Expression of a Recombinant Apolipoprotein(a) in HepG2 Cells

EVIDENCE FOR INTRACELLULAR ASSEMBLY OF LIPOPROTEIN(a)*

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Apolipoprotein(a) (apo(a)), a large glycoprotein with extensive homology to plasminogen, forms a complex with apolipoprotein B100 (apoB100), which circulates in human plasma in the form of lipoprotein(a) (Lp(a)). Evidence indicates that the association of apo(a) with apoB100 occurs in the extracellular environment. We have reevaluated the possibility that apo(a)-B100 association can also occur as an intracellular event through studies with HepG2 cells stably transfected with an apo(a) minigene. Several lines of evidence support this possibility. First, continued Lp(a) production was demonstrated following incubation of transfected HepG2 cells with anti-apo(a) antisera, conditions that effectively block the fluid-phase association of apo(a) and apoB100 in vitro. Second, an apo(a)-B100 complex was detectable in Western blot analyses of transfected HepG2 lysates following immunoprecipitation with anti-apo(a) antisera. These studies incorporated precautions to eliminate cell-surface attachment of preformed apo(a)-B100 complexes to the low density lipoprotein receptor and were conducted in the presence of the lysine analog e-aminocaproic acid, which precludes apo(a)-B100 association occurring during the isolation and analyses. Third, the presence of an intracellular apo(a)-B100 complex was demonstrated in lipoproteins isolated from microsomal contents. Of particular significance was the observation that this complex contained the precursor form of apo(a), which is not secreted, in addition to the mature, recombinant form. Finally, direct evidence was provided for the synthesis of a precursor form of apo(a) in a nascent intracellular complex with apoB100 following treatment of transfected HepG2 cells with brefeldin A plus N-acetyl-leucyl-leucyl-norleucinal. Taken together, these data suggest that apo(a)-B100 association can occur as an intracellular event in a human hepatoma-derived cell line, raising important implications for the regulation of Lp(a) secretion from human liver.

Lipoprotein(a) (Lp(a))† is a cholesterol-rich lipoprotein spe-

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The abbreviations used are: Lp(a), lipoprotein(a); apo(a), apoli-

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Eagle’s medium; PVDF, polyvinylidene difluoride; PBS, phosphate-
buffered saline; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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The opposing concept, namely that apo(a)B100 association and Lp(a) formation can occur as an intracellular process in human liver, has been difficult to examine. In one study, workers were unable to demonstrate apo(a)B100 association in homogenates of human liver samples using double-antibody enzyme-linked immunosorbent assays (ELISAs) (25). Studies from our laboratory, by contrast, demonstrated the presence of an intracellular apo(a)B100 complex in primary human hepatocytes (26), but logistical limitations, coupled with the variable and generally low secretion rates of apo(a), ~ 3 orders of magnitude lower than that of apoB (26), have largely precluded a more widespread use of this approach.

In view of the importance of hepatic Lp(a) production, we have reevaluated the question of whether apo(a)B100 association can be demonstrated to occur intracellularly. We have generated stably transfected clones of HepG2 cells expressing an apo(a) minigene containing the minimal critical structural domains of KIV required for maximal association with apoB100, as specified recently (27–29). Clones of these cells, in contrast to wild-type HepG2 cells, synthesize and secrete abundant quantities of apo(a) and Lp(a) and demonstrate the presence of apoB coimmunoprecipitating with apo(a) in lysates and in microsomal contents. In addition, the form of apo(a) found to coprecipitate with apoB100 from lysates includes the precursor species, which is not secreted. These data indicate that formation of an apo(a)B100 complex in human liver-derived cells is accounted for, at least in part, by intracellular association.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human apo(a) λ clones were a gift from John McLean (Department of Cardiovascular Research, Genentech, Inc., South San Francisco, CA). Monoclonal antibodies against human apoB were a gift from Dr. F. Ollier (Ottawa University Heart Institute, Ottawa, Canada). Triton X-100 and rabbit polyclonal antibodies against human Lp(a) were previously supplied by Gunther Fless (University of Chicago). Goat anti-human apo(a) antisera was also purchased from Biorheinger Mannheim. 

**Construction of r-Apo(a) Expression Vectors—**The recombinant apo(a) expression vector, pChA, illustrated in Fig. 1, contains the signal sequence and 6 repeatsof the KIV domain, as well as the KV and protease domains. The plasmid was constructed by partial digestion, blunt-ended, and directionally subcloned into the vector (30) and rabbit polyclonal antibodies against human Lp(a) were generated by Gunther Fless (University of Chicago). Goat anti-human apo(a) antisera was also purchased from Biorheinger Mannheim.

**siRNA—**siRNA duplexes were designed and purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were duplexed with Oligo (dT)20 and annealed. 

**Immunoblot Analysis of Intracellular ApoB-Apo(a) Association—**Confluent wild-type and transfected HepG2 cells were washed three times with cold PBS and incubated at 4 °C for 4 h with PBS containing heparin (10 mg/ml) to remove residual LDL bound to LDL receptors (35). 100 mM e-aminoacaproic acid (e-ACA) was added to the incubation mixture and all buffers. This concentration was found to be 2 log orders in excess of that required to inhibit the association of recombinant apo(a) with LDL, in vitro (see Fig. 1, lower panel). The cells were washed four times with cold PBS and spiked into cold lysis buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% bovine serum albumin, 5 mM EDTA, 0.5% Tween 20) containing protease inhibitors (leupeptin (100 μg/ml), aprotinin (450 μg/ml), pepstatin (2 μg/ml), EDTA (5 μM), phenylmethylsulfonyl fluoride (1 mM), and benzamidine (1 mM)) and 100 mM e-ACA and passed several times through a 26-gauge needle. The cell lysate was centrifuged at 16,000 × g for 15 min at 4 °C, and aliquots of supernatants were immunoprecipitated with either a polyclonal anti-apo(a) antibody in the presence of e-ACA and electroblotted or with or without DT at 4 °C. The electroblots were reacted with a PVDF membrane and immunoblotted with a monoclonal antibody to apoB (1D1).

**Antibody Incubation Experiments—**Transfected HepG2 cells were maintained in serum-free media with 0.1 times the normal concentration of methionine and cysteine, together with 150 μCi/ml Tran35S-label (1000 Ci/mmol) and, incubated with 100 mM e-ACA, followed by electrophoresis. Biotinylated apo(a)- and apoB-reactive proteins were transferred to a PVDF membrane and immunoblotted with either goat anti-human apo(a) (50 μg/ml) or normal goat serum. Culture medium was collected with protease inhibitors and 100 mM e-ACA, followed by incubation at 4 °C for 4 h with PBS containing protein G-agarose. The protein G-agarose beads were washed three times with 50 mM Tris, pH 7.4, 0.65 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and two times with distilled water. Protein G-bound apo(a) complexes were eluted in gel sample buffer and analyzed by SDS-PAGE, followed by fluorography or immunoblot analysis. 

**Cell-surface Biotinylation of Transfected HepG2 Cells—**Cells were washed three times with ice-cold PBS and biotinylated for 15 min with 0.62 mg/ml NHS-LC-biotin (Pierce) in PBS (36). The biotinylation buffer was removed, fresh biotin added, and the cells incubated for another 15 min. The cells were washed three times in cold PBS, and each wash containing 100 mM e-ACA and 10 mM heparin was added, and the cells were rotated at 4 °C for 1 h. The cells were lysed and immunoprecipitated with polyclonal antisera against either apo(a) or apoB, containing 100 mM e-ACA, followed by electrophoresis. Biotinylated apo(a)- and apoB-reactive proteins were transferred to a PVDF membrane, and the immunoblots were incubated in streptavidin-horseradish peroxidase conjugate, followed by detection with ECL.

**Analysis of Intramembranous Lipoproteins—**Cells were washed four times with ice-cold PBS and scraped in homogenization buffer (10 mM Tris, pH 7.4, 250 mM sucrose) containing 100 mM e-ACA and protease inhibitors. The cell suspension was homogenized with a tight-fitting pestle in a Dounce homogenizer at 0 °C. The homogenized sample was centrifuged two times at 10,000 rpm for 10 min at 4 °C and then subjected to ultracentrifugation at 40,000 rpm for 30 min at 4 °C in a Beckman SW60Ti rotor to remove all insoluble material (37). The membrane fraction was rinsed twice with homogenization buffer, lysed at 4 °C in hypotonic sodium carbonate buffer, pH 11.3 (37), containing 100 mM e-ACA, and dialyzed overnight at 4 °C against PBS with 100 mM e-ACA. After the density was adjusted to 1.21 g/ml with solid KBr, the sample was ultracentrifuged at 100,000 rpm for 16 h at 4 °C in a Beckman TL100 ultracentrifuge. The top 150 μl was collected.
and lipoproteins precipitated with fumed silica (38). Electrophoresis and subsequent Western blot analysis of the precipitated lipoproteins were performed as detailed above. For radiolabeling experiments, the cells were pulsed for 10 min with 250 µCi/ml of Tran35S-label and chased for another 10 min. The cells were then washed three times with cold PBS, the cells were scraped into homogenization buffer as described above and total microsomes prepared. Intramicrosomal lipoproteins were scraped into cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate) containing proteinase inhibitors and centrifuged at 10,000 rpm at 4 °C for 5 min. Aliquots of cell lysate supernatants were immunoprecipitated for apo(a).

**Incubation of Transfected McA-RH7777 Cells with Brefeldin A**—Transfected McA-RH7777 cells were incubated for 1.5 h in methionine- and cysteine-free DMEM containing brefeldin A (5 µg/ml), followed by a 10-min pulse in the same medium containing Tran35S-label (250 µCi/ml). After the pulse, chase conditions were initiated by removing labeling medium and adding chase medium containing 10 mM methionine and 3 mM cysteine and brefeldin A for up to 2 h. At each time point, the cells were washed three times with cold PBS and scraped into cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate) containing protease inhibitors and centrifuged at 10,000 rpm at 4 °C for 5 min. Aliquots of cell lysate supernatants were immunoprecipitated for apo(a).

**Incubation of Human LDL with r-Apo(a)**—Incubation of Transfected McA-RH7777 Cells with Brefeldin A-tREATED Transfected McA-RH7777 Cells—Cells were pulse-labeled and chased in the presence or absence of brefeldin A as described above. After 120 min of chase, cell monolayers were washed three times with cold PBS and scraped in 1 ml of cold PBS. Cell lysates were passed three times through a 23-gauge needle and clarified by centrifugation at 10,000 rpm at 4 °C for 5 min. Aliquots of cell lysate supernatants containing equal amounts of trichloroacetic acid-precipitable radioactivity were incubated with h-LDL (1.5 µg of protein) at 37 °C for 1.5 h, followed by immunoprecipitation with anti-apo(a) antisera, SDS-PAGE, and fluorography.

**Brefeldin A and N-Acetyl-leucyl-leucyl-norleucinal (ALLN) Treatment of Transfected HepG2 Cells**—Transfected HepG2 cells were preincubated and radiolabeled for 1 h in medium containing either brefeldin A (5 µg/ml) and/or ALLN (calpain inhibitor I; Boehringer Mannheim, 40 µg/ml) and 250 µCi/ml Tran35S-label. The cells were lysed in HBS buffer (50 mM HEPES, pH 7.5, 200 mM NaCl) containing 2% sodium cholate, 100 mM e-ACA, and protease inhibitors. Lysates were immunoprecipitated with goat anti-apo(a) antisera. For sequential immunoprecipitations, cell lysates were first immunoprecipitated with goat anti-apo(a) antisera in HBS buffer containing 1% sodium cholate, 0.5% Triton X-100, and 100 mM e-ACA, following which 0.1 ml of HBS buffer (50 mM HEPES, pH 7.5, 200 mM NaCl) containing 1% SDS was added to the protein G-agarose beads and heated at 90–100 °C for 5 min. 1 ml of HBS containing 1% Triton X-100 and 100 mM e-ACA was then added to the immunoprecipitation reaction. After centrifugation, the supernatant was used for recapture-immunoprecipitation with a rabbit polyclonal antibody to apoB100. Normal, preimmune goat serum was used as a control for the first immunoprecipitation. All immunoprecipitates were analyzed by SDS-PAGE followed by fluorography.

**TABLE I**

| Cell line                        | Apo(a) | ApoB |
|----------------------------------|--------|------|
| HepG2/pCH(a)                     | 0.077 ± 0.023 | 2.81 ± 0.057 |
| HepG2/vector                     | ND    | 0.132 ± 0.036 |
| McA-RH7777/pCH(a)               | ND    | 0.436 ± 0.050 |
| McA-RH7777/vector               | ND    | ND |

a Data are from the highest producing clones of HepG2 cells (2.15) and McA-RH7777 cells (8.2).

b Values are means ± S.D. of 5 separate flasks.

c ND, not detectable.

**FIG. 1. Apo(a) expression vector.** The 6-kringle IV-repeat apo(a) construct, pCH(a), is aligned with the apo(a) cDNA as published by McLean et al. (4), with the numbering of KIV (1–37) on top. Upper panel, the numbering system 1–10 denotes the corresponding nomenclature of KIV repeats proposed by Morisetti and colleagues (31, 32). S indicates the signal sequence, P the protease domain, and F the fusion kringle, which contains 31 amino acids from KIV, and 80 amino acids from KIV, e. Lower panel, part A details the association kinetics of the recombinant apo(a) protein following in vitro incubation with human LDL to form an apo(a)-B100 complex. Conditioned culture medium (18 h) from transfected McA-RH7777 cells was incubated with human LDL for 1–60 min at 37 °C. Part B shows the inhibition of this association by the simultaneous inclusion of increasing concentrations of e-ACA throughout the 60-min incubation. Aliquots of each incubation mixture were analyzed by nonreducing 4% SDS-PAGE, followed by immunoblotting for apo(a).

**RESULTS**

**Parameters of Apo(a) and Lp(a) Production**—The highest producing clones of stably transfected HepG2 (2.15) and McA-RH7777 cells (8.2) were selected for further study. Culture media from the HepG2 clone demonstrated the presence of both apo(a) and Lp(a) as determined by ELISA (Table I). The relative distribution of apo(a), as inferred from both the ELISA data and by Western blot analysis of conditioned culture media (Fig. 1, lower panel, A), reveals that the majority (~70%) of apo(a) is unassociated with apoB100. Accumulation of apo(a)
Apo(a)-B100 Association—Cells: Incubation with Anti-apo(a) Antisera Does Not Inhibit presence of anti-apo(a) antisera. Immune complexes isolated HepG2 cells were radiolabeled for either 6 or 20 h in the exclusively extracellular association. Accordingly, transfected HepG2 cells have demonstrated that the association of apo(a) and apoB100, is eliminated in the presence of anti-apo(a) antisera (23). These conditions were applied to transfected HepG2 cells in order to determine whether Lp(a) assembly, as judged by the criterion of coimmunoprecipitation with anti-apo(a) antisera (lane 3). This complex reduces in the presence of 100 mM DTT (+) to a product with the mobility of LDL apoB100. This is a representative illustration of three independent experiments.

from the media demonstrated a coimmunoprecipitating band with the anticipated mobility of apoB100 (Fig. 2A, lanes 2 and 3). The identity of this band was confirmed by Western blot analysis of the immunoprecipitate using an anti-apoB antibody (Fig. 2A, lane 4). To establish that the conditions used fully inhibit fluid-phase association of apo(a) and apoB100, radiolabeled culture medium from transfected McA-RH7777 cells was incubated (with or without antisera) for 2 h at 37 °C with human LDL. These conditions allow maximal association of apo(a) and apoB100 in vitro (see Fig. 1, lower panel, A). Aliquots of the medium were analyzed under nonreducing conditions, the results demonstrating that incubations performed in the presence of anti-apo(a) antisera completely blocked apo(a)-B100 association (Fig. 2B, compare lanes 2 and 3). Repeated immunoprecipitation of the supernatant from lane 3 with anti-apo(a) antisera failed to recover additional apo(a) (Fig. 2B, lane 4), indicating that the initial incubations were conducted under conditions of antisera excess. Accordingly, the results of the experiments shown in Fig. 2A are inconsistent with the hypothesis that a complex containing apo(a) and apoB100 arises exclusively from extracellular association. Comparison of apoB100 cpm coimmunoprecipitated with apo(a) from the media of radiolabeled, transfected, HepG2 cells incubated either
in the presence or absence of anti-apo(a) antisera revealed that 62 ± 16% (n = 6) of the apoB was resistant to inhibition of fluid-phase association, implying an origin of the apo(a)-B100 complex that is inaccessible to apo(a) antiserum.

**FIG. 5.** Demonstration of an intracellular apo(a)-B100 complex in microsomal d < 1.21 g/ml lipoproteins from transfected HepG2 cells. A and B, cells were grown overnight in 10% fetal bovine serum, microsomes prepared as described under “Experimental Procedures,” and intramicrosomal lipoproteins of d < 1.21 g/ml isolated by ultracentrifugation. The intramicrosomal lipoproteins (Micr.) were adsorbed to fumed silica, electrophoresed on a 4% polyacrylamide gel, and transferred onto a PVDF membrane. The membranes were probed with a polyclonal rabbit anti-apo(a) antibody (A) or with a monoclonal anti-apoB antibody (B). Serum-free culture medium (CM) from transfected HepG2 cells is used as a marker to confirm the migration of the apo(a)-B100 complex (B(a)) and apoB100, respectively. The migration of the precursor (p-apo(a)) and mature (r-apo(a)) recombinant apo(a) forms are illustrated. This is a representative illustration of three independent experiments. C, transfected HepG2 cells were radiolabeled for 10 min and chased for 10 min, followed by alkylation with 50 mM iodoacetamide. Microsomes were isolated, and intramicrosomal lipoproteins of d < 1.21 g/ml were immunoprecipitated using anti-apo(a) antisera. The mobility of the precursor (p-apo(a)), mature (r-apo(a)), and apoB100 forms is indicated. D, wild-type HepG2 cells were radiolabeled overnight and culture medium and cell lysates were immunoprecipitated using one of the following: anti-apo(a) antiserum, a mixture of anti-apoB monoclonal antibodies (1D1, 4G3, Bsol 7, and Bsol 16), normal goat serum (NGS), or normal mouse serum (NMS).

In view of the findings of White et al. (23), demonstrating that Lp(a) assembly occurs at the cell surface of baboon hepatocytes, experiments were conducted to evaluate this process in transfected HepG2 cells. Cell-surface biotinylation was undertaken in confluent monolayers of transfected HepG2 cells, which were then washed and incubated with e-ACA ± heparin as described above. Cell lysates were prepared and separate
immunoprecipitations performed for apo(a) and apoB. Under these conditions, coimmunoprecipitating bands that have a cell-surface origin will be biotinylated and can be detected in Western blots using streptavidin-HRP. Apo(a) immunoprecipitated from lysates prepared without heparin treatment demonstrated a coprecipitating apoB100 band, confirming the presence of a cell surface-associated apo(a)-B100 complex (Fig. 4). By contrast, no coprecipitating apoB100 band was found when apo(a) immunoprecipitations were performed on cells incubated in the presence of heparin (compare + and − α lanes, Fig. 4). Heparin treatment greatly reduced the recovery of apoB100 (compare aB lanes + and − heparin), confirming previous observations (35) that a large proportion of the cell-surface apoB is found in association with LDL receptors from these HepG2 cells; importantly, however, no coprecipitating apo(a) band was seen with the apoB100 immunoprecipitations. Taken together, these experiments suggest that any apo(a)-B100 complexes present on the cell surface can be virtually eliminated with a combination of heparin and e-ACA treatment. This observation, coupled with the demonstration (see Fig. 3) that an apo(a)-B100 complex is detectable in cell lysates prepared from HepG2 cells incubated under conditions (heparin + e-ACA) that minimize any possible contribution of pre-formed, surface-associated apo(a)-B100 complexes, further supports the possibility of an intracellular source of the apo(a)-B100 complex.

Demonstration of an Apo(a)-B100 Lipoprotein Complex Isolated from Microsomal Contents—In order to pursue the possibility that an apo(a)-B100 complex might represent de novo intracellular synthesis, microsomes were prepared from transfected HepG2 cells and the content lipoproteins isolated at densities less than 1.21 g/ml. These lipoproteins were adsorbed to silica and subjected to SDS-PAGE and Western blotting with antisera specific for either apo(a) or apoB100. Apo(a) was demonstrated in a covalent complex with apoB100, as evidenced by the presence of an apo(a)-immunoreactive band in the nonreduced sample (Fig. 5A, Micr. −). Interestingly, upon reduction of this complex with DTT, there appeared two apo(a)-immunoreactive bands, one of which was of a size compatible with the precursor form of the protein and was not detectable in culture medium (Fig. 5A; see also Figs. 6 and 8). A corresponding complex of apo(a)-B100 was detectable with anti-apoB100 antisera (Fig. 5B); this band disappeared upon reduction of the complex with DTT. These results, demonstrating the presence of a covalent apo(a)-B100 complex that includes the precursor form of apo(a), provide further support for the possibility of an intracellular source of this complex, since the precursor form of apo(a) is not secreted from the cell. This experiment was also undertaken following a brief (10 min) radiolabel in order to identify the newly synthesized precursor form of apo(a). In addition, cell lysates were prepared following alkylation in order to prevent disulfide bond formation during preparation of the microsomes. The results of this experiment (Fig. 5C) confirm the presence of apoB100 coimmunoprecipitating with the (newly synthesized) precursor form of apo(a) from microsomal contents. Fig. 5D shows the absence of apoB100, in control immunoprecipitations with wild-type HepG2 cells, generated with anti-apo(a) antisera.

The Precursor Form of Apo(a) Is Competent to Associate with ApoB100 in Vitro—Identification of the precursor apo(a) protein in a lipoprotein complex with apoB100, isolated from microsomal contents, strongly implies the possibility of an intra-

**Fig. 6.** The effect of brefeldin A on the distribution of the precursor and mature forms of apo(a) in transfected McA-RH7777 cells. Stably transfected McA-RH7777 cells were preincubated in methionine- and cysteine-free DMEM containing 5 μg/ml brefeldin A, pulse-labeled for 10 min using Tran35S-label, and chased for 10–120 min with serum-free medium containing brefeldin A. Control (A) and brefeldin A-treated (B) cell lysates were immunoprecipitated using an apo(a)-specific polyclonal antibody and electrophoresed under nonreducing (A) or reducing (B) conditions on 4% SDS gels. The gels were exposed for 14 days at −70°C with enhancing screens. The mobilities of the precursor (p-apo(a)), mature form (r-apo(a)), and the apo(a)-B100 complex (B(a)) are shown along with the mobility of the 200-kDa molecular size marker. This is a representative illustration of two independent experiments.

**Fig. 7.** The precursor form of apo(a) from brefeldin A-treated McA-RH7777 cells is competent to associate with LDL apoB100. Transfected McA-RH7777 cells were incubated and radiolabeled in the presence (+BFA) or absence (−BFA) of 5 μg/ml brefeldin A as detailed in the legend to Fig. 6. Cell lysates were prepared from a 120-min chase, and aliquots were incubated with 1.5 μg of human LDL for 1.5 h at 37°C. Aliquots were immunoprecipitated using anti-apo(a) polyclonal antiserum and electrophoresed under nonreducing (A) or reducing (B) conditions on 4% SDS-PAGE gels and processed for fluorography. The positions of molecular weight markers and of the mature (r-apo(a)) and precursor (p-apo(a)) forms of apo(a) are indicated. Figure is representative of four such experiments.
cellular origin for this complex. In order to investigate this possibility further, evidence was sought for the ability of the precursor form of apo(a) to associate with LDL-apoB100 following in vitro incubations. Stably transfected McA-RH7777 cells were radiolabeled in the presence or absence of brefeldin A in order to trap the precursor form of apo(a) within the endoplasmic reticulum (40). As shown in Fig. 6A, immunoprecipitation of apo(a) from control cells demonstrates the expected precursor-product distribution with time following pulse-chase. Cells radiolabeled in the presence of brefeldin A demonstrate only the precursor form of the protein (Fig. 6B). Cell lysates prepared under these conditions and examined for their ability to assemble an apo(a)-B100 complex. As shown in Fig. 7A, assembly of a covalent complex was seen with the precursor form of apo(a) as well as the mature recombinant apo(a). These data thus establish the potential for apo(a)-B100 complex formation in vivo with the precursor form of apo(a).

A Newly Synthesized, Precursor Form of Apo(a) Associates with ApoB100 in HepG2 Cells—In order to demonstrate conclusively that the precursor form of apo(a) actually assembles into a complex with apoB100 in vivo, further experiments were performed using the 2.15 clone of transfected HepG2 cells. The purpose of these experiments was to demonstrate the presence of newly synthesized apoB100 in coimmunoprecipitations performed with anti-apo(a) antisera as a measure of intracellular complex formation. In order to maximize the possibility of detecting apoB100, these experiments were performed in the presence of the calpain protease inhibitor ALLN, which has previously been demonstrated to reduce the intracellular degradation of apoB100 (41). Accordingly, cells were radiolabeled in the presence of either ALLN alone or ALLN plus brefeldin A and immunoprecipitations performed using anti-apo(a) antisera. Cells incubated with ALLN alone demonstrated both the precursor and mature recombinant apo(a) protein, while cells incubated with the combination of ALLN plus brefeldin A demonstrated only the precursor apo(a) form (Fig. 8A). In both instances a faint coimmunoprecipitating band, consistent with apoB100, was demonstrated (Fig. 8A). No apoB100 band could be detected in the absence of ALLN (data not shown). Subsequent experiments were undertaken using sequential immunoprecipitation in order to confirm the identity of the coimmunoprecipitating band as apoB100. These experiments demonstrate conclusively that apoB100 coprecipitates with the precursor form of apo(a) from cell lysates of ALLN plus brefeldin A-treated HepG2 cells (Fig. 8B). The demonstration of an apo(a)-B100 complex in brefeldin A-treated HepG2 cells implies that formation of this complex may be a very early event in the secretory pathway.

DISCUSSION

A considerable amount of information has accumulated in support of the hypothesis that Lp(a) assembly is an extracellular process (9, 42). The current results add an additional dimension to the understanding of Lp(a) assembly by demonstrating that the association of apo(a) and apoB100 can occur intracellularly in a human liver-derived cell line. It bears emphasis that the results of the current study in no way diminish earlier conclusions indicating that the association of apo(a) and apoB100 can take place in a cell-free system; indeed, the ability of our recombinant apo(a) to associate with apoB100 in this manner was confirmed. Nevertheless, the demonstration of intracellular apo(a) in complex with apoB100 raises important implications for the regulation of apo(a) secretion from human hepatocytes.

Several lines of evidence were sought to examine the possibility of intracellular association of apo(a) and apoB100. The following observations support this hypothesis. First, the association of apo(a) and apoB100 in the medium of transfected HepG2 cells could not be blocked by the addition of anti-apo(a) antiserum. Second, apoB100 was found in coimmunoprecipitate with apo(a) from cell lysates prepared from transfected HepG2 cells. Both of these observations represent critical differences from previous reports using baboon hepatocytes (22, 23) and will be discussed in more detail below. Third, lipoproteins prepared from microsomal contents were found to contain a complex of apoB100 and apo(a) and, perhaps more importantly, the form of apo(a) demonstrated in this complex included the precursor, which is not secreted. Fourth and finally, synthesis of a precursor form of apo(a) that associates with apoB100 was demonstrated in transfected HepG2 cells treated with a combination of brefeldin A and ALLN. Each of these observations merits further consideration.

As alluded to above, previous work by White and co-workers demonstrated that the addition of anti-apo(a) antiserum to primary cultures of baboon hepatocytes completely blocked Lp(a) assembly, an observation that strongly suggested apo(a)-apoB100 association was primarily an extracellular event (23). The current studies applied a similar experimental paradigm...
to transfected HepG2 cells, yielding results that indicate continued apo(a)-apoB100 association. Precautions were taken to ensure that surreptitious association of apo(a) and apoB100 was not a factor in these results, including the use of e-ACA in all the buffers. In addition, the antibody incubation conditions were demonstrated to completely block fluid-phase association of apo(a) and apoB100. The most reasonable interpretation of these results is that an antiserum-inaccessible source of apo(a) is available for association with apoB100. Among the possibilities considered in this regard are cell membrane-associated and/or intracellular pools of apo(a) and apoB100. In consideration of the first possibility, there is a clear precedent for the cell-surface association of apo(a) on HepG2 cells (39, 43). Recent studies by Tam and colleagues have demonstrated that apo(a) binds to at least two classes of receptors on HepG2 cells. These authors identified a high affinity (heparin-displaceable) binding site, which corresponds to the LDL receptor, while the second is a low affinity site, which is competed for by the presence of lysine analogs and plasminogen (39).

The presence of recombinant apo(a) in association with apoB100 on the surface of HepG2 cells was confirmed by the biotinylation studies, which demonstrated a (coimmunoprecipitating/biotinylated)-apoB100 band in immunoprecipitations of transfected HepG2 cell lysates performed with anti-apo(a) antisera. This coimmunoprecipitating species was not detectable, however, following heparin treatment, suggesting the possibility that this apo(a)-B100 complex was attached to the LDL receptor. It is important to emphasize that the Western blot analysis presented in Fig. 3, demonstrating an apo(a)-B100 complex in HepG2 cell lysates, was conducted following heparin incubation, thus rendering it unlikely that the presence of a coimmunoprecipitating apoB100 band in these lysates could be accounted for by the binding to LDL receptors, of apo(a)-apoB100 complexes formed in the media. In addition, all lysis and immunoprecipitation steps were conducted in the presence of the lysine analog e-ACA, making it unlikely that the residual, low affinity binding of apo(a) could result in the artificial generation of a complex with apoB100 liberated from within heparin-inaccessible domains in the cell membrane.

The current studies are the first to demonstrate the presence of a coimmunoprecipitating apoB100 band in transfected HepG2 cell lysates immunoprecipitated with anti-apo(a) antisera. Koschinsky and colleagues specifically examined the possibility of intracellular synthesis of Lp(a) in transfected HepG2 cells but were unable to detect coimmunoprecipitation of apoB100 in lysates immunoprecipitated with anti-apo(a) antisera (19, 20). Additionally, White and colleagues demonstrated that, despite the presence of mature apo(a) on the cell surface of baboon hepatocytes, apoB was never detectable in immunoprecipitations performed with anti-apo(a) antisera (19, 20). Koschinsky and colleagues specifically examined the possibility of intracellular synthesis of Lp(a) in transfected HepG2 cells but were unable to detect coimmunoprecipitation of apoB100 in lysates immunoprecipitated with anti-apo(a) antisera (19, 20). Additionally, White and colleagues demonstrated that, despite the presence of mature apo(a) on the cell surface of baboon hepatocytes, apoB was never detectable in immunoprecipitations performed with anti-apo(a) antisera (22, 23).

Several differences in the current experimental model may account for some aspects of this fundamental discrepancy. Earlier studies in transfected HepG2 cells examined the issue of coimmunoprecipitation of apo(a) and apoB100 using an apo(a) construct with 17 repeats of KIV (19–21). The large size of the predicted protein expressed from this cDNA makes it difficult to distinguish apoB100 from apo(a), thus creating ambiguity in the interpretation of coimmunoprecipitating bands. An additional technical limitation may be that, at least in our hands, transfection of this size construct results in considerably lower apo(a) protein secretion into the media of either HepG2 or MCA-RH7777 cells than found with the 6-KIV construct used in the current study. This impression is consistent with other studies in baboon hepatocytes by White and colleagues, who demonstrated an inverse correlation between apo(a) size and processing efficiency in the endoplasmic reticulum, resulting in lower secretion of the larger isoforms (44). Thus, an important technical advance in the present approach is the ability to express apo(a) at high levels and in a secreted form, which is readily distinguishable in size from apoB100. The sheer abundance of these protein species within lysates of transfected HepG2 cells may be one important factor in our ability to detect the presence of apoB100 in coimmunoprecipitations performed with anti-apo(a) antisera.

Further evidence in support of an intracellular origin of the apo(a)-B100 complex in transfected HepG2 cells was the demonstration of a precursor form of apo(a) in a complex with apoB100 isolated from microsomal lipoproteins. It is well established, from studies in both transfected HepG2 cells and in baboon hepatocytes, that the precursor form of apo(a) is found in the endoplasmic reticulum, where it undergoes glycosylation and additional processing to yield the mature species (22, 23). Of relevance to the current findings, however, is the observation that the precursor form of apo(a) is not secreted into the media (19–23). Proof that HepG2 cells synthesize an apo(a)-B100 complex, which includes the precursor form, was sought through experiments in which brefeldin A and ALLN were added to transfected HepG2 cells and radiolabeled cell lysates examined by sequential immunoprecipitation. The inclusion of ALLN was essential to the demonstration of an apoB100 band in co- and sequential immunoprecipitation experiments and suggests that the ability to detect small quantities of intracellular apoB in complex with apo(a) may be appreciably improved by limiting apoB degradation (41). In this context, it will be important to examine apo(a) synthesis and secretion and Lp(a) formation in this clone of HepG2 cells following exposure to oleate treatment, another mechanism proven to stabilize apoB100 (45, 46). Such studies are currently under way.

Several limitations in this experimental paradigm should also be emphasized so as to place in context the physiological significance of these findings. In particular, the relevance of intracellular apo(a)-B100 association using an apo(a) minigene containing only 6 repeats of kringle IV may have only limited significance to our understanding of Lp(a) assembly in human liver, where the minimum number of such repeats exceeds 12 (4–6). In addition, the demonstration of apoB100 coimmunoprecipitating with apo(a), despite the fact that apo(a) was never demonstrated to coimmunoprecipitate in complexes generated with anti-apoB antisera, remains unexplained. Among the possible explanations for this apparent paradox are that, compared to the intracellular pool of apoB, a relatively larger percentage of the intracellular apo(a) pool contains the apo(a)-B100 complex. This issue is difficult to resolve at present, since there are no physical or biochemical methods by which to isolate selectively the free and complexed forms of intracellular apo(a). A related issue is that the amount of apo(a) actually available for association with apoB100 is likely to be only a fraction of the total pool, based on the observation that prolonged, cell-free incubations with human LDL result in a maximum complex formation of 25–30% of the input apo(a) (Fig. 1). These values are comparable to those reported by other workers (19, 20) and have been interpreted to suggest that a large percentage of apo(a) is incorrectly folded with respect to the domains required for association with apoB100 (19, 20).

These reservations notwithstanding, the current results illustrate the feasibility of approaching questions concerning the structural requirements for the covalent association of apo(a) with apoB100, both intracellular and extracellular, and such studies form the focus of ongoing investigation.
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