Intracellular maturation of apolipoprotein[a] and assembly of lipoprotein[a] in primary baboon hepatocytes

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Abstract The glycoprotein apolipoprotein [a] (apo[a]) is present in plasma at highly variable concentrations and appears as a number of genetically determined size isoforms (400–800 kDa), disulfide linked to apoB-100 in low density lipoprotein to produce lipoprotein [a](Lp[a]). Apo[a] is synthesized by the liver, but the site of association of apo[a] and apoB and factors that regulate its production are unknown. To examine the morphogenesis of the Lp[a] particle, baboon hepatocytes expressing a single, low molecular weight isoform of apo[a] were labeled with [^{35}S]cysteine and methionine, and apo[a] was analyzed by immunoprecipitation and SDS-PAGE. Steady-state labeling revealed two molecular weight forms of apo[a] inside the cell. Only the large form was recovered from the culture medium. Pulse-chase studies and endoglycosidase treatment revealed that the lower molecular weight form of apo[a] represented a precursor with a prolonged residence time in the endoplasmic reticulum or an early Golgi compartment, after which it was processed to the mature form. A proportion of the mature form of apo[a] was rapidly secreted after synthesis, whereas the remainder had a prolonged residence time in a late Golgi compartment. In all experiments, apoB co-precipitated with apo[a] from the culture medium, but not from cell lysates. Density gradient ultracentrifugation and immunoblot analysis revealed that the majority of apo[a] was secreted into the medium in a free form, suggesting that the association between apo[a] and apoB occurred after secretion. Regulation of the movement of apo[a] between intracellular compartments may be one mechanism by which the plasma levels of Lp[a] are influenced.—**White, A. L., D. L. Rainwater, and R. E. Lanford.** Intracellular maturation of apolipoprotein[a] and assembly of lipoprotein[a] in primary baboon hepatocytes. *J. Lipid Res.* 1993. **34:** 509–517.

Supplementary key words apolipoprotein B • endoplasmic reticulum • Golgi apparatus

First described by Berg in 1963 (1), lipoprotein[a] (Lp[a]) consists of a low density lipoprotein (LDL)-like particle in which apoB-100 is disulfide-linked to an additional, high molecular weight glycoprotein, apolipoprotein[a] (apo[a]) (2). Elevated plasma levels of Lp[a] are a strong independent risk factor for the development of coronary heart disease (3, 4). However, little is known of the nature of the association of Lp[a] with this disease, the physiological role of Lp[a], or the mechanisms that control its rate of production and removal from the circulation.

Apo[a] occurs as a number of discrete, heritable size isoforms between 400 and 800 kDa (5, 6) and is highly homologous to plasminogen (7, 8). A single copy of the plasminogen kringle 5 (K5) and protease domain is preceded by a variable number of copies of plasminogen K4. The number of K4 domains encoded correlates with the size of the protein (9–12). Approximately 28% of the mass of apo[a] is contributed by N- and O-linked carbohydrate (13). Apo[a] and apoB-100 are synthesized primarily by the liver (14, 15). In vitro production of intact Lp[a] by hepatocytes in culture has been demonstrated (16). However, it is not known whether the association of apo[a] and apoB occurs before or after secretion.

Plasma Lp[a] levels show dramatic variation among individuals (ranging from undetectable to 100 mg/dl), but are relatively constant during an individual's lifetime (17). High heritability coefficients (18) and metabolic studies (19) have suggested that Lp[a] levels are governed largely at the point of biosynthesis. An inverse correlation between apo[a] size and plasma Lp[a] level exists (5, 6, 20); however, many exceptions to this relationship have been reported (6, 12, 21, 22). Furthermore, apo[a] size explains only approximately 40% of the variation in plasma Lp[a] levels (23). In macaques, differences in hepatic apo[a] mRNA concentration account for some of the variation in plasma Lp[a] level. However, this effect is independent of

Abbreviations: apo, apolipoprotein; ER, endoplasmic reticulum; HDL, high density lipoprotein; K, kringle; LDL, low density lipoprotein; Lp, lipoprotein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SFM, serum-free medium; VLDL, very low density lipoprotein.

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transcript length (24). In humans, hepatic apo[a] mRNA concentrations do not correlate with plasma Lp[a] levels (25). Clearly, post-transcriptional mechanisms play a role in determining levels of Lp[a] production. Further evidence comes from studies of "null" phenotype baboons with no detectable Lp[a] in plasma. Some of these animals were found to have an apo[a] transcript in the liver (10).

Baboons and humans are similar in terms of properties of Lp[a] and apo[a] (26, 27). Nine isoforms of apo[a] have been identified in baboons and are designated A (the largest) through L (the smallest) (21). We have developed a serum-free medium for the long term culture of primary baboon hepatocytes (28). Studies using this system were the first to demonstrate the in vitro production of intact Lp[a] by hepatocyte cultures and the production of apo[a] isoforms identical in size with those detected in the donors' sera (16). This system is uniquely amenable to the study of Lp[a] morphogenesis and factors that influence Lp[a] production, as hepatocytes can be isolated from animals with defined plasma Lp[a] levels and apo[a] isoforms.

The aim of this study was to define the sequence of events involved in the production of the Lp[a] particle. In particular, we wished *a)* to determine the cellular site of association of apo[a] and apoB; *b)* to examine the intracellular maturation of the apo[a] glycoprotein; and *c)* to compare the kinetics of Lp[a] (apo[a]) versus VLDL (apoB) secretion. The results suggest that apo[a] and apoB associate after secretion and that a substantial proportion of apo[a] remains in a free form in culture medium. Remarkably complex kinetics for apo[a] maturation and secretion were demonstrated. Regulation of the movement of apo[a] between intracellular compartments may provide one mechanism by which plasma levels of Lp[a] are influenced.

MATERIALS AND METHODS

Materials

[^{35}S]Cysteine and Expre^{35}S^{35}S label were from New England Nuclear (Boston, MA). Protein A agarose was purchased from RepliGen Corporation (Cambridge, MA). Sheep anti-human apoB and sheep anti-human apoA-I polyclonal antibodies were from Boehringer Mannheim (Indianapolis, IN), and rabbit anti-human apoE polyclonal antibody was from Calbiochem (San Diego, CA). The rabbit anti-baboon apo[a] polyclonal antibody used was as described previously (27). This antibody was prepared against purified Lp[a] and anti-apo[a] was isolated by removing antibodies that bound apoB (27). Purified Lp[a] was prepared as described previously (26). Methionine- and cysteine-free Williams medium E was purchased from GIBCO (Grand Island, NY). Amplify autoradiographic enhancer fluor was from Amersham International (Arlington Heights, IL) and Centricon-100 microconcentrators were from Amicon (Danvers, MA). N-Glycanase (peptide-N-(N-acetyl-β-glucosaminyl) asparagine amidase), O-glycanase (endo-α-N-acetylgalactosaminidase), and neuraminidase (acylneuraminyl hydrolase) were from Genzyme Corporation (Cambridge, MA) and endoglycosidase H (endo-β-N-acetylglucosaminidase H) was from ICN Immunobiologicals (Costa Mesa, CA). All other chemicals used were of analytical grade.

Hepatocyte isolation and culture

Hepatocytes were isolated and cultured in a serum-free medium (SFM) formulation (formula III) exactly as described previously (28), except for the omission of thyrotropin-releasing factor. All experiments were performed on confluent 60-mm dishes of cells that had been in culture for 7–10 days.

Radiolabeling

For steady-state labeling experiments, hepatocytes were incubated for 16 h in cysteine- and methionine-free SFM supplemented with 50 μM each of unlabeled L-methionine and L-cystine plus 125 μCi/ml each of L-[^{35}S]cysteine and Expre^{35}S^{35}S label. For pulse-chase studies, cells were preincubated for 2 h in cysteine- and methionine-free SFM, labeled for 10 min with 2 ml of the same medium supplemented with 62.5 μCi/ml each of L-[^{35}S]cysteine and Expre^{35}S^{35}S label, and then chased for between 10 min and 5 h in SFM. Labeled media samples were clarified for 5 min at 2000 *g* and protease inhibitors were added, as described previously (29). Cells were washed 3 times with phosphate-buffered saline (PBS) and 1.5 ml of extraction buffer (EB) (50 mM Tris/HCl, 100 mM NaCl, 1% NP40, pH 9.0) was added. Plates were incubated at 4°C for 10 min with gentle shaking; supernatants were collected and these were pooled with a further wash with 1.0 ml of EB. Protease inhibitors were added as above.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Media samples were adjusted to 1% NP40 and 1.0–1.5 ml of media or cell lysates were incubated with the respective antibodies at room temperature for 2 h with shaking. Protein A agarose (100 μl of a 50% suspension) was added and samples were incubated for a further 30 min at room temperature with shaking. To reduce nonspecific background, protein A agarose was preincubated for 1 h at room temperature with unlabeled conditioned culture medium and washed 3 times with EB before incubation with radiolabeled samples. Immunoprecipitates were washed 2 times with 1 ml of washing buffer (WB) (50 mM Tris/HCl, 100 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, pH 8.0), once with WB, containing 0.3 M NaCl, once with WB, and once with PBS. Im-

munoprecipitates were eluted in 100 μl of SDS disruption buffer (100 mM Tris/HCl, pH 6.8, 2% SDS, 20% glycerol, 10% mercaptoethanol, 1 mM EDTA, 0.1% bromophenol blue) for 5 min at room temperature and were denatured for 5 min at 100°C. ^{35}S-labeled proteins were resolved by electrophoresis in 3–10% concave gradient polyacrylamide slab gels with a 3% stacking gel (30). Gels were fixed in 40% methanol, 10% acetic acid, impregnated with fluor, then dried and exposed to Kodak X-AR5 film.

Immunoblotting

Aliquots of media and cell lysates were mixed with an equal volume of SDS-disruption buffer with or without 10% mercaptoethanol. Samples were separated in 3–10% SDS-PAGE gels as described above and proteins were transferred electrophoretically (100 mA for 16 h) to Gene Screen Plus membranes (New England Nuclear, Boston, MA). Blots were probed with anti-apo[a] or anti-apoB antibodies exactly as described previously (28).

Density gradient ultracentrifugation

Labeled medium (2 ml) was added to 0.86 g of solid KBr and overlaid with KBr solutions of decreasing densities as follows: 1.5 ml of d = 1.21 g/ml, 1.5 ml of d = 1.125 g/ml, 1.5 ml of d = 1.07 g/ml, 2.0 ml of d = 1.063 g/ml, 1.5 ml of d = 1.019 g/ml, and 2.0 ml of d = 1.006 g/ml. Gradients were developed by ultracentrifugation in an SW41 rotor (Beckman) at 39,000 rpm for 24 h at 20°C. Fractions (1 ml) were collected and densities were determined using a refractometer. Fractions were desalted by washing 3 times with 1 ml of H_2O in Centricon-100 microconcentrators, recovered in 200 μl of EB, and immunoprecipitated as described above.

Endoglycosidase digests

Apo[a] was immunoprecipitated from labeled cells and media as described above, and samples were eluted from protein A agarose beads in 112 μl of 0.5% SDS and 8 μl of 10% β-mercaptoethanol for 5 min at 100°C. Aliquots of the samples (20 μl) were incubated at 37°C for 16 h after addition of one of the following: *1)* 10 μl of 7.5% NP40, 30 μl of 0.4 M Tris, pH 8.6, and 5 U of N-glycanase; *2)* 40 μl of 50 mM sodium acetate, pH 5.7 and 3 mIU of endoglycosidase H; *3)* 10 μl of 7.5% NP40, 30 μl of 30 mM sodium acetate, pH 6.5, and 10 mU neuraminidase; or *4)* as in *3)* with 1 mU of O-glycanase added after 2 h at 37°C; or *5)* 40 μl of PBS (control).

RESULTS

Hepatocyte donor

To simplify analyses, all experiments were performed using hepatocytes from a single donor. The donor had very high levels of Lp[a] (45 mg Lp[a] proteins/dl, see ref. 27) and expressed a single, low molecular weight, L, isoform of apo[a] (21). The serum Lp[a] phenotypes of the sire and dam were "null" and L, respectively. From this we inferred that the donor was heterozygous, possessing an allele that encoded an L protein and an allele not expressing detectable protein ("null" allele).

Immunoprecipitation of intracellular and secreted apo[a]

To determine the site of association of apo[a] and apoB, hepatocytes were labeled metabolically with [^{35}S]methionine and [^{35}S]cysteine and apo[a] was immunoprecipitated from cell lysates and culture medium. Co-precipitation of apoB with apo[a] was used as the criterion for association. Two forms of apo[a] with marked differences in mobility on SDS-PAGE were recovered from the cell lysate, while only the larger form was found in the medium (**Fig. 1**). Immunocompetition with purified Lp[a] and immunoprecipitation with a control antibody (anti-albumin) confirmed the identity of these proteins as apo[a] (Fig. 1). A protein co-migrating with apoB-100 on SDS-PAGE co-precipitated with apo[a] from the medium, but not from the cell lysate (Fig. 1), which suggests that apo[a] and apoB associate after entering the medium. Alternatively, association could occur just prior

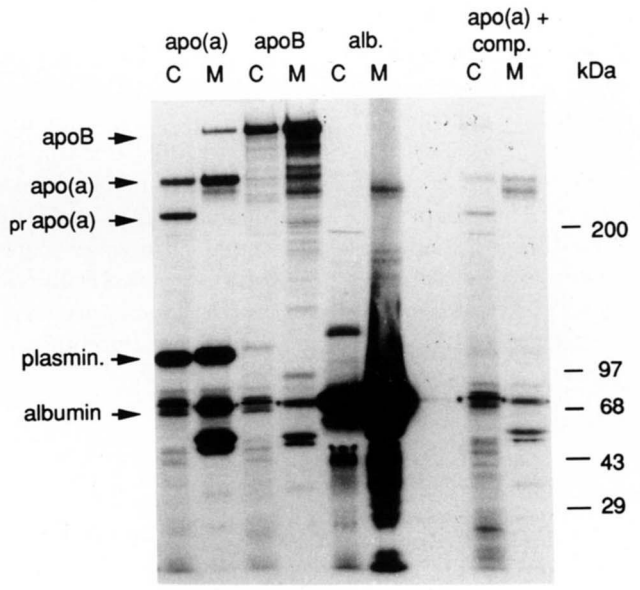

Fig. 1. Immunoprecipitation of intracellular and secreted apo[a], apoB and albumin after steady-state labeling with [^{35}S]cysteine and [^{35}S]methionine. Baboon hepatocytes expressing a single, low molecular weight (L) isoform of apo[a] were labeled for 20 h with 250 μCi/ml [^{35}S]cysteine and [^{35}S]methionine. Apo[a], apoB, and albumin were immunoprecipitated from cell lysates (C) and culture medium (M) and analyzed by SDS-PAGE and fluorography (see Materials and Methods). The two lanes on the far right represent apo[a] immunoprecipitation reactions where purified Lp[a] (10 μg) was added at the antibody incubation step as a competitor. The positions of molecular weight standards and those of apo[a], apoB, albumin, and a smaller intracellular form of apo[a] (pr apo[a]) are shown. The anti-apo[a] antibody cross-reacts with domains in plasminogen (plasmin.) due to homology with apo[a].

to secretion so that at steady-state the amount of labeled apoB co-precipitating with apo[a] from the cell lysate would be too small to be detected.

To determine the presence of a disulfide-linked apo[a]-apoB complex, immunoblotting was performed in the presence and absence of reducing agent (16). In the absence of reducing agent, an apo[a]/apoB complex could be detected in the medium after immunoblotting with antibodies to apo[a] or apoB. The unreduced form of apo[a] had a greater mobility than reduced apo[a] on SDS gels (**Fig. 2A**). The increase in mobility of unreduced apo[a] has been observed with other size isoforms (data not shown) and has been reported previously (31). Immunoblotting of unreduced cell lysate failed to detect an association of apo[a] and apoB inside the cell, although an apo[a]/apoB complex was again observed in the medium (Fig. 2B).

Pulse-chase studies

In an attempt to reveal any association of apo[a] and apoB occurring late in the secretory pathway and to examine the relationship between the two intracellular forms of apo[a], pulse-chase studies were performed. The kinetics of apo[a] and apoB secretion were also compared. Hepatocytes were pulse-labeled for 10 min with [^{35}S]methionine and [^{35}S]cysteine and chased for various periods between 10 min and 5 h. Intracellular and secreted apo[a] and apoB were analyzed by immunoprecipitation and SDS-PAGE. At 10 and 30 min of chase, only the small form of apo[a] was apparent inside the cell. At 60 min, the larger form of the protein appeared intracellularly, concomitant with secretion of apo[a] into the medium (**Fig. 3A**). The smaller intracellular form of apo[a] thus appears to represent a precursor of the mature protein. These data suggest that maturation of apo[a] is a rate-limiting step in apo[a] secretion. Moreover, since greater than 50% of apo[a] immunoprecipitated from cell lysates after steady-state labeling was in the mature form (Fig. 1), the data would suggest a second rate-limiting step occurs prior to secretion.

Radiolabeled apoB co-precipitated with apo[a] from the medium, but not from the cell lysates, which again suggests extracellular association (Fig. 3A). However, association of apo[a] and apoB immediately prior to secretion cannot be ruled out by these results.

The kinetics of apoB and apo[a] secretion were similar, with radiolabeled apoB first appearing in the medium at 60 min of chase (Fig. 3B); however, a substantial proportion of apo[a] remained intracellular after a 5-h chase, while apoB was almost completely secreted. The baboon hepatocytes secrete apoB-100 as VLDL (see below). The time required for apoB-100 secretion is 45 min in primary rat hepatocytes (which secrete apoB as VLDL) (32) and 30–35 min for HepG2 cells (which secrete apoB in an LDL-like particle) (33). No time points between 30 and 60 min were examined in the present study. However, apoB secretion kinetics appeared similar to those observed in the other systems.

Density gradient ultracentrifugation and immunoblotting of secreted apo[a]

Crucial to the distinction between intracellular versus extracellular association of apo[a] and apoB is the determination of whether apo[a] can be secreted in a free form. Two approaches were adopted to examine this issue. In the first, hepatocytes were labeled overnight with [^{35}S]methionine and [^{35}S]cysteine, and the conditioned medium was fractionated on KBr gradients from 1.008 to 1.3 g/ml. Gradient fractions were immunoprecipitated with antibodies to apoB, apo[a], apoE, or apoA-I and analyzed by SDS-PAGE and fluorography. Greater than 99% of apoB-100 was found at $d < 1.030$ g/ml, with approximately 78% at $d \leq 1.008$ g/ml, consistent with the density of VLDL (**Fig. 4**). As expected, a small peak (8%) of apoE immunoreactivity was found in VLDL ($d \leq 1.008$ g/ml). A small fraction of apoE (10%) was recovered at the density of soluble protein (1.299 g/ml) with the remainder at $d = 1.078 - 1.171$ g/ml, within the HDL density range. ApoA-I was distributed over the entire gradient

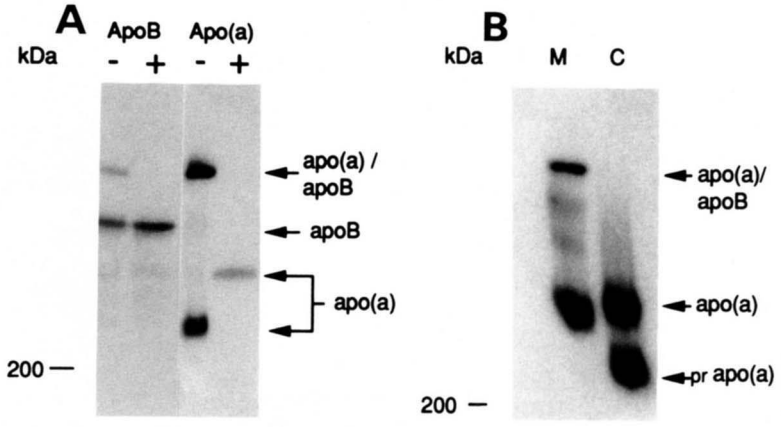

Fig. 2. Immunoblotting of apo[a] and apoB in the presence and absence of reducing agent. (A) Medium was collected after incubation with hepatocytes for 48 h and concentrated. Aliquots were SDS-treated in the presence (+) or absence (−) of 10% β-mercaptoethanol and then electrophoresed on a 3–10% concave gradient SDS-PAGE gel. Proteins were transferred to a nylon membrane and blots were reacted with antibodies to apoB (left) or apo[a] (right) as described in Materials and Methods. Detection of bound antibodies was with ^{125}I-labeled protein A. (B) Aliquots of medium (M) and cell extract (C) were electrophoresed in the absence of reducing agent and immunoblotted for apo[a], as described for (A). The positions of molecular weight markers and of free and complexed apo[a] and apoB are indicated.

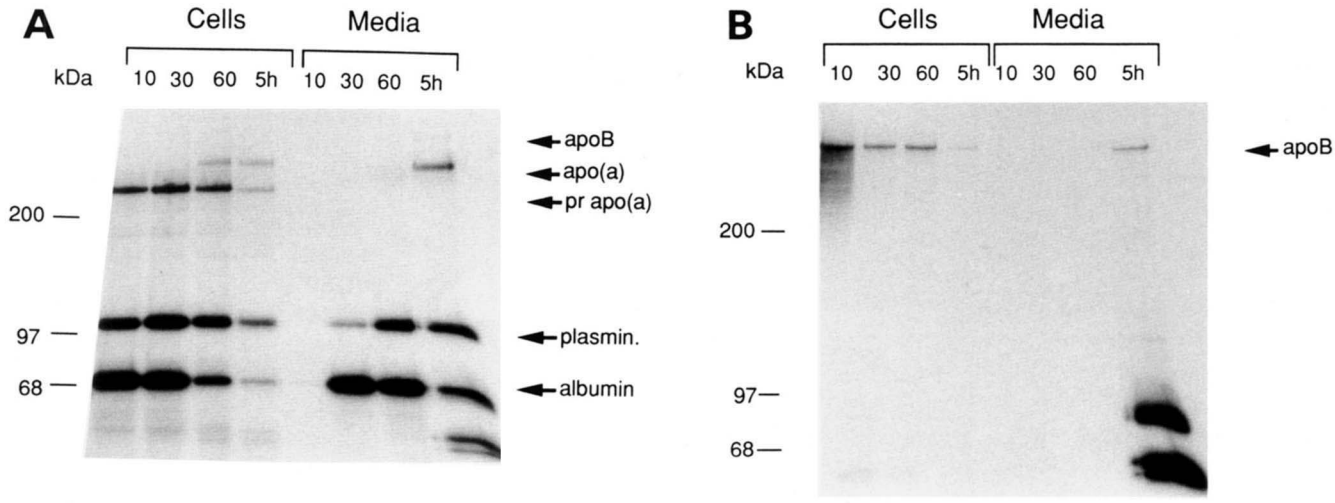

Fig. 3. Pulse-chase studies to examine the kinetics of the intracellular maturation and secretion of apo[a] and apoB. Baboon hepatocytes (see Fig. 1) were labeled for 10 min with 125 μCi/ml [^{35}S]cysteine and [^{35}S]methionine and chased for 10, 30, 60 min, or 5 h in an excess of unlabeled amino acids. Cells and media were immunoprecipitated with antibodies to apo[a] (A) or apoB (B) and were analyzed by immunoprecipitation, SDS-PAGE and fluorography (see Materials and Methods). The positions of molecular weight markers and of apo[a], apoB, and the apo[a] precursor (pr apo[a]) are indicated. In the original fluorographs, mature apo[a] (A) and apoB (B) could be seen in the medium at 60 min of chase and apoB could be seen co-precipitating with apo[a] at 5 h of chase (A). However, due to the loss of resolution in reproduction, these bands may only be faintly visible.

with a peak at 1.078–1.137 g/ml (Fig. 4). These results confirm those previously reported for baboon hepatocytes cultured under these conditions (28) and confirm the ability of these cells to secrete bona fide lipoprotein particles. Surprisingly, greater than 75% of apo[a] was found in a free form at the bottom of the gradient (d = 1.299 g/ml), with a minor proportion (< 2%) at d ≤ 1.008 g/ml associated with apoB. Small amounts of apo[a] immunoreactivity were found in all other fractions of the gradient. Immunoblotting of gradient fractions for apo[a] under nonreducing conditions confirmed that the majority of apo[a] was in a free form (data not shown).

Immunoblotting of total medium in the presence and absence of β-mercaptoethanol was a second approach used to examine free and complexed apo[a]. Under reducing conditions, a single band representing free apo[a] was seen (Fig. 2A). In the absence of reducing agent, a lower mobility band appeared with both apo[a] and apoB immunoreactivity that represents the apo[a]/apoB complex (Fig. 2A). Free unreduced apo[a] migrated with a mobility greater than reduced apo[a] (Fig. 2A). The apparent proportion of apo[a] in complex with apoB was 34% across five experiments (range 23 to 59%). The proportion of apo[a] in complex determined by immunoblotting was higher than that observed by density gradient ultracentrifugation (Fig. 4). This discrepancy may be due to preferential loss of lipid-associated apo[a] during ultracentrifugation. Results from both methods, however, demonstrate that a substantial proportion of apo[a] in the medium is in a free form.

Endoglycosidase treatment of precursor and mature forms of apo[a]

Apo[a] has a high content of both N- and O-linked carbohydrate (13). Addition and processing of carbohydrate chains occurs at specific points along the secretory pathway (35). To determine the structural relationship between the precursor and mature forms of intracellular apo[a] and their approximate locations in the cell, endoglycosidase digests were performed. N-glycanase, which removes both complex and high mannose N-linked sugars leaving an unsubstituted polypeptide chain (36), and endoglycosidase H (endo H), which cleaves high mannose but not complex N-linked structures leaving a charged N-acetylglucosamine residue attached to the polypeptide chain (37), were used to examine N-linked carbohydrate. N-glycanase increased the mobility of both precursor and mature apo[a] on SDS-PAGE confirming the presence of N-linked carbohydrate (**Fig. 5,** lanes 1). Endo H did not affect the mobility of mature apo[a] but increased the mobility of the precursor to a greater degree than did N-glycanase (Fig. 5, lanes 2). The N-acetylglucosamine residue left by endo H presumably accounts for the difference in mobility of N-glycanase and endo H-treated precursor. Resistance of N-linked carbohydrate to endo H is acquired in the medial Golgi, where processing by mannosidase II occurs (35). Results of endo H digestion, therefore, suggest that the precursor protein is a pre- or early-Golgi form of apo[a] whereas the mature form has at least traversed the medial Golgi.

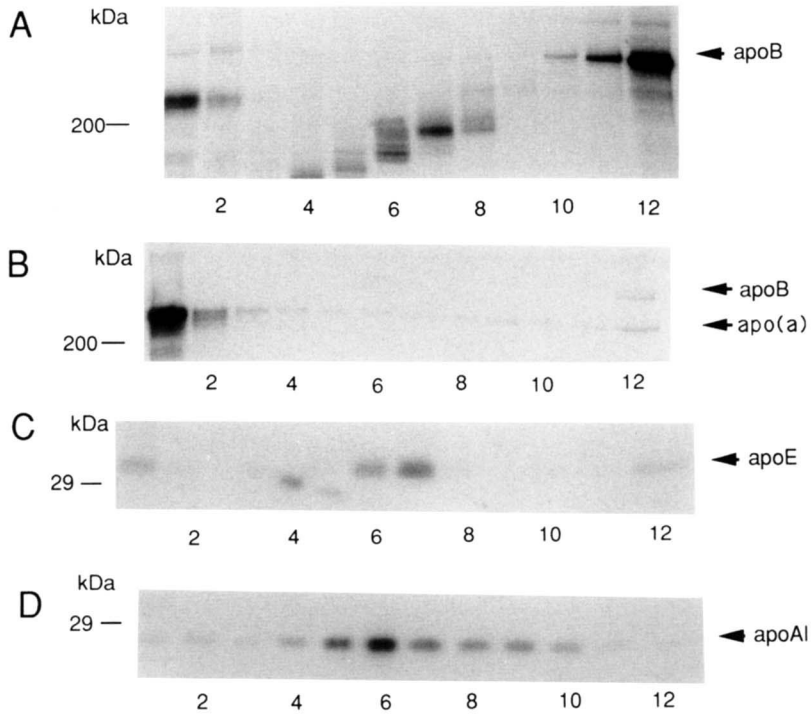

Fig. 4. Density gradient ultracentrifugation of secreted lipoproteins. Hepatocytes were labeled as described in the legend to Fig. 1. The conditioned medium was ultracentrifuged in a continuous KBr gradient from d 1.299 to 1.008 g/ml, fractions were collected and immunoprecipitated with antibodies to apoB (A), apo[a] (B), apoE (C), or apoA-I (D) (see Materials and Methods). Immunoprecipitates were analysed by SDS-PAGE and fluorography. The density of each fraction (g/ml) was as follows: 1 = 1.299, 2 = 1.246, 3 = 1.209, 4 = 1.171, 5 = 1.137, 6 = 1.106, 7 = 1.078, 8 = 1.056, 9 = 1.037, 10 = 1.023, 11 = 1.015 and 12 = 1.008. The series of lower molecular weight bands recovered in the apoB immunoprecipitates most likely represent degraded fragments of apoB associated with some lipid (34). The positions of molecular weight markers are indicated.

Neuraminidase removes terminal sialic acid residues from both N- and O-linked sugars (38). O-Glycanase removes O-linked sugars after sialic acid residues have been removed (39). Treatment with neuraminidase resulted in a large increase in the mobility of the mature form of apo[a] (both intracellular and secreted) but did not affect the precursor (Fig. 5, lanes 3). These data are consistent with biochemical data that showed a high sialic acid content for plasma apo[a] (13) and demonstrate that mature apo[a] has reached the *trans*-Golgi apparatus, where sialyl transferase is located (35). Treatment with neuraminidase and O-glycanase increased the mobility of mature apo[a] to that of the precursor, confirming the presence of O-linked sugar on mature apo[a]. O-Glycanase did not affect the mobility of the precursor form of apo[a], consistent with a pre-Golgi location of this form of apo[a] (Fig. 5, lanes 4). The lack of difference in mobility of the precursor and mature forms of apo[a] after treatment with neuraminidase and O-glycanase is probably due to the removal of sialic acid residues from N-linked sugars on the mature form, to the anomalous effect of carbohydrate on protein mobility, and to the limitations of the gel system used for resolving large proteins.

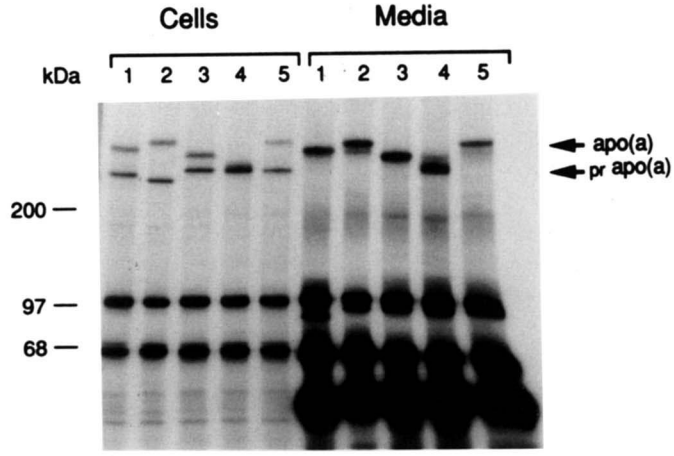

Fig. 5. Endoglycosidase treatment of precursor and mature forms of apo[a]. Baboon hepatocytes were labeled as described in the legend to Fig. 1, and apo[a] was immunoprecipitated from the cell lysate and culture medium. Aliquots of each immunoprecipitate were treated with the following endoglycosidases, as described in Materials and Methods: Lanes 1, N-glycanase; lanes 2, endoglycosidase H; lanes 3, neuraminidase; lanes 4, neuraminidase and O-glycanase; and lanes 5, PBS control. Digests were analyzed by SDS-PAGE and fluorography. The positions of molecular weight markers are indicated. In medium samples, the protein migrating below apo[a] in lanes 2 and 5 and above apo[a] in lane 4 is a protein which binds to protein A agarose and is not specific to the anti-apo[a] antibody.

DISCUSSION

High plasma levels of Lp[a] are a strong, independent risk factor for the development of coronary heart disease (3, 4). Levels are highly heritable and remain remarkably constant throughout an individual's lifetime (17). In vivo metabolic studies suggest that the concentration of plasma Lp[a] is determined by the rate of Lp[a] production rather than its rate of removal from the circulation (19). However, little is known of the processes involved in the assembly of the Lp[a] particle or of factors which determine production levels. In the present study we have examined the intracellular maturation and secretion of apo[a] and the morphogenesis of Lp[a] in primary cultures of baboon hepatocytes. Results demonstrated a remarkably complex series of events involved in the synthesis and secretion of apo[a]. In addition, apo[a] appeared to be secreted in a free form and to associate with apoB only after entering the culture medium.

A combination of steady-state and pulse-chase labeling studies demonstrated that apo[a] was synthesized as a lower molecular weight precursor. After a prolonged period of time apo[a] was processed to a mature form and secreted. Although the minimum time for apo[a] maturation was 30–60 min, a significant fraction (52%) remained in the immature form after a 5-h chase. Endoglycosidase digests determined that the precursor form of apo[a] represented an incompletely glycosylated, ER- or early-Golgi-associated protein, whereas the mature form contained mature carbohydrate and had at least traversed the *trans*-Golgi apparatus. The data therefore suggest that movement out of the ER is one of the rate-limiting steps in apo[a] secretion resulting in accumulation of the precursor form of apo[a]. The structure of apo[a] requires the formation of a large number of intramolecular disulfide bonds. Only properly folded proteins are permitted to exit the ER (40). The time required for folding of apo[a] may therefore retard its movement to the Golgi apparatus. In addition, the data suggest that a second rate-limiting step in apo[a] secretion results in the accumulation of the mature form of apo[a] in a late Golgi compartment, since at steady-state, greater than 50% of apo[a] inside the cell is in this form. To our knowledge, no other secretory protein has been shown to have a prolonged residence time in the *trans*-Golgi apparatus.

In a recent study by Koschinsky and co-workers (31), the kinetics of recombinant apo[a] maturation and secretion from a human embryonic kidney cell line were studied. Results obtained were very different to those reported in the present study; after a 1-h pulse and 20-min chase period, approximately 50% of the radiolabeled apo[a] protein was already secreted into the medium and virtually all of the protein had been secreted by 1 h of chase. Of the radiolabeled apo[a] still inside the cell at each time period, the majority was in the immature form (31). These data suggest that the recombinant apo[a] is secreted much more rapidly and that the residence time in the Golgi is much shorter than that observed in the present study. The reason for this discrepency is unclear at this time; the data in the current study may reflect a hepatocyte-specific event, or the recombinant clone used by Koschinsky and co-workers (31) may not reflect the behaviour of a natural apo[a] allele. Both experimental approaches, however, will provide useful information on the factors which influence the rate of apo[a] secretion.

In the present study, the synthesis and secretion of a single isoform of apo[a] was examined. Preliminary studies in baboon hepatocytes expressing other isoforms of apo[a] have revealed similar characteristics of apo[a] maturation and secretion. However, the residence time of apo[a] in the ER was found to vary substantially between allelic variants (data not shown). Further experimentation will be required to determine whether apo[a] is quantitatively secreted or whether significant intracellular degradation of the protein occurs. If degradation of apo[a] does occur, the ability of different isoforms of apo[a] to move through cellular compartments could influence the proportion of the protein degraded and such a mechanism could have a large influence on plasma levels of Lp[a]. ApoB-100, the other protein component of Lp[a], has been shown to undergo extensive degradation in an endoplasmic reticulum-related compartment (34).

Observations made in this study are consistent with the hypothesis that apo[a] is secreted as a free protein which is then disulfide-linked with apoB in the culture medium; in both steady-state and pulse-chase labeling experiments, apoB co-immunoprecipitated with apo[a] from the culture media, but not from the cell lysates. In addition, density gradient ultracentrifugation and analysis of unfractionated culture medium by immunoblot, in the absence of reducing agent, demonstrated that the majority of apo[a] present in the culture medium was in a free form. These results are similar to those reported by Koschinsky and co-workers (31), where recombinant apo[a] secreted from transiently transfected HepG2 cells was shown to be present as a free soluble protein as well as in complex with apoB in the LDL and HDL density ranges. Free apo[a] was also secreted from a human kidney cell line stably transfected with the same apo[a] construct (31).

Small amounts of lipid-poor apo[a]-apoB complex and free apo[a] are found in human plasma and a small proportion of apo[a] is in the VLDL density range. However, the majority of apo[a] in plasma is at a density of 1.05–1.12 g/ml in association with apoB in Lp[a] (41–43). The concentration of apoB-containing lipoproteins (and in particular, LDL) in plasma is very high, which may drive the interaction between apo[a] and apoB to completion. Conversely, the serum-free culture medium contains a very low concentration of apoB-containing lipoproteins, and almost exclusively VLDL,

which may cause a high proportion of apo[a] to remain in a free form. Metabolic studies involving injection of radiolabeled VLDL and LDL into human subjects, however, failed to show the incorporation of any of this radioactivity into plasma Lp[a] (44). The discrepancy between the in vivo and in vitro data cannot be explained from the current studies. Further work will be required to elucidate the nature of the apoB-apo[a] interaction.

Currently virtually nothing is known about factors that determine the level of Lp[a] production by the liver. A great need therefore exists for an in vitro system to study Lp[a] morphogenesis. Results from the present study demonstrate the value of the baboon hepatocyte system for such analyses. Baboons show characteristics similar to humans in terms of the physicochemical properties of Lp[a], plasma Lp[a] levels, and apo[a] isoform sizes. The Southwest Foundation for Biomedical Research has a large colony of baboons, more than 700 of which are of known pedigree and have been characterized for Lp[a] phenotype (isoform size and Lp[a] level). The ability to isolate and culture hepatocytes from these animals in a serum-free medium for extended periods represents a valuable resource to study the effect of allelic variation at the apo[a] locus on levels of Lp[a] production. Problems in obtaining and culturing hepatocytes from selected donors would make such a study in humans extremely difficult.

In conclusion, the results from this study reveal a remarkably complex series of events involved in the assembly and secretion of Lp[a]. A number of questions, however, remain unanswered, such as the mechanism of association of apo[a] and apoB, the processes involved in regulating apo[a] maturation and secretion, and the effect of these parameters on the regulation of plasma levels of Lp[a]. Clearly, extensive future study will be required to unravel the complex web of events involved in the formation and metabolism of this unusual lipoprotein.

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