhibitor CP-113,818 (acyl-CoA:cholesterol acyltransferase inhibitor) containing either 3 μCi/ml [3H]cholesterol for 24 h or 5 μCi/ml [3H]cholesterol chloride for 48 h (single labeling) (41). Double labeling was achieved by incubation with 20 μCi/ml [3H]cholesterol chloride and 3 μCi/ml [3H]cholesterol for 48 h. For preparation of cholesterol-enriched monolayers, the cells were labeled with the same medium that also contained 25 μg/ml acetylated density lipoprotein (LDL) and incubated for 48 h. All media were supplemented with 50 μg/ml gentamycin. Following the 24–48-h labeling period, the cells were washed and incubated with 25 μg/ml CP-113,818 in RPMI medium buffered with 13 mM CPT-CAMP for 12 h (41, 42). Some wells were then washed with phosphate-buffered saline, dried, and extracted with isopropl alcohol (43). From the extracted lipids, cell radioactivity at time 0 was measured by liquid scintillation counting (PL was extracted before counting as described in detail below). FC assay was assayed by gas-liquid chromatography, and the mass of choline-containing PL was determined by an enzymatic assay (Wako Bioproducts, Richmond, VA).

**Efflux of Cellular Cholesterol and Phospholipid—CPT-cAMP-treated J774 cell monolayers were washed with Hepes-buffered minimal essential medium, and lipid efflux was initiated by the application of 1.0 ml/well RPMI 1640 medium in the presence or absence (background) of CPT-cAMP. J774 or mutant apoA-I at the indicated concentrations or time points. For lipid efflux experiments, unlabeled proteins were used, whereas for gel filtration chromatography analysis of the lipid particles released into the extracellular medium, [3H]apoA-I and unlabeled apoA-I were used with single- and double-labeled cells, respectively. FC efflux was quantified by removing aliquots of the incubation medium, followed by filtration through a 0.45-μm multiscreen plate (Millipore Corp., Bedford, MA) to remove floating cells. The radioactivity present in the incubation medium was determined by liquid scintillation counting, and the percent of radiolabeled FC released (percent efflux) was calculated as follows: (cpm in medium/cpm at time 0) × 100. Background cholesterol efflux (in the absence of apoA-I) was subtracted. For FC efflux after the medium was filtered, the lipidic medium was extracted by the procedure of Bligh and Dyer (44); the aqueous phase was aspirated, and the chloroform phase was washed three times with 10:9 (v/v) methanol/water. The chloroform phase was dried under nitrogen in liquid scintillation vials, and radioactivity was quantitated by liquid scintillation counting. The cells were extracted with isopropl alcohol to a final volume of 50 μl. The extracts were dried under nitrogen, and the free [3H]cholesterol was separated by the Bligh and Dyer procedure. PL efflux was calculated as described for FC efflux.

**Mass Analysis of the Extracellular Medium**—To determine lipid mass, it was necessary to concentrate the conditioned medium from ~250 ml to 5 ml. The conditioned medium was treated as described above (5) by passage through a BSA-washed 0.22-μm filter (Millipore Corp.). This filtered medium was then dialyzed extensively (M, 35,000 cutoff) at 4°C against Tris buffer and then concentrated 10–20-fold by passage.
Northern Blot Analysis—RNA was extracted from J774 mouse macrophage cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. 20 μg of this total RNA was electrophoretically separated on a agarose gel containing formaldehyde. The RNA was then transferred to a nylon membrane and UV-cross-linked. A 453-bp actin probe extending from residues 452 to 905 on the open reading frame of murine β-actin cDNA, a 580-bp ATP-binding cassette transporter G1 (ABCG1) probe extending from residues 413 to 993 on the murine ABCG1 cDNA open reading frame, and a 324-bp ABCA1 probe extending from residues 971 to 1295 on the murine ABCA1 cDNA open reading frame were generated by reverse transcription-PCR and used to detect and measure expression levels. The probes were 32P-radiolabeled using the Prime-iT™ 11 random priming kit (Stratagene) and hybridized overnight to the Northern blot. This was then exposed to a phosphor screen and scanned with a PhosphorImager (Storm, Amer sham Biosciences), and the band intensities were quantified using Image analysis software (ImageQuant). The blots were probed sequentially for ABCG1, ABCA1, and actin, and all of the mRNA levels were normalized to that of actin.

RESULTS

Lipids Removed from cAMP-stimulated J774 Cells—In agreement with previous studies (41, 42), pretreatment of J774 cells with cAMP as described under “Experimental Procedures” increased the level of ABCA1 mRNA by ~6-fold; there was no change in the expression of ABCG1 (compare with Ref. 48) (data not shown). This induction of ABCA1 increased efflux of PL and FC to 20 μg/ml of extracellular apoA-I in 6 h by ~6- and 10-fold, respectively, relative to control cells (data not shown). The data in Table I show that cAMP-stimulated J774 cells released cholesterol-rich lipid particles into the extracellular medium. The overall FC/PL molar ratio of 0.84 ± 0.21 is much higher than the value of ~0.2 seen for human HDL2a (49). The mass analysis revealed that, in addition to the radiolabeled FC and choline-containing PL, the medium contained phosphatidylethanolamine and desmosterol that were not radiolabeled by the procedures used. The distribution of PC, SM, and phosphatidylethanolamine reported in Table I for control J774 cells is consistent with an earlier analysis of the PL released from macrophages (28). The fact that PC is the major class of PL transported out of cells by ABCA1 activity is also established already (10).

Characterization of Lipid Particles Present in the Extracellular Medium—Fig. 1 shows the fractionation by passage through a Superdex 200 gel filtration column of the lipid particles present in the extracellular medium after incubation of J774 cells with apoA-I. Typical recoveries of radiolabeled apoA-I, PL, and FC after filtration of the extracellular medium through a 0.45-μm filter were >80, 75, and 65%, respectively; much of this loss was due to removal of floating cells. The equivalent recoveries after passage through the Superdex 200 column were 95, 65, and 80%, respectively. Thus, the elution profile in Fig. 1 is characteristic of most of the apoA-I and at least 50% of the PL and FC initially present in the extracellular

### Table I

| Cell condition          | FC/PL % of total PL<sup>a</sup> | PC       | SM       | PE       | Lysop-PC | % of total PL<sup>a</sup> |
|-------------------------|---------------------------------|----------|----------|----------|----------|---------------------------|
| Control<sup>b</sup>     | 0.84 ± 0.21 (n = 5)             | 45       | 22       | 22       | 11       | 30% ± 0.25                |
| FC-enriched<sup>c</sup> | 4.28 ± 0.84 (n = 6)             | 36       | 19       | 22       | 23       | 30% ± 0.25                |

<sup>a</sup> Values are the averages that differed by <5% from duplicate experiments.

<sup>b</sup> These cells contained initially 14.9 ± 4.7 and 5.3 ± 2.5 μg of PC and desmosterol/mg of cell protein, respectively (n = 4). The PL content was 83 ± 2.6, 10.9 μg/mg of cell protein at time 0.

<sup>c</sup> The J774 cells were incubated with 10 μg/ml apoA-I for 12 h. Control cells also released desmosterol, and the desmosterol/PL molar ratio was 0.37 ± 0.25.

<sup>d</sup> These cells were not treated with an hcy-CoA: cholesterol acyltransferase inhibitor and initially contained 41.9 ± 10.2 and 83.4 ± 19.4 μg of FC and total cholesterol (the cell cholesterol ester content is the difference between these two values/mg of cell protein, respectively (n = 5). The PL content was 164.9 ± 26.5 μg/mg of cell protein (n = 3). There was no detectable desmosterol.

through a BSA-washed YM filter (M<sub>w</sub> 10,000 cutoff) using an Amicon ultrafiltration 8050 cell pressurized at 20 p.s.i. N<sub>2</sub>. If necessary, further concentration was achieved using a BSA-washed Ultrafree-15 centrifugal filter device (M<sub>w</sub> 10,000 cutoff, Biomax low protein-binding membrane, Millipore Corp.). The typical ratio of FC and PL after this procedure were close to 55%. Lipids were extracted from aliquots of the concentrated medium by the method of Bligh and Dyer (44); cholesteryl methyl ether was added as an internal standard for the gas-liquid chromatographic assay (45) of cholesterol. PL was measured as inorganic phosphorus (46). Protein was determined using a modified Lowry assay for FC. The PL subclasses present in the lipid extract were separated by TLC using silica gel plates with a mobile phase of chloroform/methanol/water (65:25:4, v/v). The PL bands were visualized by iodine staining and quantified by assaying for phosphorus (47).

**Gel Filtration Chromatography**—The medium was collected after incubating CPT-cAMP-treated cells with or without apoA-I. The medium was filtered through a 0.45-μm filter, loaded (2.5 ml) onto a Superdex 200 column (60 × 1.6 cm) using the Akta FPLC system, and eluted with 10 mM Tris buffer at a flow rate of 1 ml/min. With single-labeled cells, 1-ml fractions were collected and counted to determine the [3H]cholesterol and [14C]apoA-I radioactivity. Lipids from 2-ml fractions of the medium from double-labeled cells were extracted by the procedure of Bligh and Dyer (44) and then counted for [3H]phosphatidylcholine and [14C]cholesterol radioactivity. Thus, at least two experiments (one to measure the [3H]cholesterol/[14C]apoA-I ratio and the other to measure the [3H]-choline-containing PL/[14C]cholesterol ratio) were conducted to obtain complete column profiles for FC, PL, and apoA-I. After each run, the Superdex 200 column was washed with 1 column volume each of 1 M NaOH, water, and elution buffer to remove the radioactivity retained and then preserialized with 1 ml of BSA solution (5 mg/ml) before the next run to enhance the recovery of radiolabeled materials.

The particle sizes of the various fractions were determined by comparing their K<sub>av</sub> values with those of proteins of known diameter (particle diameter range, 6.1–17 nm; and protein mass range, 29–669 KDa). K<sub>av</sub> was calculated using the following equation: K<sub>av</sub> = (V<sub>C</sub>−V<sub>V</sub>)/(V<sub>c</sub>−V<sub>V</sub>), where V<sub>C</sub> is the void volume, V<sub>c</sub> is the total column volume, and V<sub>V</sub> is the elution volume. For this purpose, a plot of log particle size or log molecular mass against K<sub>av</sub> was constructed, and the points were fitted using a linear regression analysis. The apparent molecular mass (in kilodaltons) and particle size (hydrodynamic diameter in nanometers) were calculated using the following equations: log<sub>10</sub> mass = −2.64 K<sub>av</sub> + 0.94, and diameter = 0.95 K<sub>av</sub> + 1.27.

The compositions (FC, PL, and apoA-I) of the various fractions were calculated using the measured counts of each fraction and the specific radioactivity for each component. Initial lipid specific activities were derived from time 0 cell samples for which the radioactivity and mass of lipid (FC or choline-containing PL) were determined. The specific activity for FC at time 0 was typically 50,000–80,000 cpm/μg and did not change significantly over a 6-h cell incubation. The specific activity for choline-containing PL at time 0 was typically 125,000–180,000 cpm/μg and decreased <15% over 6 h. The subclasses of choline-containing PL released into the extracellular medium were determined by a one-dimensional TLC procedure. After extraction of free [3H]choline, the samples were applied to Silica Gel G plates, which were then developed in chloroform/methanol/acetic acid/water (65:25:8:4, v/v/v). The bands corresponding to lyso-PC, FC, and SM standards were scraped from each sample lane and analyzed by liquid scintillation counting to determine the percentage of radioactivity associated with each PL subclass.
medium. It is apparent that the use of $[^{14}C]$apoA-I allows effective monitoring of the protein at the low concentrations (5 μg/ml) needed in this type of experiment. All of the apoA-I entered the column, and ~70% of it eluted at the position (peak IV) expected for the lipid-free protein (Fig. 1). The shoulder on the right of peak IV ($V_e = 83$ ml) corresponds to the elution position of lipid-free apoA-I added directly to the column without exposure to the J774 cells; the hydrodynamic diameter for this position is 6 nm (Table II). The major $V_e$ for peak IV is 79 ml, which reflects an ~0.5-nm increase in diameter. This existence of apoA-I in two states may be due to the interaction of small amounts of PL with the protein; ~10% of the PL that eluted from the column was in peak IV (Table II). However, the occurrence of PL at this elution position may also reflect some interaction of PL with the Superdex matrix and delayed elution from the column. Fig. 1 shows that apoA-I also eluted in peaks II and III, which correspond to larger particles (Table II) that are similar in size to HDL$_{3}$ and bovine serum albumin, respectively. The number of apoA-I molecules in each of these particles is not known at this stage; it will be necessary to isolate material free of any secreted cellular proteins in sufficient amounts for a structural characterization. The PL and FC contents of the peak II and III species are listed in Table II. After a 6-h efflux period, peak II contained ~43% of the PL (Table II) and 35% of the FC, and the FC/PL ratio was essentially equimolar. The particles eluting as peak III were smaller and relatively PL-enriched. The extracellular medium also contained FC/PL particles that were too large (>19-nm diameter) to enter the Superdex 200 matrix and that eluted in the void volume (peak I). The particles are at least as large as human LDL because this lipoprotein also elutes at $V_e$. The peak I material also appeared in the void volume of a Superose 6 column (data not shown); this gel fractionates globular proteins that are 10 times larger than those fractionated by Superdex 200. The larger peak I particles, which do not contain apoA-I, require separate characterization, but most likely represent membrane vesicles shed by the J774 cells (50, 51).

The experiment summarized in Fig. 2 was conducted to see how the acquisition of cellular PL and FC by apoA-I proceeds with time. Fig. 2 (compare A and B) shows that the amount of radiolabeled PL and FC appearing in peaks II and III increased as the apoA-I-cell contact period was increased from 2 to 8 h. Comparison of the distributions of radiolabeled PL and FC between peaks II and III relative to peak I in Fig. 2 (A and B) indicates that the former material accumulated ~2.5 times more than the peak I material in the 2–8-h period. The ratio of the PL contents of peaks II and III was ~1.3 at 2 and 8 h, indicating that the distribution of PL between the two peaks is constant over this period. The FC/PL ratios for peaks II and III were unchanged in Fig. 2 (A and B); this suggests that the FC/PL stoichiometry is constant over the 2–8-h period and that sequential release of PL and FC does not occur in this time frame. The above results are consistent with the two types of apoA-I-containing (HDL) particles corresponding to peaks II and III being formed simultaneously, with the peak II material being relatively FC-enriched.

The distribution of choline-containing PL released by apoA-I from cAMP-stimulated control J774 cells into the extracellular medium was 55% PC and 36% SM as measured by $[^{3}H]$choline radioactivity (Table III); these values agree with the PL mass analysis presented in Table I. Significantly, the PC/SM ratio was different for the material eluting in peak I and in peaks II and III: the PC/SM molar ratios were ~1.1 and 2.1, respectively (Table III). Thus, the fraction of PL that was SM was lower in the HDL particles formed in the extracellular medium than in the non-HDL particles eluting in the void volume.

The fact that the PL and FC radioactivity associated with peaks II and III increased more rapidly over time than that associated with peak I (Fig. 2) raises the possibility that there is a precursor/product relationship between the particles eluting in peak I and in peaks II and III. The experiment shown in Fig. 3 was conducted to determine whether apoA-I could create HDL-like particles from the peak I material. When J744 cells were incubated in the absence of apoA-I, PL and FC were released and eluted as large particles found exclusively in the void volume of the Superdex 200 column (Fig. 3A). Stimulation of the cells with cAMP increased the release of this material by ~60% over 6 h relative to unstimulated cells incubated in the absence of apoA-I (data not shown). Similarly, the presence of apoA-I (5 μg/ml for 6 h) enhanced the release of this void volume material by ~3-fold (data not shown), perhaps due to the stabilizing effect of apoA-I on ABCA1 (52). Fig. 3B shows that incubation of the void volume material with apoA-I at 37 °C overnight did not lead to formation of any HDL particles. This result indicates that the peak I material was not a precursor and that there was a parallel release from J774 cells of FC/PL particles eluting in peak I and in peaks II and III (Figs. 1 and 2).
Experimental Procedures.

J774 mouse macrophages were labeled with [3H]choline chloride and [14C]cholesterol and treated with CPT-cAMP as described under "Experimental Procedures." Data are shown as means ± S.D. from three to four independent experiments. The molecular mass and particle diameter of an equivalent globular protein were derived using the calibration equations described under "Experimental Procedures." Data are shown as means ± S.D. from three to four independent experiments.

The composition of peak fractions was calculated using the count/min of fractions and the specific activities of lipids and apoA-I (see "Experimental Procedures"). Values are means from two to three independent experiments. The FC/apoA-I and FC/PL stoichiometries were measured separately and varied by ~10% for peak II; the equivalent variability for peaks III and IV was ~40%.

Characteristics of the extracellular particles formed after incubation of J774 macrophages with apoA-I

| Column fraction | K_m (mM) | Molecular mass (kDa) | Diameter (nm) | Composition (PL/FC/apoA-I) | Fraction PL in peak |
|-----------------|----------|----------------------|---------------|-----------------------------|-------------------|
| I               | 0        | >1175                | >19           |                             | 0.26              |
| II              | 0.20 ± 0.03 | 324 ± 31  | 12 ± 0.4     | 6:7:1                       | 0.43              |
| III             | 0.36 ± 0.02 | 125 ± 15   | 9 ± 0.5      | 3:1:1                       | 0.18              |
| IV              | 0.49 ± 0.01 | 54 ± 2     | 6 ± 0         | 0.1-0.05:1                  | 0.13              |

"The cells were treated and the medium was fractionated as described in the legend to Fig. 1.

"K_m was calculated as described in under "Experimental Procedures." Data are shown as means ± S.D. from three to four independent experiments.

The molecular mass and particle diameter of an equivalent globular protein were derived using the calibration equations described under "Experimental Procedures." Data are shown as means ± S.D. from three to four independent experiments.

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“Fraction PL in peak” was calculated by integrating each peak using Prism (GraphPad) and expressing the area as a fraction of the total area under peaks I-IV. Values are the means that did not differ by >15% from two independent experiments.

Comparison of the distributions of choline-containing PL measured in the extracellular media of control and FC-enriched cells (Table III) indicates that FC efflux was enhanced relative to SM efflux upon FC loading of the cells; this effect was also observed with fibroblasts (15). As was observed with control cells, the PC/SM ratio for peak I was apparently lower than that for peaks II and III (Table III). However, the relatively poor resolution of the elution profile in Fig. 4 makes quantitation difficult for the FC-enriched cells.

Effects of the C-terminal α-Helix of ApoA-I—The comparison of PL and FC efflux to native apoA-I and apoA-I lacking the C-terminal 21 amino acids (apoA-I-(Δ223–243)) presented in Fig. 5 confirms that the C-terminal α-helix plays a critical role in apoA-I-mediated efflux via ABCA1 (20–24). Efflux to native apoA-I was higher than to the truncated apoA-I molecule, and the concentration dependence is characterized by a high affinity process. The hyperbolic dependence of FC and PL efflux on apoA-I concentration shown in Fig. 5 (A and B) can be fitted to the Michaelis-Menten equation, yielding a K_m value of 3–5 μg/ml. Under the conditions used, the V_max values for FC and PL efflux are 18 and 2.5%/4 h, respectively. In marked contrast, apoA-I-(Δ223–243) gave a low affinity effect with a linear dependence of both FC and PL efflux on protein concentration (Fig. 5). The reduction in efflux to the apoA-I variant was particularly marked at concentrations ≤10 μg/ml.

The effects of the C-terminal deletion of apoA-I on the particles present in the extracellular medium after a 6-h incubation with J774 cells are revealed in the elution profiles depicted in Fig. 6. Comparison of Figs. 1 and 6A shows that the two apoA-I-containing particles corresponding to peaks II and III in Fig. 1 were no longer formed when the last helix in the apoA-I molecule was deleted. Instead, larger particles were formed, so much of the released PL and FC was in the void volume (peak I), with no well defined particles eluting between 50 and 70 ml. There was a small peak (peak II) of apoA-I-(Δ223–243) (Fig. 6A), which had the same elution volume as peak III of native apoA-I (Fig. 1); but there was no corresponding peak of PL.

Effects of Cholesterol Enrichment of Cells—Loading of J774 cells with FC by incubation with acetyl-LDL before stimulation with cAMP increased the 6-h efflux of cellular FC from ~20 to ~30% (data not shown). The effect of FC loading on ABCA1 expression was small relative to the effect of cAMP stimulation (data not shown). Because the initial content of FC was typically increased by ~5-fold in loaded cells, the mass of FC released to the medium in 6 h was 7–8 times higher than that released from control cells. This effect is reflected in the FC/PL molar ratio of 4 observed for the extracellular medium from FC-enriched cells (Tables I and III). Comparison of Fig. 4 with Fig. 1 shows that the extracellular apoA-I/FC/PL particles created by incubating apoA-I with FC-enriched cells had lower elution volumes, indicating their increased size. Peak III in Fig. 4 eluted at the same position as peak II in Fig. 1, and a new peak II (15–16-nm diameter) is apparent in Fig. 4. Compared with Fig. 1, a greater proportion of the apoA-I in the Fig. 4 profile is associated with the larger lipid particles eluting at 45–60 ml.

Comparison of the distributions of choline-containing PL measured in the extracellular media of control and FC-enriched cells (Table III) indicates that FC efflux was enhanced relative to SM efflux upon FC loading of the cells; this effect was also observed with fibroblasts (15). As was observed with control cells, the PC/SM ratio for peak I was apparently lower than that for peaks II and III (Table III). However, the relatively poor resolution of the elution profile in Fig. 4 makes quantitation difficult for the FC-enriched cells.

Effects of the C-terminal α-Helix of ApoA-I—The comparison of PL and FC efflux to native apoA-I and apoA-I lacking the C-terminal 21 amino acids (apoA-I-(Δ223–243)) presented in Fig. 5 confirms that the C-terminal α-helix plays a critical role in apoA-I-mediated efflux via ABCA1 (20–24). Efflux to native apoA-I was higher than to the truncated apoA-I molecule, and the concentration dependence is characteristic of a high affinity process. The hyperbolic dependence of FC and PL efflux on apoA-I concentration shown in Fig. 5 (A and B) can be fitted to the Michaelis-Menten equation, yielding a K_m value of 3–5 μg/ml. Under the conditions used, the V_max values for FC and PL efflux are 18 and 2.5%/4 h, respectively. In marked contrast, apoA-I-(Δ223–243) gave a low affinity effect with a linear dependence of both FC and PL efflux on protein concentration (Fig. 5). The reduction in efflux to the apoA-I variant was particularly marked at concentrations ≤10 μg/ml.

The effects of the C-terminal deletion of apoA-I on the particles present in the extracellular medium after a 6-h incubation with J774 cells are revealed in the elution profiles depicted in Fig. 6. Comparison of Figs. 1 and 6A shows that the two apoA-I-containing particles corresponding to peaks II and III in Fig. 1 were no longer formed when the last helix in the apoA-I molecule was deleted. Instead, larger particles were formed, so much of the released PL and FC was in the void volume (peak I), with no well defined particles eluting between 50 and 70 ml. There was a small peak (peak II) of apoA-I-(Δ223–243) (Fig. 6A), which had the same elution volume as peak III of native apoA-I (Fig. 1); but there was no corresponding peak of PL.

Effects of Cholesterol Enrichment of Cells—Loading of J774 cells with FC by incubation with acetyl-LDL before stimulation with cAMP increased the 6-h efflux of cellular FC from ~20 to ~30% (data not shown). The effect of FC loading on ABCA1 expression was small relative to the effect of cAMP stimulation (data not shown). Because the initial content of FC was typically increased by ~5-fold in loaded cells, the mass of FC released to the medium in 6 h was 7–8 times higher than that released from control cells. This effect is reflected in the FC/PL molar ratio of 4 observed for the extracellular medium from FC-enriched cells (Tables I and III). Comparison of Fig. 4 with Fig. 1 shows that the extracellular apoA-I/FC/PL particles created by incubating apoA-I with FC-enriched cells had lower elution volumes, indicating their increased size. Peak III in Fig. 4 eluted at the same position as peak II in Fig. 1, and a new peak II (15–16-nm diameter) is apparent in Fig. 4. Compared with Fig. 1, a greater proportion of the apoA-I in the Fig. 4 profile is associated with the larger lipid particles eluting at 45–60 ml.

Comparison of the distributions of choline-containing PL measured in the extracellular media of control and FC-enriched cells (Table III) indicates that FC efflux was enhanced relative to SM efflux upon FC loading of the cells; this effect was also observed with fibroblasts (15). As was observed with control cells, the PC/SM ratio for peak I was apparently lower than that for peaks II and III (Table III). However, the relatively poor resolution of the elution profile in Fig. 4 makes quantitation difficult for the FC-enriched cells.

Effects of the C-terminal α-Helix of ApoA-I—The comparison of PL and FC efflux to native apoA-I and apoA-I lacking the C-terminal 21 amino acids (apoA-I-(Δ223–243)) presented in Fig. 5 confirms that the C-terminal α-helix plays a critical role in apoA-I-mediated efflux via ABCA1 (20–24). Efflux to native apoA-I was higher than to the truncated apoA-I molecule, and the concentration dependence is characteristic of a high affinity process. The hyperbolic dependence of FC and PL efflux on apoA-I concentration shown in Fig. 5 (A and B) can be fitted to the Michaelis-Menten equation, yielding a K_m value of 3–5 μg/ml. Under the conditions used, the V_max values for FC and PL efflux are 18 and 2.5%/4 h, respectively. In marked contrast, apoA-I-(Δ223–243) gave a low affinity effect with a linear dependence of both FC and PL efflux on protein concentration (Fig. 5). The reduction in efflux to the apoA-I variant was particularly marked at concentrations ≤10 μg/ml.
Measurements, where peaks I–IV and radioactivity was determined by liquid scintillation counting. The extracellular medium that contained radiolabeled FC and PL after a 6-h incubation with apoA-I was fractionated by gel filtration, and the elution peaks for control and FC-enriched cells are identified in Figs. 1 and 4, respectively.

The FC/PL ratios were very dependent on cell FC content; the values indicated are representative for cells containing FC in the range of 10–20 μg/mg of cell protein for control cells and 70–90 μg/mg of cell protein for FC-enriched cells (and treated with an acyl-CoA:cholesterol acyltransferase inhibitor).

Values are means ± SD from three independent experiments, with the exception of the last two rows, which are the average of two measurements, where peaks I–III are poorly resolved (see Fig. 4).

**DISCUSSION**

The mechanism of ABCA1-mediated efflux of cellular lipids is not understood fully, but a consensus is emerging that it involves a membrane microsolubilization process (10, 15, 21, 53, 54) whereby there is simultaneous release of PL and FC to apoA-I molecules present in the extracellular medium. The data in Tables I and III show that more PC than SM was released into the total medium, in agreement with prior reports (15, 25, 28). The FC/PL ratios are also consistent with earlier studies (27, 28), given that the FC content is dependent upon the initial degree of cholesterol loading of the cells. Although the extracellular concentration of apoA-I was only 5 μg/ml, we were able to monitor its lipidation without using either ultracentrifugation or ultrafiltration to concentrate the particles because the protein was 14C-labeled. It should be noted that apoA-I was the only apolipoprotein present in the extracellular medium because J774 cells do not synthesize apoE. Fig. 1 and Table II show that a heterogeneous collection of lipid particles was released upon incubation of J774 cells (in which ABCA1 is up-regulated) and apoA-I. There were three types of apoA-I-containing particles (peaks II–IV) with diameters in the range of 6–12 nm; because this is the HDL size range, these particles are designated as HDL hereafter. When the J774 cells were not preloaded with FC, peak I did not contain apoA-I (Fig. 1); these particles, which have diameters in the range of 20–450 nm, are referred to as membrane vesicles. The compositions and likely origins of the various HDL and membrane vesicle particles are considered in turn below.

**HDL Particles**—The 9- and 12-nm HDL particles (Table III) created by interaction of apoA-I with ABCA1-expressing J774 cells are similar in size to the particles observed in related experiments with Chinese hamster ovary cells (27) and THP-1 macrophages (28). The sizes are consistent with those of typical discoidal PL:apoA-I complexes containing 2–3 apoA-I molecules and 20–100 PL molecules/apoA-I molecule (55–57). Indeed, Forte et al. (27) visualized such discoidal complexes by electron microscopy in their experiments using Chinese hamster ovary cells. Because there was no lecithin:cholesterol acyltransferase activity present in the extracellular medium to convert FC to cholesteryl ester, the particles eluting in peaks II and III (Fig. 1) are also most likely to be discoidal. As mentioned under “Results,” peak IV represents excess unreacted apoA-I probably mixed with some minimally lipidated apoA-I molecules.

**FIG. 3.** Gel filtration elution profiles of medium collected after incubation of J774 macrophages in medium alone. J774 mouse macrophages were labeled with [3H]cholesterol and treated with CPT-cAMP as described under “Experimental Procedures.” RPMI 1640 medium only (no apoA-I) was added to the cells and incubated for 6 h. After the 6-h incubation, the medium was collected and filtered, and half of the medium (2.5 ml) was subjected to gel filtration chromatography directly (A). [3H]apoA-I (5 μg/ml) was added to the other half of the medium and incubated at 37°C overnight, and then the medium was subjected to gel filtration chromatography (B). Fractions were collected, and radioactivity was determined by liquid scintillation counting. ○, FC; ●, apoA-I. The void volume of the Superdex 200 column was 54 ml, and the total volume was 136 ml.

With FC-enriched cells, the particles created in the extracellular medium after incubation with apoA-I-(Δ223–243) had relatively high FC/PL molar ratios; in the case of peak I, this ratio was ~2.5:1 (Fig. 6B), which can be compared with the value of ~1.5 for the peak I material released by cells that were not loaded with cholesterol (Fig. 6A). Fig. 6B shows that some apoA-I-(Δ223–243) appeared as a peak in the void volume; this effect is most likely due to the formation of very large apoA-I-(Δ223–243)/FC/PL particles. Interestingly, this apoA-I variant led to formation of a discrete FC/PL particle eluting like HDL3 after incubation with FC-enriched cells (peak II in Fig. 6B); this peak was not apparent when apoA-I-(Δ223–243) was incubated with control cells (Fig. 6A).

**Table III. Compositional analysis of different lipid particles released from J774 cells to apoA-I**

| Cell condition | Medium fraction | FC/PL | % of choline-containing PL | Lipid (mol/mol) |
|---------------|----------------|-------|---------------------------|-----------------|
|               |                |       |                           | PC    | SM    | Lyso-PC |
| Control       | Total          | 1.5   | 55 ± 6                    | 36 ± 10 | 9 ± 4 |
|               | Peak I         | 2     | 42 ± 2                    | 41 ± 8  | 17 ± 9 |
|               | Peaks II + III | 0.5   | 58 ± 9                    | 30 ± 3  | 12 ± 6 |
| FC-enriched   | Total          | 4     | 61 ± 7                    | 23 ± 4  | 17 ± 10|
|               | Peak I         | 7     | 50                        | 40      | 10     |
|               | Peaks II + III | 3     | 60                        | 35      | 5      |

* Expression of ABCA1 was up-regulated by treatment with cAMP as described under “Experimental Procedures.”

* The 9- and 12-nm HDL particles were created by interaction of apoA-I with ABCA1-expressing J774 cells in medium alone. J774 mouse macrophages were labeled with [3H]cholesterol and treated with CPT-cAMP as described under “Experimental Procedures.” RPMI 1640 medium only (no apoA-I) was added to the cells and incubated for 6 h. After the 6-h incubation, the medium was collected and filtered, and half of the medium (2.5 ml) was subjected to gel filtration chromatography directly (A). [3H]apoA-I (5 μg/ml) was added to the other half of the medium and incubated at 37°C overnight, and then the medium was subjected to gel filtration chromatography (B). Fractions were collected, and radioactivity was determined by liquid scintillation counting. ○, FC; ●, apoA-I. The void volume of the Superdex 200 column was 54 ml, and the total volume was 136 ml.
Macrophages to apoA-I and apoA-I-(Δ223–243) were labeled with [3H]cholesterol or with [3H]choline chloride plus [14C]cholesterol in the presence of acetyl-LDL and treated with CPT-cAMP as described under “Experimental Procedures.” [13C]ApoA-I (5 μg/ml) or unlabeled apoA-I (20 μg/ml) was added to the [3H]cholesterol-labeled and [3H]choline chloride/[14C]cholesterol-labeled cells, respectively. The medium was collected after a 6-h incubation, filtered, and subjected to Superdex 200 gel filtration chromatography as described in the legend to Fig. 1. ■ PL, ○ FC; ●, apoA-I.

The larger diameter of the apoA-I molecule is a consequence of its flexible conformation (58). Rather than forming a tightly packed tertiary structure, it adopts a range of conformations and exists in a relatively expanded state. The looser intramolecular packing apparently allows incorporation of some lipid molecules without much change in hydrodynamic diameter.

It is important to note that the 9- and 12-nm HDL particles had different lipid compositions (Table II) and that they were created concurrently (Fig. 4). The fact that they had different FC/PL and FC/apoA-I ratios implies that they are formed from different domains of the cell plasma membrane (see below). The compositions of the peak II and III materials listed in Table II suggest that the degree of lipidation of apoA-I after a 6-h incubation with J774 cells is low. The PL contents are underestimated because non-choline PL were not included, and there was some decrease in the specific radioactivity of the cellular choline-containing PL during the experiment (see “Experimental Procedures”). A complete explanation for the surprisingly low PL and FC contents of these HDL particles must await a more detailed structural characterization. Among other things, this will necessitate the determination of the number of apoA-I molecules/particle and the presence of any cellular proteins.

The data in Figs. 4 and 6 indicate that the sizes of the HDL particles formed are dependent upon both the FC content of the plasma membrane and the apolipoprotein structure. Thus, increasing the FC content of the cells gives rise to larger particles; HDL particles with a diameter of 15–16 nm (peak II in Fig. 4) were created at the expense of the 9-nm particles seen with control cells (peak III in Fig. 1), and the HDL FC/PL ratio was increased to 3:1 (mol/mol) (Table II). A higher FC/PL ratio also leads to larger particles when discoidal apoA-I/PC HDL particles are reconstituted using the cholate solubilization and removal method (59). Removal of the C-terminal α-helix of apoA-I also has a parallel effect on the size of HDL particles formed by either ABCA1 action or reconstitution using detergent. Thus, discoidal HDL particles reconstituted with apoA-I-(Δ223–243) are larger than those formed by wild-type apoA-I under the same conditions, and the nascent HDL created by ABCA1 activity in J774 cells are also bigger than those formed by plasma apoA-I (unlike plasma apoA-I (Figs. 1 and 4), apoA-I-(Δ223–243) eluted in the void volume (Fig. 6)). This similarity between ABCA1-mediated and non-ABCA1-mediated formation of HDL particles is presumably due to a common effect in both situations, most probably involving apolipoprotein/lipid interaction. It seems that reduced apoA-I/lipid binding affinity

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(a) S. T. Thuahnai and M. C. Phillips, unpublished data.
is associated with the formation of larger HDL particles. Thus, increasing the FC content decreases the affinity of apoA-I binding to PL particles (60), and removal of the C-terminal /H9251 -helix of apoA-I does the same thing (61). This finding of a link between apolipoprotein/lipid affinity and nascent HDL particle size implies that apoA-I/ABCA1 interaction (54) does not have a direct effect on the size of the HDL particles formed.

**Membrane Vesicles**—The membrane vesicle fraction that eluted in the void volume (Fig. 1) did not contain any apoA-I, but comprised about one-quarter of the PL released from the cells (Table II). The peak I material was relatively enriched in FC and SM relative to the HDL fractions (peaks II and III) (Table III). This variation in lipid composition is consistent with the origins of the membrane vesicle and HDL particles being in different plasma membrane domains. Because lipid raft subdomains of the plasma membrane have average diameters in the 100–200-nm range and contain high concentrations of FC and SM (62), both the size and composition of the peak I material support the classification as membrane vesicles originating from lipid rafts. Membrane shedding of lipid raft components is known to occur (50, 51), and it will be important to identify some of the protein constituents of the released particles. It is possible that ABCA1 is involved in this process because lipid rafts contain the transporter (63), and it promotes secretory vesicle trafficking (64). Release of the peak I membrane vesicles does not require apoA-I to be present in the extracellular medium, and these vesicles are apparently not a source of lipid for formation of HDL particles (Fig. 3).

As mentioned above, a plausible explanation for the concurrent formation of 9- and 12-nm HDL particles with different compositions (Table II) is that they originate from distinct microdomains of the plasma membrane. ABCA1 is located primarily in non-lipid raft domains (63, 65), so the lipids transported to apoA-I from this relatively fluid environment may be expected not to be highly enriched in SM and FC. This trend is apparent from a comparison of the compositions of HDL peaks II and III relative to the peak I membrane vesicles (Table III). However, lipid rafts can be heterogeneous and contain some ABCA1 that is not uniformly distributed, so apoA-I preferentially depletes FC and choline-containing PL from these subdomains of the lipid rafts (63). It is also possible that the lipids acquired by apoA-I come from intracellular sites because ABCA1-containing vesicles rapidly recycle between the plasma membrane and late endosomes (66), and it has been suggested that cholesterol in the latter compartment is a preferential source for ABCA1-mediated efflux (67). Clearly, more work is required to establish the exact origins of the cellular lipids that are solubilized to create the 9- and 12-nm HDL particles described in Table II.

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