Mechanism of Prebeta-HDL Formation and Activation†

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ABSTRACT: The mechanism of formation of functional high-density lipoprotein (HDL) from secreted lipid-free apolipoprotein A1 (apo A1) was determined using human liver-derived (HepG2) cells, human intestine-derived (CaCO2) cells, and CHO cells stably expressing full-length human apo A1 (CHO-A1 cells). In each cell line, a significant proportion of secreted apo A1 had a Stokes radius of 2.6 nm and was inactive in binding phospholipids (PL) or free cholesterol (FC). Extracellularly, in a reaction dependent on membrane transporter ABCA1, prealpha-migrating 2.6 nm apo A1 was converted to a prebeta-migrating product that was able to bind PL. Both forms were reactive with mAb55201, a monoclonal antibody specific for native plasma lipid-poor (prebeta$_1$) HDL [Nakamura, Y., et al. (2004) *Biochemistry 43,* 14311−14318]. The physical properties of precursor and product apo A1 suggested that both are monomers, with Stokes radii of 2.6 and 3.6 nm, respectively, consistent with the absence of intermolecular cross-linking of apo A1 in lipid-poor HDL, reported previously. Product but not precursor apo A1 promoted reverse cholesterol transport (RCT) from human aortic smooth muscle cells. These studies suggest an important contribution of secreted lipid-free apo A1 to HDL formation.

Human apolipoprotein A1 (apo A1),[1] the major protein of serum high-density lipoprotein (HDL), is synthesized, mainly in hepatic and intestinal cells, as a 267-amino acid (aa) pre-proprotein (*1*). The 18 aa leader sequence is cleaved during transit through the Golgi secretion pathway and a 249 aa proprotein secreted. After cleavage of its N-terminal hexapeptide by an extracellular metalloproteinase (*2*), the mature 243 aa apo A1 polypeptide (molecular mass of 28.1 kDa) recycles as part of HDL between the plasma compartment and the extracellular space (*3*). Mature apo A1 isolated from delipidated serum HDL has been used for almost all biophysical and biochemical studies of the protein. It self-associates readily in aqueous media in the absence of lipid (*4*, *5*). In the presence of PL vesicles, lipid-free serum apo A1 forms large, phospholipid (PL)-rich discoidal HDL particles in which a belt of two to three apo A1 molecules surrounds a lipid core (*6*).

In contrast to this, several studies have shown that a small lipid-poor HDL particle in plasma is a key early biological acceptor of peripheral cell lipids in the reaction sequence that promotes cell-to-medium ("reverse") cholesterol transport (RCT) (*7*−*9*). This particle has prebeta electrophoretic migration, compared to the alpha migration of most other HDL particles. Apo A1, the only protein in prebeta-HDL (*3*), is present in a unique conformation recognized by monoclonal antibodies that are unreactive with other HDLs (*10*, *11*). We recently showed that free cholesterol (FC) in prebeta$_1$-HDL was the preferred substrate for downstream plasma FC metabolism, specifically esterification by lecithin:cholesterol acyltransferase (LCAT), and transfer of LCAT-derived cholesteryl ester (CE) to low-density lipoprotein via the cholesteryl ester transfer protein (CETP) reaction. This selectivity increased the efficiency of FC transport ∼8-fold (*12*). In native plasma, prebeta$_1$-HDL is regenerated from mature, alpha-migrating HDL particles via the action of PL transfer protein (*12*−*14*).

Much less is known of the steps by which new HDL is formed. Both PL-poor and PL-rich HDL fractions have been purified from the culture medium of hepatocytes (*15*−*17*), intestinal cells (*18*), and CHO cells stably expressing apo A1 (*19*). In human Tangier disease, the ATP-binding cassette A1 (ABCA1) transmembrane lipid transporter, responsible for the transfer of PL from cell membranes to circulating HDL, is genetically defective. Little or no HDL is then present in the circulation (*20*−*22*), and Tangier cells in vitro secrete an unusual form of prebeta-HDL in which phosphatidylcholine (PC) is largely replaced by lyso-PC (*23*). PL is also associated with intracellular fractions of apo A1. In ABCA1 −/− cells, the level of lipidation of both intra- and extracellular fractions is reduced. These data indicate that ABCA1 plays a complex role in the lipidation of apo A1 but has left open the identity of the major primary secretion products and the steps involved in forming HDL that is able to promote RCT. Our studies here report that a lipid-free monomeric apo A1 particle is a significant initial secretion

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[1] Abbreviations: ABCA1, ATP-binding cassette protein A1; apo A1, apolipoprotein A1; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CHO, Chinese hamster ovary; FC, free cholesterol; HDL, high-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; PC, phosphatidylcholine; PL, phospholipids; RCT, reverse cholesterol transport; SMC, smooth muscle cell; SPH, sphingomyelin.

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product of HepG2, CaCO2, and CHO-A1 cells and is converted extracellularly to a biologically active prebeta-HDL.

EXPERIMENTAL PROCEDURES

Cell Culture. Human liver-derived HepG2 cells, a gift from B. Knowles, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human colon-derived (CaCO2) cells were obtained from ATCC. They were cultured in Eagle's MEM/Earles BSS containing 20% FBS. When confluent in 3.5 cm wells, CaCO2 and HepG2 cells were transferred to 0.3 mL of serum-free medium containing high-molecular weight dextran, 1 mg/mL (T500, Pharmacia). CHO cells expressing mature human apo A1 polypeptide under control of the metallothein gene promoter (CHO-A1 cells) were a gift from A. Protter. These cells were grown to confluency in 3.5 cm plastic wells in a 50% DMEM/50% F-12 medium supplemented with 10% (v/v) FBS. Apo A-1 expression was induced by the addition for 24 h of 30 μM $ZnSO_4$ and 30 μM $FeSO_4$. Wild-type CHO cells lacking the human apo A1 insert (CHO-WT cells) were cultured in DMEM/F-12 medium without Fe^{2+} and Zn^{2+}. For individual experiments, CHO-A1 cells were washed and then transferred to 0.3 mL of serum-free medium containing Zn^{2+}, Fe^{2+}, and dextran. At intervals, samples of medium were taken for immediate assay. Apo A1 levels were determined by a solid phase immunoassay using a goat polyclonal antibody against human apo A1 (Rockland Immunochemicals, Gilbertsville, PA) (*12*). Human primary aortic smooth muscle cells (SMC) were cultured as previously described (*24*). They also were transferred to serum-free medium containing high-molecular weight dextran for 24 h before being used in individual experiments.

Inhibitors of vesicular transport (brefeldin A1 and ilimaquinone), ABC transporter activity (glyburide), and fumonisin B1 and pravastatin, which block the synthesis of sphingolipids and FC, respectively, were from CalBiochem (San Diego, CA). Diethanolamine, an inhibitor of phosphatidylcholine synthesis and transport, was from Sigma (St. Louis, MO).

Isotopic tracers of FC ([1,2-^{3}H]FC) or of PL synthesis ([^{3}H]choline) (both at 60−65 Ci/mmol) were from Pharmacia-NEN (Boston, MA). Each label was equilibrated into cultured cells (10−20 μCi per dish) via incubation for 24 h (37 °C). Cell FC mass was determined with cholesterol oxidase; PL mass was determined with phospholipase A2 and choline oxidase (*12*). The amount of ^{3}H label was determined by liquid scintillation spectrometry. The specific activities determined were used to calculate the lipid content of secreted apo A1 species.

Preparation of Lipid-Free Serum Apo A1. HDL was isolated from human serum by sequential ultracentrifugation between density limits of 1.063 and 1.21 g/mL. Following delipidation with ethanol and diethyl ether, apo A1 was purified (>98%) by molecular sieve chromatography (*25*).

Physical Properties of Secreted Apo A1. Estimates of the Stokes radius (SR) of secreted apo A1 were made relative to the migration rates of globular protein standards (*26*) using nondenaturing PAGE and precast 10−20% (w/v) polyacrylamide gels (Bio-Rad, Hercules, CA). Samples of CHO-A1 cell medium at 4 °C were brought to 15.5% (w/v) sucrose and 0.03% Na_2-EDTA at pH 8.25 (*12*). Electrophoresis was carried out for 20 h (40 V and 4 °C). Following electrotransfer to nitrocellulose membranes (0.2 μm), apo A1 was identified by Western blotting, using either goat polyclonal antibody to human apo A1 or mouse monoclonal antibody mAb55201 specific for human prebeta$_1$-HDL (gift from Daiichi Pure Chemicals, Tokyo, Japan).

Estimates of the sedimentation coefficient ($s_{20,w}$) of secreted apo A1 were made with reference to globular protein standards (catalase, lactate dehydrogenase, serum albumin, ovalbumin, and lysozyme) (*27*). Density gradient ultracentrifugation was carried out using 5 to 20% linear sucrose gradients in the SW-55 rotor of a Beckman L-7 ultracentrifuge. Fractions (0.1 mL) collected after the run (48 K for 22 h) were assayed for apo A1 or protein. The molecular weight was determined from the SR and $s_{20,w}$ (*28*).

Fractionation of secreted apo A1 on the basis of net charge was carried out in 0.8% (w/v) agarose gels developed in 25 mM Tricine, 63 mM Tris, and 10 mM Ca^{2+}-lactate (pH 7.4). Electrotransfer and immunoblotting with apo A1 antibody were carried out as described above for nondenaturing PAGE.

Assay of HDL Lipids. Using cells prelabeled (24−48 h) with [^{3}H]choline or FC (10 μCi per 3.5 cm well in each case), lipid bound to secreted apo A1 was assayed by liquid scintillation spectrometry following nondenaturing PAGE. Recovery of ^{3}H was assayed by comparing the amount of label in individual species before and after blotting to nitrocellulose. Additional analysis of [^{3}H]choline-labeled lipids was carried out after extraction with chloroform and methanol (1/1, v/v). Thin-layer chromatography of PL was carried out using Whatman LK-6 silica gel layers developed in a chloroform/methanol/2-propanol/0.25% (w/v) aqueous KCl/triethylamine mixture (30/9/25/6/18, v/v) (*29*). Separation of FC and CE was carried out on Silica G plates developed in a petroleum ether/diethyl ether/acetic acid mixture (80/20/1, v/v) (*12*).

ABCA1 Knockdown. There is strong sequence conservation among mammalian ABCA1 mRNAs. We first determined whether rodent (RAW cell) ABCA1 siRNA inhibited the expression of ABCA1 in human-derived and CHO cells. Total RNA was transcribed into cDNA with Superscript III RNase H. PCR was carried out at 94 °C for 1 min, and then 35 cycles at 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 40 s, and finally an extension step for 6 min at 72 °C, using the sense primer 5′-taa tac gac tca cta tag gga gag aat ggg caa ttc gca aac t-3′ and the antisense primer 5′-taa tac gac tca cta tag gga gat tcc cgg aaa cgc aag tc-3′ (100 nM each) (*30*). A Silencer siRNA kit (Ambion, Austin, TX) was used to produce dsRNA and then siRNA for the transfections. CHO-WT cells were plated in 3.5 cm wells in medium containing FBS for 8 h. They were then transfected with 200 pmol of siRNA and 8 μL of siPORT Amine (Ambion) according to the manufacturer's instructions. Control transfections contained negative control siRNA (Ambion). After 18 h at 37 °C, fresh complete medium containing [^{3}H]choline was added (20 μCi per well), and the incubation continued for 20 h. The transfected labeled cells were washed and incubated with 16 h medium from unlabeled CHO-A1 cells for 8 h. After nondenaturing PAGE, the distribution of apo A1 and [^{3}H]-PL was determined as described above. Comparable experi-

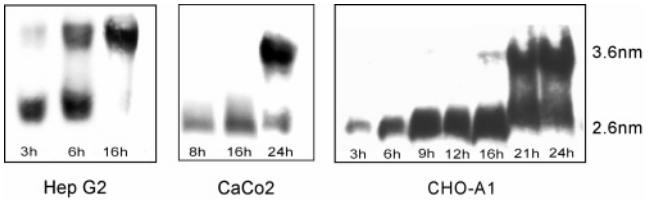

FIGURE 1: Nondenaturing PAGE of apo A1 secreted from HepG2 cells, CaCO2 cells, and CHO-A1 cells. Medium samples were collected at the intervals shown from washed cells transferred to serum-free medium. Following electrotransfer, apo A1 species were identified with polyclonal antibody. The Stokes radius (nanometers) was determined relative to protein standards, as described in Experimental Procedures.

ments were carried out in which HepG2 cells were transfected with ABCA1 siRNA or scrambled RNA (*30*) except that 12 μL of siPORT Amine was used. ABCA1 silencing was expressed relative to ABCA1 protein levels with immunopurified rabbit polyclonal antibody (Genetex, San Antonio, TX) and in terms of the reduction of the rate of [^{3}H]PL efflux from [^{3}H]choline-labeled cells to apo A1. A degree of inhibition of >70% was achieved in each case.

RESULTS

Nondenaturing PAGE of Apo A1 from HepG2, CaCO2, and CHO-A1 Cells. Cells were washed and transferred to serum-free medium. Samples were taken as a function of time and then immediately fractionated by nondenaturing PAGE. After electrotransfer and immunoblotting with apo A1 polyclonal antibody, two fractions were identified in HepG2 cell medium (Figure 1A). By comparison with the migration of globular protein standards with known SRs (*26*), these bands were assigned values of 2.6 and 3.6 nm. In contrast, in CaCO2 and CHO-A1 cell medium, the 2.6 nm apo A1 species was the major or only species present at early time points. With a more extended incubation, 3.6 nm apo A1 became predominant (Figure 1). Under these conditions, little or no higher-MW material was found. The PL content of both species was determined by repeating these experiments in cultures including [^{3}H]choline. In each case, 3.6 nm apo A1 exhibited significant lipid binding. In contrast, no [^{3}H]PL was recovered with the 2.6 nm species of HDL from HepG2, CaCO2, or CHO-A1 cells.

Secretion of Apo A1. A more detailed characterization of secreted lipid-free 2.6 nm apo A1 was next made using CHO-A1 cells, under conditions (16 h) in which little if any of the larger (3.6 nm) form was present. The classical protein secretory pathway requires an intact Golgi apparatus, where a hydrophobic leader sequence is cleaved prior to the transfer of newly synthesized protein to the cell surface (*31*). Inhibitors of Golgi vesicle assembly block protein secretion by this pathway. In contrast, the secretion of "leaderless" proteins is Golgi-independent but blocked by inhibitors of ABC transporter proteins, including glyburide (*32*). Brefeldin A1 and ilimaquinone, early and late inhibitors, respectively, of Golgi-dependent transport (*33*, *34*), strongly inhibited (>95%) the accumulation of apo A1 in CHO-A1 culture medium (Figure 2). Unexpectedly, glyburide (100 nM) also blocked secretion of apo A1 from CHO-A1 cells, suggesting roles for both Golgi-dependent and -independent processes in apo A1 secretion. Other inhibitors of cellular lipid synthesis and transport (*35*−*37*) were without significant

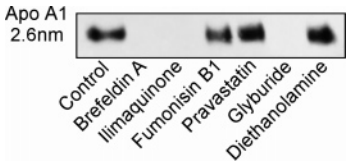

FIGURE 2: Effects of inhibitors of Golgi-mediated secretion and cellular lipid metabolism and transport on secretion of apo A1 (8 h) from CHO-A1 cells: brefeldin A1 (10 μg/mL), ilimaquinone (25 μM), glyburide (100 nM), fumonisin B1 (25 μM), pravastatin (500 μg/mL), and diethanolamine (500 μg/mL). Data shown are from medium characterized by nondenaturing PAGE, followed by electrotransfer and immunoblotting with polyclonal apo A1 antibody. Only 2.6 nm apo A1 was detected under these conditions.

Table 1: Characteristics of Apo A1 Secreted by CHO-A1 Cells[a]

	S (nm)	$s_{20,w}$ ($\times 10^{-13}$)	MW[b] $\times 10^3$
CHO-A1, 16 h	2.6 ± 0.1	2.4 ± 0.1	27.1 ± 2.0
CHO-A1, 24 h	3.6 ± 0.2	2.2 ± 0.1	34.4 ± 1.9

[a] Values shown are means ± the standard deviation of three independent determinations. [b] The molecular weight (MW) was estimated using a partial specific volume for apo A1 of 0.75 mL/g.

effect. Neither dithiothreitol (1 mM) nor 1,10-phenanthroline (0.2 mM), inhibitors of the plasma conversion of pro-apo A1 to its mature species (*2*), affected apo A1 secretion (data not shown). No apo A1 was detected in the medium of CHO-WT cells.

Properties of Apo A1 Particles. The cells were induced with Zn^{2+} and Fe^{2+} to activate the metallothein promoter driving apo A1 transcription, and the medium was collected after 16 h. The concentration of apo A1 in the culture medium (12 h) was 3 ± 0.5 μg/mL, comparable to that previously reported (*19*). The $s_{20,w}$ of apo A1 was 2.4 ± 0.1, and the SR was 2.6 ± 0.1 nm (Table 1). Its calculated molecular mass was 27.1 ± 2.3 kDa, not significantly different from that predicted from the primary sequence (28.1 kDa). The molecular properties of this particle were unchanged if the medium was stored (3 days) at 4 °C. The 2.6 nm initial secretion product of CHO-A1 cells was therefore an apo A1 monomer.

Upon further incubation (24 h), apo A1 with a Stokes radius of 3.6 nm (compared to 3.5 nm for albumin) (*26*) became the major form present. Its $s_{20,w}$ was 2.2 ± 0.1, similar to that of the 2.6 nm form of apo A1 (Table 1). The calculated molecular mass of the 3.6 nm particle containing apo A1 (34.4 ± 1.9 kDa) was slightly (>20%) greater than that of the 2.6 nm form.

To determine the relationship between the 2.6 and 3.6 nm forms of apo A1, medium from CHO-A1 cells containing the 2.6 nm species was transferred to washed CHO-WT cells. While CHO-WT cells did not secrete apo A1, they did promote conversion of the 2.6 nm particles to 3.6 nm particles (Figure 3). No effect was seen if CHO-A1 medium was incubated in empty culture dishes. These data indicate that a cell-surface factor present on both CHO-A1 and CHO-WT cells promoted the conversion of 2.6 nm apo A1 particles to 3.6 nm apo A1 particles. Also, the molecular parameters of 3.6 nm apo A1 (Table 1) suggest that this represents not a dimer but possibly a rod-shaped or ellipsoidal monomer whose dimensions are responsible for its anomalous migration rate relative to that of albumin during nondenaturing PAGE, a recognized property of rod-shaped proteins (*28*, *38*, *39*).

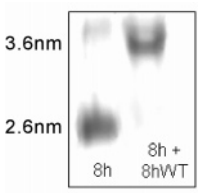

FIGURE 3: Conversion of 2.6 nm apo A1 particles to 3.6 nm apo A1 particles. Medium (8 h) was removed from CHO-A1 cells and transferred to CHO-WT cells for 8 h. The migration rate of apo A1 was determined as described in the legend of Figure 1. A representative experiment (of three) is shown.

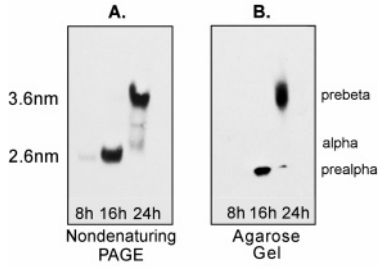

FIGURE 4: Electrophoretic migration and immunoreactivity of 2.6 and 3.6 nm forms of secreted apo A1. (A) Medium from CHO-A1 cells 8, 16, and 24 h after induction, fractionated by nondenaturing PAGE. Following electrotransfer, membranes were reacted with mAb55201. (B) Same medium fractionated by agarose gel electrophoresis, followed by electrotransfer and immunoblotting as described for panel A. PAGE migration rates are relative to those of globular protein standards. Agarose gel migration rates are shown relative to those of the prebeta- and alpha-migrating lipoprotein fractions of native plasma.

Both 3.6 and 2.6 nm forms of apo A1 were reactive in blots of nondenaturing PAGE gels with a monoclonal antibody (mAb55201) specific for lipid-poor HDL (Figure 4A). When the same samples were fractionated by agarose gel electrophoresis, the 3.6 nm fraction had prebeta migration (Figure 4B). In contrast, the 2.6 nm apo A1 migrated much more rapidly, ahead of HDL and almost coincident with albumin. These data confirm the existence of major differences in the physical structure of the precursor (2.6 nm) and product (3.6 nm) forms of secreted apo A1.

Binding of PL and FC to Secreted Apo A1. To investigate if apo A1 secretion was dependent on prior lipidation by PL, CHO-A1 cells were labeled with [^{3}H]choline. Secretion of apo A1 from CHO-A1 cells was induced, and medium was collected for 8−24 h. No significant ^{3}H label copurified with the 2.6 nm apo A1 secretory product that first appeared. In contrast, substantial levels of [^{3}H]PL comigrated with the larger (3.6 nm) species (Figure 5A) generated from its precursor, the 2.6 nm form (Figure 5B). The major part (92 ± 4%) of the [^{3}H]choline label recovered was in PC, consistent with the specificity of PL transporter ABCA1 (*40*). Less was in SPH (6 ± 2%) and little if any in LPC (2 ± 2%). PC label associated with 3.6 nm apo A1 reached a maximum of 2.0 ± 0.1 mol of PL/mol of apo A1 as conversion from the precursor to product form became complete (Figure 5C). This result suggested that the 3.6 nm apo A1 had a limited binding capacity for PL, similar to that described for plasma prebeta-HDL (*41*).

The role of ABC transporter activity in the transfer of PL to secreted apo A1 could not be determined directly using CHO-A1 cells, since apo A1 secretion was inhibited in the presence of glyburide (Figure 2). A possible role for ABCA1 in the conversion to 3.6 nm product and PL binding was therefore studied in medium (16 h) from CHO-A1 cells that was then incubated in the presence of CHO-WT cells labeled with [^{3}H]choline. In some cases, the CHO-WT cells had first been transfected with ABCA1 siRNA, or with a negative control siRNA. In other experiments, CHO-WT cells were incubated in the presence of glyburide (100 nM) with medium from labeled CHO-A1 cells. The rate of conversion of 2.6 nm apo A1 to 3.6 nm apo A1 was determined as a function of time, together with the level of associated [^{3}H]-PC.

Transfection with ABCA1 siRNA reduced ABCA1 levels by 70% and the level of binding of [^{3}H]PL to apo A1 to a similar extent (79 ± 5%). The level of conversion of 2.6 nm apo A1 to 3.6 nm apo A1 (84 ± 5%) was also substantially reduced (Figure 6A). These data show that ABCA1 was downregulated by rodent ABCA1 siRNA and confirm the role of this transporter in the transfer of PL to secreted apo A1. In contrast, glyburide (100 nM), which also inhibited the transfer of PL to 3.6 nm apo A1 to an extent similar to that by ABCA1 siRNA, maintained or even increased the rate of formation of 3.6 nm apo A1 from 2.6 nm apo A1 (Figure 6B). These data showed that ABCA1 plays multiple roles in HDL formation de novo, but its effect on the conversion of 2.6 apo A1 to 3.6 nm apo A1 must be independent of ABCA1-dependent PL transfer. ABCA1 may promote unfolding of the 2.6 nm apo A1 species to a 3.6 nm product, though further research will be needed to establish the pathway that is involved.

In parallel to our findings with PL, no [^{3}H]FC was associated with the initial (2.6 nm) fraction of secreted apo A1. In contrast, the 3.6 nm fraction formed a stable labeled

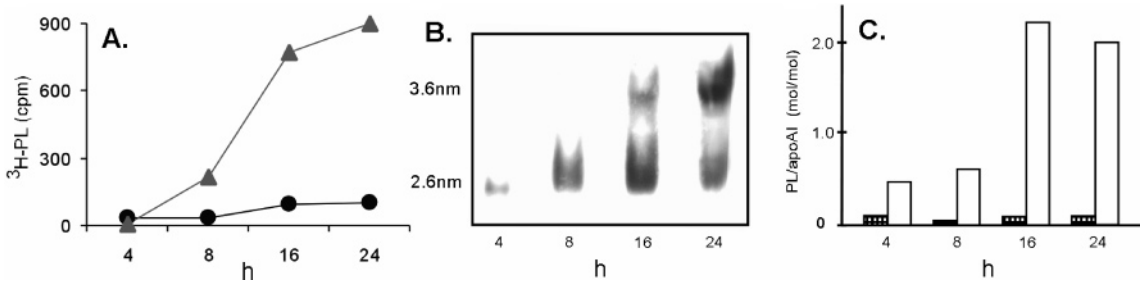

FIGURE 5: Incorporation of [^{3}H]PL into HDL as a function of incubation time. Medium from CHO-A1 cells was fractionated by nondenaturing PAGE. After electrotransfer, immunoblotting was carried out as described in the legend of Figure 1. (A) Label associated with each apo A1 species, corrected for recovery (85 ± 4%): (●) 2.6 nm apo A1 and (▲) 3.6 nm apo A1. (B) Levels of 2.6 and 3.6 nm apo A1 during the same time course. (C) PC/apo A1 molar ratio in apo A1 in the same experiments. Molecular weights of 750 (for PC) and 28.4 × 10^3 (for apo A1) were used in these calculations: hatched bars, 2.6 nm apo A1; and white bars, 3.6 nm apo A1.

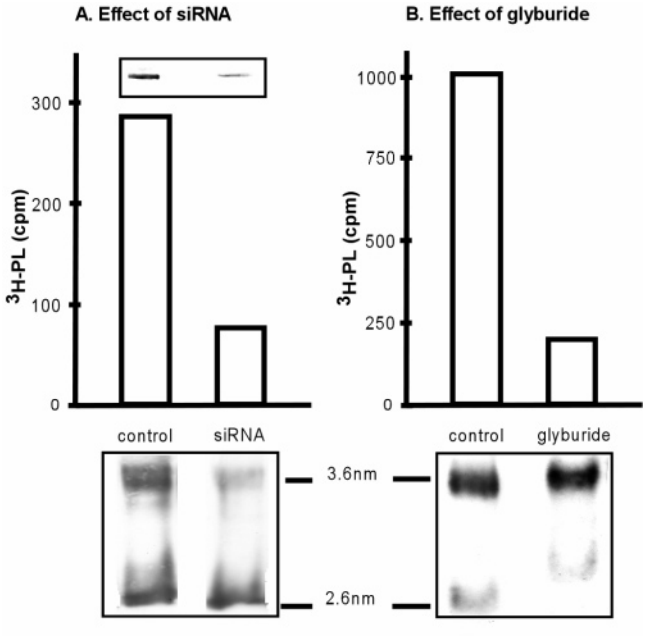

FIGURE 6: Conversion of 2.6 nm apo A1 to 3.6 nm apo A1 and PL binding in medium from unlabeled CHO-A1 cells transferred to [^{3}H]choline-labeled CHO-WT cells. (A) [^{3}H]PL content of 3.6 nm apo A1 in the medium of CHO-WT control cells and cells transfected with ABCA1 siRNA fractionated by nondenaturing PAGE (top). The inset shows cell ABCA1 protein levels determined with polyclonal antibody. Levels of 2.6 and 3.6 nm apo A1 in the same experiment (bottom). (B) [^{3}H]PL content of 3.6 nm apo A1 in the presence and absence of glyburide (100 nmol) (top) and levels of 2.6 and 3.6 nm apo A1 under the same conditions (bottom).

complex in the medium of CHO-A1 cells. Under conditions where the level of PL binding had reached its maximum, the FC content of 3.6 nm apo A1 was 0.9 ± 0.2 mol/mol. No CE was recovered with apo A1 in any experiment. These data indicate that the capacity of 3.6 nm apo A1 for FC, like that for PL, is limited.

Secreted Apo A1 and Lipid Transport. The 2.6 nm form of secreted apo A1, which was unable to bind PL or FC from CHO-A1 or CHO-WT cells, was also inactive in promoting the transfer of FC from SMC (Figure 7A). In contrast, 3.6 nm apo A1 stimulated the efflux of both FC and PL from [^{3}H]FC or [^{3}H]choline-labeled SMC (Figure 7B). However, it stimulated FC efflux 28-fold more effectively (moles per mole) than that of PL. The reverse was the case for purified, serum-derived apo A1 under the same conditions, when the molar ratio of lipid efflux was only 0.22 (FC/PL), consistent with the ratio of 0.25 previously reported (*42*).

Addition of 3.6 nm apo A1 stimulated RCT from SMC even when it was added to native plasma (Figure 7C). These data indicate that the 3.6 nm form of secreted apo A1 had biological properties resembling those of authentic prebeta$_1$-HDL. They also showed that the concentration of lipid-poor HDL was rate-limiting for FC efflux in native plasma under these conditions.

DISCUSSION

In this study, we sought to identify if apo A1, secreted lipid-free, could generate lipid-poor HDL that had biological properties similar to those of circulating prebeta$_1$-HDL, an early acceptor of cell-derived FC and intermediary in RCT.

Precursor (2.6 nm) Apo A1. The initial apo A1 product of CHO-A1, HepG2, and CaCO2 cells had a Stokes radius of 2.6 nm. Though it is lipid-free and reactive with both polyclonal apo A1 and mAb55201 antibodies, its properties differed in major respects from those of lipid-free apo A1 purified from serum HDL. It was not a substrate for ABC transporter-derived PL, nor did it bind FC from CHO cells or SMC. Unlike serum-derived apo A1, the 2.6 nm secreted apo A1 species did not self-associate. In contrast to the prebeta electrophoretic migration of lipid-free serum-derived apo A1, the newly secreted species was prealpha-migrating. Because apo A1 with properties similar to those of apo A1 from CHO-A1 cells was also the initial product of CaCO2 cells and was a significant component of apo A1 secreted by HepG2 cells, we suggest that the 2.6 nm species represents a significant contributor to newly secreted apo A1 in vivo.

Product (3.6 nm) Apo A1. The 2.6 nm apo A1 was the precursor of a distinct, 3.6 nm particle. This was shown not only by the increased proportion of the latter as a function of time in the medium of CHO-A1 cells but also by the

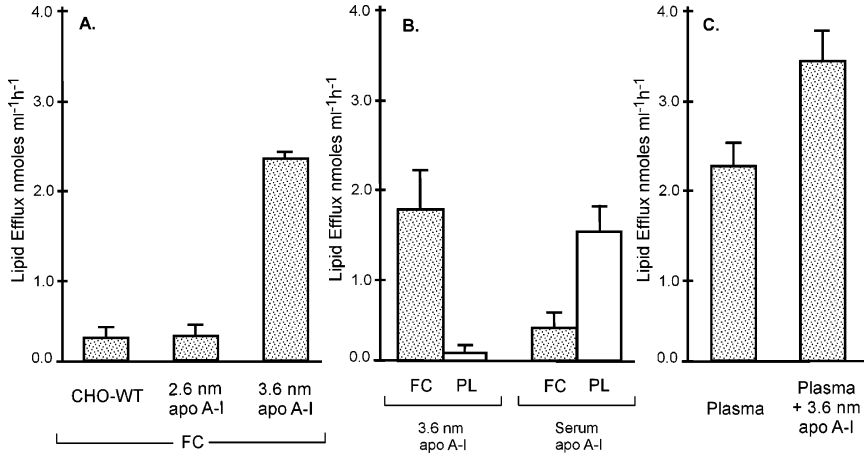

FIGURE 7: Efflux of FC and PL from labeled SMC to newly secreted apo A1, serum-derived lipid-free apo A1, and native plasma. (A) Rates of efflux of FC to CHO-WT medium, to CHO-A1 medium containing 2.6 nm secreted apo A1, and to converted CHO-A1 medium containing 3.6 nm apo A1 at the same concentration. (B) Molar rates of efflux of FC and PL from labeled SMC to the 3.6 nm apo A1 (left bars) or serum-derived lipid-free apo A1 under the same conditions (right bars). (C) Efflux of FC to native plasma (1/1, v/v, with CHO-WT culture medium) and plasma with 3.6 nm secreted apo A1 in CHO culture medium (1/1, v/v). Data shown are means ± the standard deviation ($n = 3$).

conversion of 2.6 nm apo A1 to 3.6 nm apo A1 in the presence of CHO-WT cells. The change was dependent on ABCA1, as seen by the effect of ABCA1 siRNA, which inhibited conversion of 2.6 nm apo A1 to 3.6 nm apo A1 in a manner proportional to PL efflux. However, it was independent of the PL efflux activity of ABCA1; glyburide strongly inhibited PL binding, but formation of 3.6 nm apo A1 from the 2.6 nm species was unmodified. These data indicate that the change in physical properties of secreted apo A1 was not dependent on PL lipidation, though it was ABC transporter-dependent. ABCA1-mediated redistribution of phosphatidylserine across the plasma membrane, unlike efflux of PL to lipid-free apo A1, was not inhibited by glyburide (*43*). It is possible that the conversion of 2.6 nm apo A1 to 3.6 nm apo A1 is driven by local differences in cell-surface PL composition. Our experiments suggest that ABCA1 may play at least three separate roles in HDL formation: in Golgi-mediated transport, in the conversion of 2.6 nm lipid-free apo A1 to 3.6 nm lipid-free apo A1, and in the lipidation of 3.6 nm particles.

Like both serum-derived apo A1 and plasma prebeta$_1$-HDL, the 3.6 nm form of secreted apo A1 had prebeta electrophoretic migration and was reactive with mAb55201. It bound PL via an ABC transporter-dependent pathway. It resembled prebeta$_1$-HDL but differed from serum-derived apo A1 in its limited capacity for PL and its greater activity in promoting RCT from SMC (*42*). These data indicate that 3.6 nm HDL, produced by the reaction of ABCA1 with 2.6 nm particles, resembled plasma prebeta$_1$-HDL and differed from both serum-derived apo A1 and the 2.6 nm lipid-free form. The increased Stokes radius of 3.6 nm compared to that of 2.6 nm apo A1 most likely reflects conversion of the particle from a spheroidal to a rodlike or tubular form, though more detailed physical analysis will be needed to establish this.

Physiological Significance. Addition of 3.6 nm apo A1 particles to native plasma stimulated RCT, indicating that under these conditions, the concentration of prebeta-migrating HDL was rate-limiting for FC transport. This is consistent with the correlation between levels of prebeta$_1$-HDL and RCT in native plasma previously noted (*44*). Intermolecular cross-linking was not detected in native prebeta-HDL (*12*), suggesting that like the 3.6 nm species studied here, it may contain a single molecule of apo A1. Taken together, our data suggest a model in which lipid-free 2.6 nm secreted apo A1 is first converted to a 3.6 nm form (Figure 8) followed by PL binding, to produce a biologically active lipid-poor particle similar to prebeta$_1$-HDL. The promotion of such unfolding may be a novel function of ABCA1. These particles have a relatively limited capacity for lipid (2 mol of PL/particle) and, probably, a single mole of FC. Plasma prebeta$_1$-HDL has also been identified as a direct substrate for LCAT and CETP (*12*). The study presented here provides strong additional data that prebeta-migrating, lipid-poor HDLs are likely to be major intermediates of FC transport by both newly synthesized and recycling HDLs. Two previous studies of HDL secretion by HepG2 cells also reported a preponderance of small, lipid-poor particles (*15*, *16*) with a Stokes radius similar to that of albumin (*15*, *16*). In the former case, a smaller particle was identified, though not further characterized. This may correspond to the 2.6 nm apo A1 fraction described here. The data are consistent

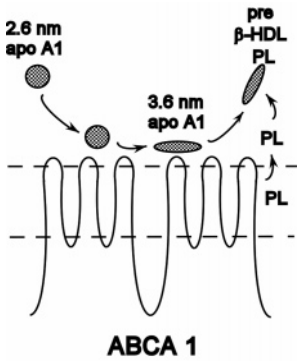

FIGURE 8: Model for the binding and activation of secreted lipid-free apo A1 at the cell surface. A compact 2.6 nm precursor is converted to a 3.6 nm product at the cell surface in an ABCA1-dependent reaction. Following unfolding, PL is transferred to generate a biologically active, prebeta-HDL.

in indicating that lipid-poor and lipid-free apo A1 particles are the major products of these cells, in a proportion probably depending on the details of culture conditions, and perhaps the level of cell surface ABCA1.

A possible biological role for 2.6 nm apo A1 as an inactive precursor of HDL is suggested by the dominance of the liver as a source of HDL proteins. The RCT pathway is considered to be one of the major functions of HDL. Binding of FC by newly secreted, lipid-binding HDL would short-circuit their ability to promote RCT from peripheral tissues (*45*). The distribution of newly secreted (2.6 nm) apo A1 within the extracellular space prior to induction of their lipid binding properties would maximize the ability of newly secreted apo A1 to sequester lipids from peripheral tissues. The presence of relatively high levels of lipid-poor HDL in the extracellular space (*46*) would be consistent with such a hypothesis.

In summary, this research identified a novel, PL-resistant monomer as an initial secretion product of apo A1 secreting cells that may play an important biological role in RCT.

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