Lipoprotein(a) (Lp(a)) consists of a low density lipoprotein particle in which apolipoprotein(a) (apo(a)), is disulfide linked to apoB. Lp(a) is produced by the liver, and high plasma levels represent an independent risk factor for cardiovascular diseases. However, pathways of production and metabolism of Lp(a) are poorly understood. We used primary cultures of baboon hepatocytes to analyze the steps involved in Lp(a) biogenesis. The results demonstrated that Lp(a) assembly was extracellular, since it was inhibited when anti-apo(a) antiserum was present in the culture medium. In addition, free apo(a) produced by hepatocytes could associate extracellularly with apoB in either very low density or low density lipoproteins. Lp(a) assembly required lysine-binding pockets in apo(a) kringle domains, as it was inhibited by the lysine analog, 6-amino hexanoic acid. A portion of apo(a) was also bound to the cell surface via its kringle domains and could be released into the medium by 6-amino hexanoic acid or proline. In add-back experiments, apo(a), but not Lp(a), bound to the cell surface. Addition of low density lipoprotein or very low density lipoprotein to hepatocyte cultures released apo(a) from the cell surface into the lipoprotein fraction of culture medium. We conclude that assembly of Lp(a) can occur at the cell surface. This represents one potential mechanism of Lp(a) production in vivo.

Lipoprotein(a) (Lp(a))1 was first described by Berg (1) in 1963 as an antigenic variant of human low density lipoprotein (LDL). Lp(a) is composed of an LDL-like particle in which apolipoprotein (apo) B100 is disulfide-linked to a unique protein component, apo(a) (2). High levels of Lp(a) in the plasma represent a strong independent risk factor for the development of cardiovascular diseases (3). However, the in vivo function of this lipoprotein, its role in disease, and the pathways involved in its production and metabolism remain poorly understood.

Apo(a) is synthesized by the liver (4–6) and is highly glycosylated, with 28% of its weight contributed by carbohydrate (7). Apo(a) is also remarkably polymorphic in size. As many as 34 apo(a) isoforms have been identified in the human population, which range from <300 to >800 kDa in molecular mass (8–11). Apo(a) is highly homologous to the plasma zymogen, plasminogen, and contains a single copy of the plasminogen kringle 5 and protease domains preceded by multiple domains with homology to plasminogen kringle 4 (12). The number of kringle 4 domains encoded in the apo(a) gene varies from approximately 12 to 51 (13) and determines the size of the apo(a) protein (10, 13–16).

Plasma concentrations of Lp(a) show tremendous variation between individuals, from <1 to >100 mg/dl (17) and are heritable, with >90% of the variation attributable to the apo(a) gene locus (18). Plasma Lp(a) levels are determined by the rate of Lp(a) production (19, 20). In cynomolgus monkeys, differences in hepatic apo(a) mRNA concentration account for only some of the variation in Lp(a) production rate (21). Using primary cultures of baboon hepatocytes, we have shown that posttranslational mechanisms play a major role in determining levels of Lp(a) production (22–24). We have demonstrated that apo(a) is synthesized as a lower molecular weight precursor that has a prolonged residence time in the endoplasmic reticulum before processing to the mature form. Maturation involves O-linked glycosylation and processing of N-linked glycans. Mature apo(a) is also associated with the cells for a prolonged period of time before release into the medium (22, 23). Apo(a) allelic variants exhibit differences in their ability to undergo posttranslational processing, accounting for the inverse correlation between apo(a) size and plasma Lp(a) level (24). In addition, some apo(a) proteins are unable to exit the endoplasmic reticulum, resulting in “null” Lp(a) phenotypes where no Lp(a) is detectable in plasma (24).

Very little is known of the pathways involved in the assembly of Lp(a). One long standing question is whether the association between apo(a) and apoB occurs inside the cell or after the proteins have been secreted (25). Studies from this laboratory using baboon hepatocytes suggest that Lp(a) assembly occurs extracellularly, since no apo(a)-apoB complex is detectable in cell lysates, and a substantial proportion of apo(a) in culture medium is present as a free protein (22, 23). This contention is supported by recent studies that demonstrate that free recombinant human apo(a) secreted by kidney cells (26) or in the plasma of transgenic mice (27) can form an Lp(a) particle with LDL extracellularly. However, definitive evidence that the association between endogenously synthesized apo(a) and apoB occurs after secretion is lacking.

The aim of the current study was to analyze the processes involved in Lp(a) formation and to determine the requirements for the interaction between apo(a) and apoB. Results confirmed that Lp(a) assembly occurred extracellularly and that the interaction between apo(a) and apoB required the kringle domains of apo(a). In addition, a substantial proportion of cell-associated apo(a) was found to be bound to the hepatocyte surface via its kringle domains and could be removed from the cell and recovered in the lipoprotein fraction of culture medium by the addition of LDL. We conclude that one pathway of Lp(a) production may be assembly at the cell surface.

EXPERIMENTAL PROCEDURES

Materials—[35S]Cysteine and Expre38256S label were from DuPont NEN. Protein A agarose was purchased from Repligen Corporation.

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+ To whom correspondence should be addressed.

1 The abbreviations used are: Lp, lipoprotein; 6AHA, 6-amino hexanoic acid; apo, apolipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; PAGE, polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein.
Cell Surface Assembly of Lp(a)

(Cambridge, MA). Goat anti-human Lp(a) was from Biodiagn (Kennebunkport, ME). This antibody was raised against Lp(a), and anti-apoB reactivity was removed by absorption with human LDL. Methionine- and cysteine-free Williams medium E was purchased from Life Technologies, Inc. Heparin, human plasminogen, amino acids, and 6-amino hexanoic acid (6AHA) were from Sigma. Human very low density lipoprotein (VLDL), and LDL were from Athens Research and Technology (Athens, GA). All other chemicals were of analytical grade.

Hepatocyte Donors—Experiments were performed using hepatocytes isolated from four baboons, 1X4612, 1X4696, 1X8837, and 1X9296. Baboon Lp(a) shows similar characteristics to human Lp(a). We've apo(a) isoform size classes have been identified in the baboon and are designated A (the largest) through L (the smallest). Animal 1X4696 was homozygous for an apo(a) allele that gave rise to a circulating I apo(a) isoform. Animals 1X4612 and 1X9296 were heterozygous for alleles encoding B and E, and A and L isoforms, respectively.

Hepatocyte Isolation and Culture—Hepatocytes were isolated as described previously (28). Lobectomy was performed according to institutional guidelines under general anesthesia; ketamine hydrochloride was used as an immobilizing and preanesthetic agent, and anesthesia was maintained with sodium pentobarbital. Analgesics were provided intraoperatively. Cells were cultured in a serum-free medium formulation (formula III) as described previously (28), except for the omission of thyrotropin releasing factor. All experiments were performed using confluent 60-mm dishes of cells, which had been in culture for 5–7 days.

Radio labeling—Hepatocyte cultures were incubated for 20 h in SFM containing 0.1 times the normal concentration of methionine and cysteine, plus 125 μCi/ml each of [35S]methionine and [35S]cysteine. This antibody was raised against Lp(a), and anti-apoB was used as the criterion for association of the two proteins, and the proteins are easily resolved by gel electrophoresis (Fig. 1). For control samples, two forms of apo(a) were recovered from cell lysates, whereas only the larger protein was recovered from the culture medium (Fig. 1A). The smaller intracellular apo(a) protein represents a non-specific product of the immunoprecipitation (see "Results").

RESULTS

Inhibition of Lp(a) Assembly—Noncovalent interactions (29), involving lysine-binding pockets in the kringle 4 domains of apo(a) (27, 30, 31), contribute to the interaction between apo(a) and apoB in addition to a single intermolecular disulfide bond. To determine whether the lysine-binding pockets were involved in interactions required for the assembly of Lp(a), we examined the ability of the lysine analog, 6AHA, to inhibit the association of apo(a) and apoB in hepatocyte cultures. In addition, to obtain direct evidence that Lp(a) assembly occurred extracellularly, we examined the ability of a polyclonal anti-apo(a) antibody to prevent the association between apo(a) and apoB. Baboon hepatocytes were labeled to steady-state with [35S]cysteine and [35S]methionine in the presence or absence of goat anti-human apo(a) antisera or 200 μg/ml 6AHA. Apo(a) was immunoprecipitated from the cell lysates and culture media and analyzed by SDS-PAGE (Fig. 1A). Co-immunoprecipitation of apoB with apo(a) was used as the criterion for association of the two proteins. Hepatocytes used in this experiment expressed a D isoform of apo(a). This isoform is substantially larger than apoB, and the proteins are easily resolved by gel electrophoresis (Fig. 1). For control samples, two forms of apo(a) were recovered from cell lysates, whereas only the larger protein was recovered from the culture medium (Fig. 1A). The smaller intracellular apo(a) protein represents a precursor of the mature, secreted

serum-free medium supplemented with 0.5 mM cycloheximide. The medium was removed, and the cells were washed once with phosphate-buffered saline. Conditioned medium labeled 20 h containing 0.5 μg/ml cycloheximide was then added, and the cells were incubated at 37 °C for 4 h. For indicated experiments, the conditioned medium was supplemented with amino acids (50–500 μM), or with human plasminogen or heparin (10–1000 μg/ml). The culture medium was harvested as described previously (22). Apo(a) in these fractions or in total medium and in the cell lysate was then examined by immunoprecipitation, as described above.

Inhibition of Lp(a) Assembly—Using Lp(a) assembly was performed as described above.

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Inhibition of Lp(a) Assembly—Using Lp(a) assembly was performed as described above.
form (22). ApoB co-immunoprecipitated with apo(a) from the culture media but not from the cell lysates (Fig. 1A), consistent with an extracellular association of the proteins. When anti-apo(a) antisera was present during the labeling period, the precursor and mature forms of apo(a) could be seen in the cell lysates, and the mature form of apo(a) was recovered from the culture media. However, apoB did not co-precipitate with extracellular apo(a) (Fig. 1A). The anti-apo(a) antibody thus inhibited the interaction between apo(a) and apoB, confirming that the association between the proteins occurs outside the cell. The interaction between apo(a) and apoB was also inhibited by GAHA, confirming that lysine-binding pockets were required (Fig. 1A). In addition, 6AHA caused a drastic reduction in the amount of mature apo(a) recovered from cell lysates. The amount of apo(a) precursor in these samples was unchanged (Fig. 1A). This suggests that 6AHA either influences the kinetics of apo(a) secretion, or releases a cell surface-associated pool of apo(a) into the culture medium. Experiments described below were designed to distinguish between these possibilities.

A protein of lower molecular weight than apo(a) (labeled with an asterisk in Fig. 1) was also recovered in immunoprecipitates from each culture medium (Fig. 1A). This protein is nonspecific since it was seen in immunoprecipitates using preimmune goat antisera (Fig. 1B) and has been described by other investigators using liver cell lines (32, 33). Control immunoprecipitations with normal serum and anti-apoB antibody, confirmed the identity of the apo(a) and apoB proteins (Fig. 1B).

**Extracellular Assembly of Lp(a)—**In previous studies, we demonstrated that a high proportion of apo(a) in baboon hepatocyte culture medium is present as a free protein (22, 23). In plasma, however, only small amounts of free apo(a) are observed (34–36). We hypothesized that this difference was due to the low concentration of apoB in the culture medium, thus reducing the potential for interaction with apo(a) (22). In addition, apoB in the culture medium is present as VLDL (22), which may have a lower affinity for apo(a) than LDL (27). To determine whether all free apo(a) produced by hepatocytes was capable of forming an Lp(a) particle, cells were labeled in steady-state in the presence or absence of 10% baboon serum to provide a high level of LDL. The culture media were then fractionated on KBr gradients from 1.006 to 1.3 g/ml, and apo(a) was immunoprecipitated from each fraction. The hepatocytes used for this experiment expressed an I isoform of apo(a). Due to its smaller size, this isoform is secreted at higher levels than the D isoform in Fig. 1 (24), making detection easier but making its migration on gels only slightly faster than apoB.

Consistent with previous results (22), a high proportion of apo(a) from the control culture was recovered in the soluble fraction at the bottom of the gradient (Fig. 2A, fraction 1). A smaller proportion of apo(a) was recovered at VLDL density and co-immunoprecipitated with radiolabeled apoB (Fig. 2A). In the presence of 10% serum, however, the vast majority of apo(a) was recovered at a peak density of 1.052 g/ml, which is within the characteristic density range of plasma Lp(a) (34–36). Only small amounts of apo(a) were recovered at the bottom of the gradient or in the VLDL density range (Fig. 2B). In addition, the amount of radiolabeled apoB, which co-immunoprecipitated with apo(a) from cultures labeled in the presence of serum, was reduced indicating that exogenously added LDL competed with endogenous apoB for complexing with apo(a).

Analysis of gradient fractions on nonreducing gels demonstrated that the apo(a) in fractions 7–10 of serum-treated cultures was disulfide-linked to apoB (Fig. 2C). This experiment demonstrates that free apo(a) secreted by the hepatocytes is capable of forming an Lp(a) particle and that the formation of Lp(a) can occur extracellularly.

To determine whether the affinity of apo(a) was similar for different apoB-containing lipoproteins, the density of apo(a)-containing particles produced by hepatocytes labeled in the presence or absence of human VLDL or LDL was examined as above (Fig. 3). In this experiment, virtually all apo(a) secreted by control cells was recovered from the bottom fraction of the gradient. In the presence of VLDL, a substantial portion of apo(a) was recovered in the VLDL/IDL density range (1.009–1.020 g/ml), and in the presence of LDL, an approximately equal amount was recovered in the LDL/HDL density fractions (1.052–1.073 g/ml) (Fig. 3). Thus, apo(a) can bind with similar affinity to different apoB-containing lipoproteins. The presence of free apo(a) in hepatocyte cultures is therefore probably due solely to the low concentration of apoB available.

**Mature Apo(a) Is on the Cell Surface—**To investigate the mechanism by which 6AHA decreased the amount of mature apo(a) associated with cell lysates (Fig. 1A), radiolabeled cell cultures expressing an I isoform of apo(a) were incubated with fresh unlabeled medium containing increasing concentrations of 6AHA for 30 min at 0 °C, and the amount of apo(a) in the media and cell lysates was examined by immunoprecipitation (Fig. 4A). Protein secretion is prevented at 0 °C, and thus any apo(a) recovered from the culture media must have been released from the cell surface. In the absence of 6AHA, virtually no apo(a) was recovered from the medium. At increasing concentrations (50–200 μM) of 6AHA, however, progressively more apo(a) could be seen in the medium and progressively less mature apo(a) was recovered from the cell lysate (Fig. 4A). Thus, at least a portion of mature apo(a) recovered from cell...
lysates was on the cell surface. Since this cell-surface association was disrupted by 6AHA, it appears to be dependent on the kringle domains of apo(a).

To determine whether apo(a) size influenced the concentration of 6AHA required for the removal of apo(a) from the cell surface, the experiment in Fig. 4A was repeated using hepatocytes heterozygous for an A and an L apo(a) allele (Fig. 4B). At 50 mM GAHA, more than 50% of the mature form of the L isoform was released into the medium, whereas, even at 200 mM 6AHA, the majority of the A isoform remained cell associated (Fig. 4B). However, at higher concentrations of 6AHA, substantial amounts of a mature A isoform could be released from the cells (47% at 1 M data not shown). When all 3 isoforms in Fig. 4 were compared, at each concentration of 6AHA examined, the proportion of apo(a) released from the cell surface was inversely correlated with apo(a) size (Fig. 4, A and B). This trend held true when cells expressing a D isoform were examined (data not shown). This suggests that the avidity with which apo(a) binds to the cell surface is a function of the number of kringle 4 domains encoded. When hepatocytes were labeled in the presence of 200 mM 6AHA, no mature form was associated with the cells (Fig. 1A). 6AHA may prevent apo(a) binding more efficiently than it releases apo(a) from the cell surface due to inaccessibility of binding pockets once apo(a) is cell surface bound.

The kringle 4 domains in apo(a) are not identical (37), and molecular modeling studies suggest that their amino acid-binding pockets have different specificities (38). We therefore examined the effect of a number of amino acids on the cell surface association of apo(a) (Fig. 5). Steady-state labeled hepatocytes expressing an I isoform of apo(a) were incubated for 30 min at 0°C with fresh medium containing no addition (control) or containing 50 mM 6AHA, lysine, arginine, or proline or 100 mM leucine. Apo(a) in the cell lysates and supernatants was examined by immunoprecipitation (Fig. 5). Again, for control samples, no apo(a) was recovered from the culture medium. As before, in the presence of 6AHA, a portion of mature apo(a) was released from the cell surface and recovered from the culture medium. Proline also removed substantial amounts of apo(a) from the cell surface and was in fact more effective than 6AHA (Fig. 5). This is consistent with studies that predict the repeated kringle 4 domain in apo(a) to bind proline better than lysine (38) and with studies in which the interaction between apoB-containing lipoproteins and apo(a) was inhibited by proline (39). From a longer exposure of the autoradiograph shown in Fig. 5, it was apparent that small amounts of apo(a) were also removed from the cell surface by 50 mM lysine and 50 mM arginine. At 200 mM, lysine and arginine were capable of removing almost 50% of mature apo(a) into the medium (data not shown). The \( k_f \) of plasminogen for lysine is higher than its \( k_f \) for 6AHA (40, 41). This difference thus appears to be exhibited by apo(a) as well. This result also suggests that the lysine-binding pockets involved are equally able to accommodate the similar structure of arginine. As a control, the effect of 100 mM leucine was examined. At this concentration, leucine did not remove any apo(a) from the cell surface (Fig. 5).

**Apo(a), But Not Lp(a), Binds to the Hepatocyte Cell Surface**—Although at least a portion of mature apo(a) resides on the cell surface, no apoB co-immunoprecipitates with apo(a) in cell lysates. This suggests that either apo(a) is surface-bound during or immediately after secretion before it can interact with apoB, or that free apo(a), but not the apo(a)-apoB complex, can bind to the hepatocyte cell surface at a level that is detectable in our assay. To determine whether apo(a) recovered from conditioned media could rebind to the cell surface and to examine whether the association of apo(a) with apoB could prevent this binding,
radiolabeled conditioned culture medium was added to unla-
beled hepatocyte cultures and incubated for 4 h at 37 °C. Hepat-ocytes expressing a D isoform of apo(a) were used as this
allowed increased resolution of the apo(a) and apoB proteins on
SDS-PAGE (Fig. 1). Total lipoproteins (d < 1.25 g/ml) were then
prepared from the culture medium, and apo(a) in the lipopro-
tein and soluble (infranatant) fractions and in the cell lysate
was analyzed by immunoprecipitation (Fig. 6A). Apo(a) in the
lipoprotein and infranatant fractions of radiolabeled condi-
tioned culture medium, which had not been added back to cells,
was also examined.

A substantial portion of apo(a) added back to hepatocytes
was recovered from the cell lysate. No apoB co-immunopreci-
pitated with apo(a) from the cell lysate suggesting that only free
apo(a) became cell-associated (Fig. 6A). Densitometric scan-
ing of a lighter exposure of the autoradiograph in Fig. 6A
demonstrated that 29% of apo(a) added-back to hepatocytes
was recovered in the cell lysate. Of that remaining in the me-
dium, 36% was in the lipoprotein fraction, and 35% was in the
infranatant. In the control medium, 43% of apo(a) was in the
infranatant fraction, and 57% was in the infranatant. Virtually
all of the apo(a) that bound to the cells was therefore accounted
for by loss of free apo(a) from the culture medium. Thus, the
affinity of free apo(a) for the hepatocyte cell surface was much
greater than that of Lp(a).

To confirm that apo(a) in this experiment bound to the cell
surface by the same mechanism as defined in Figs. 4 and 5, the
ability of 6AHA, lysine and proline to prevent apo(a) binding to
the hepatocyte was examined. Unlabeled hepatocytes were in-
cubated for 4 h at 37 °C with 35S-labeled conditioned culture
medium containing between 50 and 500 mM 6AHA, lysine, or
proline. Radiolabeled apo(a), which bound to the hepatocyte
surface, was analyzed by immunoprecipitation and SDS-PAGE
(Fig. 6B). At 50 mM, 6AHA and proline reduced the amount of
apo(a) recovered from the cell lysate to 16 and 10% of the
control, respectively. Lysine had little effect at 50 mM, but at
500 mM lysine reduced cell-associated apo(a) by 90% (Fig. 6B).
This is consistent with the relative abilities of these compounds
to remove apo(a) from the cell surface (Fig. 5).

Lp(a) is known to bind to the plasminogen receptor and also
to components of the extracellular matrix such as glycosami-
noglycans. To examine the nature of the receptor for apo(a), the
ability of plasminogen and heparin to compete with apo(a) for
cell surface binding was determined (Fig. 6C). Neither plas-
mogen nor heparin prevented association of apo(a) with the
hepatocyte, even at 1 mg/ml.

Assembly of Lp(a) Can Occur at the Cell Surface—The fact
that apo(a), but not Lp(a), binds to the cell surface suggests
that the association of apo(a) with apoB changes the conforma-
tion of apo(a) such that it can no longer bind to its "receptor." To
determine whether apoB added to cell cultures could capture
apo(a) from the cell surface, labeled hepatocytes were incu-

FIG. 5. Cell surface association of apo(a) involves lysine- and proline-
binding pockets in apo(a) kringles. Hepatocytes expressing an I isoform of
apo(a) were labeled to steady state, as de-
scribed in the legend to Fig. 1. The cells
were then cooled to 0 °C, and fresh me-
dium was added containing no addition (control) or 50 mM 6AHA, lysine, arginine
or proline, or 100 mM leucine. Cells were
then incubated for a further 30 min on ice,
and apo(a) was immunoprecipitated from
cell lysates (c) and culture medium (m) and
analyzed by SDS-PAGE, as described
under "Experimental Procedures." The
positions of apo(a), its precursor (pr apo(a)), and myosin (200) are indicated.

FIG. 6. Binding of apo(a) to the cell surface. A, hepatocytes ex-
pressing a D isoform of apo(a) were labeled to steady state, as described
in the legend to Fig. 1, and the culture medium was collected. The
labeled medium was supplemented with 0.5 mM cycloheximide and was
added to fresh unlabeled cultures that had been pre-treated for 10 min at
37 °C with 0.5 mM cycloheximide. Cells were then incubated at 37 °C
for 4 h. Lipoprotein and infranatant fractions were prepared from this
medium (add-back) and from 2 ml of labeled medium that had not been
added back to hepatocytes (control) as described under "Experimental
Procedures." Apo(a) was immunoprecipitated from the cell lysate (c)
and the lipoprotein (lp) and infranatant (I) fractions and analyzed by
SDS-PAGE, as described under "Experimental Procedures." The
positions of apo(a) and apoB are indicated. B, the experiment was repeated
as above, except that culture media were supplemented with 0–500 mM
6AHA, lysine, or proline. Apo(a) bound to the cell surface was analyzed
by immunoprecipitation from cell lysates and SDS-PAGE. Panel C, as in
B, except the labeled medium was supplemented with 0–1000 µg/ml
plasminogen (Plg) or heparin.

bated for 30 min at either 4 or 37 °C in the presence or absence
of human VLDL or LDL. Lipoprotein and infranatant fractions
were prepared from the culture medium, and apo(a) in each
fraction and in the cell lysates was analyzed by immunopre-
icipitation (Fig. 7A). At 4 °C, a portion of mature apo(a) was
removed from the cell by both VLDL and LDL, and was recov-
ered in the lipoprotein fraction of the culture medium (Fig. 7A).
No apo(a) was recovered from culture medium of cells incu-
bated in the absence of lipoproteins. At 37 °C, LDL released
almost 50% of mature apo(a) into the medium, which was again
recovered entirely in the lipoprotein fraction. In other exper-
iments, as much as 70% of mature apo(a) was released into the
medium by LDL in 30 min at 37 °C (data not shown). The
greater portion of apo(a) released from the cell at 37 versus 4 °C
may reflect structural differences in the lipoprotein particle and/or cell surface at different temperatures. At 37 °C, a small amount of apo(a) was recovered from the culture medium of control cells, which represents protein secretion during the 30-min incubation period. The majority of this apo(a) was in the infranatant fraction, consistent with previous results (Ref. 22 and Fig. 2).

Analysis by nonreducing SDS-PAGE of apo(a) recovered from the cell surface by LDL at 37 °C demonstrated that only a small portion of apo(a) was disulfide linked to apoB (Fig. 7B). This was also the case when 10% serum was used to remove apo(a) from the cell surface (Fig. 7B). However, when 10% serum was present during an overnight labeling of hepatocytes, all apo(a) associated with LDL was disulfide linked to apoB (Fig. 2C). These contrasting results may reflect modification of the free cysteine residue in apo(a) after secretion when sufficient apoB is not available for interaction. Metal ions present in tissue culture media can promote oxidation of free thiols (42). Thus, apo(a) secreted directly into the presence of high concentrations of LDL may covalently link to apoB, whereas apo(a), which has been exposed to the culture medium for a prolonged period, either on the cell surface or in the supernatant, may become modified and unable to form a disulfide when mixed with LDL. This theory is consistent with studies in which a maximum of 60% of recombinant apo(a) secreted into the culture medium of transfected kidney cells was able to covalently link with LDL in vitro (26). Phillips et al. (30) were unable to demonstrate any covalent linkage between recombinant apo(a) and LDL when the two were mixed in vitro in phosphate-buffered saline, although a tight noncovalent association was formed. In contrast, essentially all recombinant apo(a) circulating free in the plasma of transgenic mice could disulfide link with injected human LDL (27). The plasma may provide a less oxidizing environment in comparison with culture medium and allow the cysteine in apo(a) to remain free. Further experimentation will be required to determine the requirements for covalent association of apo(a) and apoB.

These experiments demonstrate that apoB, in the form of either VLDL or LDL, can bind to and remove apo(a) from the cell surface. Lp(a) assembly may therefore occur at the hepatocyte surface. This may be one pathway by which Lp(a) is produced in vivo.

**DISCUSSION**

The aim of this study was to analyze the mechanisms involved in Lp(a) assembly and to examine the requirements for the interaction between apo(a) and apoB. The results establish that formation of Lp(a) occurs extracellularly and is dependent on the kringle domains in apo(a). In addition, we demonstrate that a portion of apo(a) resides on the cell surface and can be recovered in the lipoprotein fraction of culture medium by the addition of apoB-containing lipoproteins. We conclude that cell surface assembly of Lp(a) may be one pathway by which Lp(a) production in vivo.

Previous studies from this (22-24) and other (26, 27) laboratories support but provide no direct evidence that Lp(a) assembly occurs extracellularly. Results from the current study conclusively demonstrate that assembly of Lp(a) in primary cultures of baboon hepatocytes occurs after secretion. This conclusion is based on the following observations. (a) The association between endogenously synthesized apo(a) and apoB could be blocked by incubation of hepatocytes with anti-apo(a) anti-

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**Fig. 7. Cell surface assembly of Lp(a).** A, hepatocytes were labeled to steady state as described in the legend to Fig. 1. Fresh unlabelled medium was then added, containing no addition (control) or human VLDL or LDL, each at 25 μg/ml apoB protein, and the cells were incubated for a further 30 min at 0 or 37 °C. Total lipoproteins were prepared from the culture media by single-step ultracentrifugation at a density of 1.25 g/ml, as described under "Experimental Procedures." Apo(a) was immunoprecipitated from the lipoprotein (lp) and infranatant (l) fractions and from cell lysates (c) and was analyzed by 3-10% SDS-PAGE under reducing conditions. The positions of apo(a), its precursor (pr apo(a)), and myxen (200 kDa) are indicated. B, hepatocytes were labeled to steady state as described in the legend to Fig. 1. Fresh unlabelled medium was then added supplemented with human LDL (25 μg/ml apoB) or 10% baboon serum and incubated for 30 min at 37 °C. Total lipoproteins were then prepared from the culture media, as above. Apo(a) was immunoprecipitated and analyzed by SDS-PAGE under nonreducing conditions, as described under "Experimental Procedures." The positions of free apo(a) and apo(a) complexed to apoB (apo(a)/apoB) are indicated.

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**Cell Surface Assembly of Lp(a)**

28721
serum (b) Addition of apoB-containing lipoproteins to hepatocyte cultures competed endogenous radiolabeled apoB from co-immunoprecipitation with secreted apo(a). (c) Free apo(a) produced by hepatocytes could associate with apoB-containing lipoproteins to produce Lp(a) extracellularly, and (d) despite the presence of mature apo(a) on the cell surface, apoB never co-immunoprecipitated with apo(a) in cell lysates.

We observed no difference in the ability of apo(a) to bind VLDL compared with LDL. This suggests that the characteristic density of Lp(a) in vivo (1.05–1.12 g/ml) is due to the preponderance of LDL over other apoB-containing lipoproteins in plasma. In addition, this result could explain the small amount of apo(a) seen in the triglyceride-rich fraction of plasma (34, 36), and is consistent with the postprandial increase in this fraction (43, 44). Our results are in contrast with studies where free recombinant apo(a) in the plasma of transgenic mice bound much less efficiently to VLDL than LDL (27). Our study demonstrates that assembly of Lp(a) can occur at the cell surface. One explanation for the difference between the studies in transgenic mice and our hepatocyte cultures may be that immobilization of apo(a) on the cell surface facilitates its interaction with apoB. Alternatively, the results may reflect differences between baboon and human apo(a), or between native and recombinant apo(a) protein or may be a reflection of apo(a) allelic variation.

Other investigators have demonstrated that, in addition to the disulfide bond between apo(a) and apoB, noncovalent interactions contribute to the stability of the Lp(a) particle (27, 29–31). These interactions may involve lysine and proline-binding pockets in the kringle domains of apo(a) (27, 39). In the current study, we provide additional evidence that lysine-binding pockets are essential for the interaction between apo(a) and apoB, since association of the proteins could be prevented by the lysine analog, 6AHA. Lysine-binding pockets in apo(a) may play two important roles in Lp(a) assembly. Firstly, association of apo(a) with lysine residues in apoB may help position the proteins so that the disulfide linkage can form. This is supported by studies where association of apo(a) with apoB in the test tube could be inhibited by 6AHA (27). Secondly, in the current study, we demonstrate that at least a portion of mature apo(a) in cell lysates is associated with the cell surface via its kringle domains. From this location, apo(a) could be captured by apoB-containing lipoproteins resulting in Lp(a) formation. Attachment of apo(a) to the cell surface may present the protein in a better conformation for association with apoB and ensure efficient interaction between the proteins. In addition, association with the hepatocyte cell surface may prevent secretion of free apo(a) into the plasma and reduce potentially detrimental associations of apo(a) with other cell surfaces (45). In pulse-chase experiments, mature apo(a) accumulates to a relatively high concentration in hepatocyte cell lysates before appearance in the culture medium and remains associated with the cell after long chase times (22, 23). This is presumably due to the low concentration of apoB in the culture medium since supplementation of the medium with LDL during the labeling period results in a lack of detectable cell surface-associated apo(a). The specificity of the removal of apo(a) from the cell surface by apoB is emphasized by the fact that incubation with anti-apo(a) antibodies does not remove apo(a) from the cell surface despite the ability of the antibodies to bind apo(a).

The association of apo(a) with the cell surface was of high avidity, as high concentrations of amino acids were required to remove mature apo(a) from the hepatocyte. This may reflect the large number of kringle domains in apo(a), since removal of large isoforms required higher concentrations of 6AHA than small isoforms. The size polymorphism of apo(a) is due to variation in the number of kringle 4 type 2 repeats (10, 16). Our data therefore implicate a role of kringle 4 type 2 in cell surface binding. However, other studies suggest that only kringle 4 type 10 contains a pocket capable of high affinity lysine binding (38, 46). The discrepancy could be explained if low affinity lysine-binding pockets in kringle 4 type 2 are masked when apo(a) is associated with apoB but are available in the free protein. In support of this hypothesis, in add-back experiments, apo(a), but not apo(a) complexed to apoB bound to the cell surface. Due to the sheer number of type 2 kringle present, these low affinity sites may make a significant contribution to the interaction of free apo(a) with the cell surface. The importance of kringle 4 type 2 is also implicated by the fact that proline, which is predicted to bind well to kringle 4 type 2, was more effective at removing apo(a) from the cell surface than 6AHA.

The identity of the hepatocyte cell surface receptor for apo(a) is unknown. Our data suggest that heparin sulfate proteoglycans and the plasminogen receptor are not involved, since neither heparin nor plasminogen prevented cell surface binding of apo(a). Lp(a) binds to a number of components of the extracellular matrix, including glycosaminoglycans (47) and proteins such as fibronectin (48) and fibrin (49). Lp(a) can also bind the plasminogen (50, 51) and LDL receptors (52, 53). Recombinant apo(a) has also been shown to bind fibrinogen and with an apparently higher affinity than Lp(a) (54). Relatively high concentrations of Lp(a) are used in binding studies. The concentration of apo(a) and Lp(a) in our hepatocyte cultures is low. This may allow us to detect high affinity apo(a) binding but not lower affinity Lp(a) binding to the hepatocyte cell surface. Future studies will be designed to identify the nature of the hepatocyte cell surface receptor for apo(a).

Other recent studies support an expanding role of the cell surface in lipoprotein metabolism. Lipoprotein lipase is located at the endothelial cell surface by interaction with heparin sulfate (55). This may facilitate rapid internalization of fatty acids released from lipoproteins (56) and may also enhance uptake of lipoproteins, including Lp(a) (57). Recently, apoE has been shown to be present on the hepatocyte cell surface (58). This association may also be mediated by heparin sulfate (59, 60). The cell surface pool of apoE may enhance the cellular uptake of VLDL remnants (60) and HDL cholesterol (61) and may account for the increase in apoE secretion from HepG2 cells incubated in the presence of LDL (62).

From these studies, we propose the following model for Lp(a) assembly. Apo(a) is secreted from the hepatocyte as a free protein after which it may follow one of two fates. (a) Apo(a) may bind to apoB to produce an Lp(a) particle. (b) Secreted apo(a) may bind back to the cell surface. ApoB-containing lipoproteins may then associate with cell surface-bound apo(a) inducing a conformational change in the apo(a) protein and releasing it from its cell surface receptor as Lp(a). Alternatively, apo(a) may be bound to the cell surface during secretion. This alternative is supported by the fact that incubation of hepatocytes with anti- apo(a) antiserum prevented the association of apo(a) with apoB, but did not prevent apo(a) from binding to the cell surface (Fig. 1A). The predominant pathway would then be assembly at the hepatocyte surface. In either case, the preponderance of LDL in plasma over other apoB-containing lipoproteins dictates that the majority of apo(a) ends up at the characteristic Lp(a) density.

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