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Cys^{4057} of apolipoprotein(a) is essential for lipoprotein(a) assembly

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ABSTRACT **Lipoprotein(a) contains one copy each of apolipoprotein B-100 and apolipoprotein(a). It has been hypothesized that a disulfide bond might exist between Cys^{4057} of apolipoprotein(a) and Cys^{3734} in apolipoprotein B-100. To investigate the role of Cys^{4057} for lipoprotein(a) assembly, wild-type and *in vitro* mutagenized apolipoprotein(a) cDNA plasmids were expressed in the human hepatocarcinoma line HepG2. The mutant plasmids encoded apolipoprotein(a) species with Cys^{4057} exchanged to either serine or glycine. Untransfected HepG2 cells, although able to secrete apolipoprotein B-100-containing lipoproteins, do not synthesize detectable amounts of apolipoprotein(a). After transfection of wild-type plasmid, almost all apolipoprotein(a) in the culture supernatant was present in lipoprotein(a)-like particles as demonstrated by immunoblotting, density-gradient centrifugation, and ELISA. The same analysis performed with supernatants of cells transfected with plasmids mutated in codon 4057 revealed free apolipoprotein(a) glycoprotein without detectable amounts of lipoprotein-associated apolipoprotein(a). Our results strongly suggest the existence of a disulfide bridge between Cys^{4057} of apolipoprotein(a) and apolipoprotein B-100 within recombinant lipoprotein(a) particles. Furthermore, they indicate that disulfide bridge formation is essential for assembly of the lipoprotein(a)-like complex produced by HepG2 cells and suggest a similar role of Cys^{4057} during lipoprotein(a) assembly *in vivo*.**

Lipoprotein(a) [Lp(a)] is a low density lipoprotein (LDL)-like particle composed of a cholesterol ester-rich lipid core, one molecule of apolipoprotein B-100 (apoB), and a glycoprotein called apolipoprotein(a) [apo(a)] (1). Plasma apo(a) is derived almost exclusively from the liver as shown by mRNA analysis (2) and liver transplantation studies (3). High plasma concentrations of Lp(a) represent an independent risk factor for the development of premature atherosclerosis (4, 5). Mice transgenic for human apo(a) develop atherosclerosis upon cholesterol feeding (6).

Cloning and sequencing of the first complete apo(a) cDNA from human liver (7) revealed a striking homology between apo(a) and plasminogen. Both proteins belong to a protein family characterized by the presence of one or more kringle domains with six highly conserved cysteine residues, which are believed to be involved in three intradomain disulfide bridges. Plasminogen contains single copies of five different kringle domains and a serine protease domain. Referring to the numbering of plasminogen kringles, the first sequenced apo(a) cDNA encoded a glycoprotein with 37 tandemly repeated kringle IV-like domains, a single kringle V domain, and a serine protease domain. Ten distinct kringle IV domains were encoded by the sequenced apo(a) cDNA. One of them occurred in 28 identical copies; the others were unique.

In human plasma, almost all apo(a) is present as a macromolecular Lp(a) complex. As the dissociation of the LDL moiety and apo(a) in Lp(a) requires reducing conditions, a disulfide bridge between apo(a) and apoB has been postulated (8, 9). In addition to the six cysteines putatively involved in intradomain disulfide bridges, a seventh unpaired cysteine has been found in the penultimate kringle IV domains of human and rhesus monkey apo(a) (7, 10). It has been hypothesized that this cysteine occurring in codon 4057 (kringle 36) might be involved in disulfide linkage to apoB (7).

To directly address the role of Cys^{4057} for the assembly of Lp(a) we have used the human hepatocarcinoma cell line HepG2. These cells, although capable of secreting different apoB-containing lipoprotein particles (11, 12), do not produce detectable amounts of Lp(a). Here, we have characterized HepG2 cells transfected with wild-type or mutant apo(a) plasmids. The mutant plasmids encode apo(a) species that differ from wild-type apo(a) by single amino acid substitutions at position 4057.

MATERIALS AND METHODS

Cell Lines and Cell Culture. HepG2 cells (13) were obtained from the American Type Culture Collection. Cells were cultured in minimal essential medium (MEM) with Earle's salts supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, and 1% MEM nonessential amino acids (Boehringer Mannheim).

Poly(A)$^+$ RNA Isolation. A biopsy sample, shock-frozen in liquid nitrogen, was used for isolation of total human liver RNA by the guanidinium isothiocyanate/CsCl method (14). Poly(A)$^+$ RNA was purified with oligo(dT)-coupled magnetic beads [Dynabeads from Dynal (Oslo)].

Oligonucleotides and PCR. Oligonucleotides were synthesized with a model 392 DNA/RNA synthesizer (Applied Biosystems) and purified by oligonucleotide purification columns (15). PCR amplifications were achieved by using an AmpliTaq PCR kit (Perkin–Elmer/Cetus) and a model 480 thermocycler (Perkin–Elmer/Cetus).

Construction of Apo(a) cDNA Expression Plasmids. Standard cloning techniques (16) were used to assemble apo(a) cDNA fragments into an expression vector containing the cytomegalovirus (CMV) promoter/enhancer/splice donor, immunoglobulin splice acceptor, and the simian virus 40 polyadenylylation signal (Fig. 1). Briefly, the 5′ end of the apo(a) cDNA, encoding the signal peptide and kringle 1 sequences, was derived from short synthetic fragments. Human placenta DNA served as starting material to assemble the kringle 2A repeats contained in the pCMV-A18 vector: PCR amplification of the two exons encoding kringle 2A and subsequent PCR ligation of the amplification products gave rise to a kringle 2A fragment. A *Bst*XI fragment encoding four tandem repeats of kringle 2A was generated by oligomerization of *Bst*XI kringle 2A repeats and subsequent introduction of silent mutations to destroy internal *Bst*XI sites. Two *Bst*XI fragments were assembled in the pCMV-A18 vector to en-

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Abbreviations: apo(a), apolipoprotein(a); Lp(a), lipoprotein(a); LDL, low density lipoprotein; apoB, apolipoprotein B-100; CMV, cytomegalovirus; r, recombinant; HDL, high density lipoprotein; EACA, ε-aminocaproic acid; PDI, protein disulfide isomerase.

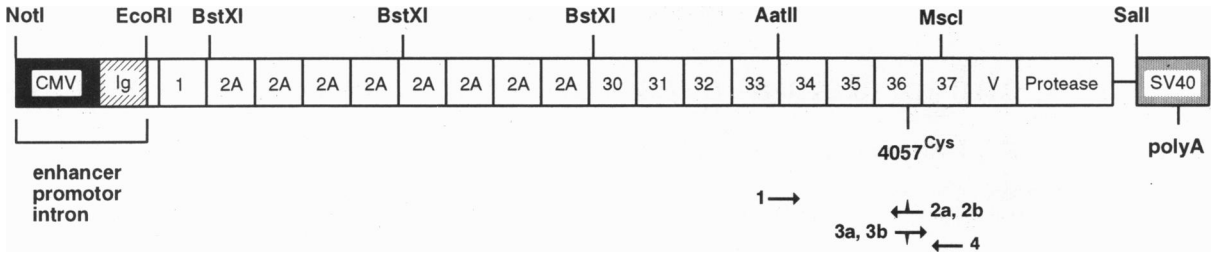

FIG. 1. Apo(a) cDNA expression construct. A 6849-bp apo(a) cDNA is flanked by the CMV enhancer promoter/splice donor (solid box) and immunoglobulin splice acceptor (hatched box) at the 5′ end and a simian virus 40 (SV40) polyadenylylation site (stippled box) at the 3′ end. The recombinant cDNA encodes the signal sequence, eight copies of the repetitive kringle 2A, all unique kringle IV domains (kringle 1 and kringles 30–37, kringle V), the protease domain, and some 3′ untranslated sequences of apo(a). Arrows indicate primers used for site-directed PCR mutagenesis.

code eight kringle 2A repeats. The unique 3′ sequences of the apo(a) cDNA, encoding kringles 30–37 and kringle V, were obtained from human liver poly(A)$^+$ RNA by reverse transcription/PCRs. Five overlapping fragments were PCR amplified from the first-strand cDNA product and successively aligned by further PCRs and subcloning steps. DNA prepared from a human liver cDNA library (Clontech) was used as a template to amplify a 700-bp fragment encoding the apo(a) protease domain and 50 bp of 3′ untranslated apo(a) sequences. A detailed description of the pCMV-A18 construction is available on request.

Site-Directed PCR Mutagenesis. To introduce single point mutations into the plasmid pCMV-A18, a 1.1-kb *Aat* II/*Msc* I fragment was replaced with fragments containing the desired nucleotide exchanges. The mutagenized fragments were prepared by two rounds of PCR (Fig. 1). Oligonucleotides 1 (GATCGAATTCGTGTCCTTGCGACGT), 2A (CCTCACAGACGGATCGGTTG), 3A (CCGATCCGTCTGTGAGGTGG), 2B (CCTCACACCCGGATCGGTTG), 3B (CCGATCCGGGTGTGAGGTGG), and 4 (GATCGTCGACTGGCCATTACCATGA) were used to amplify two overlapping fragments for each mutation. Subsequent alignment of the overlapping PCR fragments was achieved by second rounds of PCR with external primers 1 and 4. The identity of the desired mutations as well as the correctness of all PCR-derived sequences were verified by DNA sequence analysis.

DNA Sequence Analysis. Double-stranded plasmid DNAs were sequenced (17) using Sequenase version 2.0 (United States Biochemical).

Transient Transfection. Transient expression of apo(a) plasmids was achieved by liposome-mediated gene transfer (18). One day before transfection, 10^6 cells were seeded in 50-mm tissue culture dishes. At the day of transfection, cells were washed once with 4 ml of transfection medium [Opti-MEM (GIBCO/BRL) supplemented with 10% fetal calf serum, 2 mM glutamine, and 1 mM sodium pyruvate]. One hundred microliters each of solution A [25 μl of CsCl purified plasmid DNA (200 ng/μl in $T_{10}E_1$) and 70 μl of Hepes-buffered saline (HBS)] and solution B [30 μl of Lipofectin (GIBCO/BRL) and 70 μl of HBS] were preincubated for 10 min at room temperature to form DNA–liposome complexes. After addition of 1.8 ml of transfection medium, the mixture was transferred to the washed cells; 4–5 h later the medium was aspirated and the cells were incubated for 48 h in 4 ml of culture medium. Prior to analysis, cell culture supernatants were adjusted to 1 mM phenylmethylsulfonyl fluoride and centrifuged 10 min each at 300 × *g* and 4000 × *g* in order to remove detached cells and cell debris.

SDS/PAGE and Immunoblotting. Cell culture supernatants were fractionated by reducing or nonreducing SDS/PAGE (19). Polyacrylamide concentrations were 3% for the stacking gel and 4.5% for the separating gel. A size marker containing four apo(a) isoforms was included. After electrophoresis, proteins were transferred to enhanced chemiluminescence (ECL) nitrocellulose filters (Amersham) (20). Immunodetections using apo(a)-specific antibody 1A2 (21) or a rabbit antiserum raised against human LDL were performed with the ECL Western blot detection kit (Amersham).

Ultracentrifugation. Culture supernatants (2 ml) were adjusted to a density of 1.21 g/ml with potassium bromide (KBr) and centrifuged 90 min at 85,000 rpm and 10°C in a Beckmann TL-100 centrifuge equipped with a TLV-100 rotor. The top 500 μl was collected as lipoprotein fraction. Density-gradient centrifugation was performed as described (22). Tubes were unloaded from the bottom into 22 fractions.

ELISA. Lp(a)/apo(a) levels were measured by a sandwich ELISA. Lp(a) reference standard (Immuno, Vienna) was used for standardization. Sheep anti-human Lp(a) (Immuno) was used to coat the solid phase. Incubation with apo(a)-specific monoclonal antibody 1A2 (21) as second antibody was followed by detection with anti-mouse IgG (Fab fragment) conjugated to horseradish peroxidase (Boehringer Mannheim) and 2,2′-azinobis(3-ethylbenzthiazoline sulfonate) substrate. To detect apoB–apo(a) complexes, anti-human apoB (Boehringer Mannheim) coating reagent was used in combination with monoclonal antibody 1A2. Alternatively, sheep anti-human Lp(a) was used together with apoB-specific monoclonal antibody mab012 (Chemicon) as second antibody.

RESULTS

Expression of Wild-Type Recombinant (r)-Apo(a) in HepG2 Cells Yields Apo(a)-Containing Lipoproteins. As a basis for the construction of apo(a) expression plasmids, we used various cloning techniques to assemble a cDNA plasmid encoding a r-apo(a) isoform with 17 kringle IV units, a single kringle V domain, and a protease domain. To achieve high expression of r-apo(a), we introduced the CMV promoter/enhancer and a heterologous intron 5′ to the apo(a) coding sequence. The resulting plasmid pCMV-A18 was transiently transfected into HepG2 cells. Untransfected HepG2 cells neither secrete apo(a) (see Fig. 3 *A* and *B*, lanes 4) nor express detectable amounts of apo(a) mRNA or intracellular protein (data not shown). Forty-eight hours after transfection of pCMV-A18 into HepG2 cells, culture supernatant was analyzed by reducing SDS/PAGE and subsequent immunoblotting with an apo(a)-specific monoclonal antibody. pCMV-A18-encoded r-apo(a) (calculated polypeptide mass, 250 kDa) expressed in HepG2 cells migrated to a position corresponding to an apparent molecular mass of >550 kDa (Fig. 2, lane 2). The observed electrophoretic mobility of pCMV-A18-encoded apo(a) indicates extensive posttranslational modification of HepG2-derived r-apo(a) as described for human plasma apo(a) (23) and for r-apo(a) derived from transfection of a similar construct in HepG2 and 293 cells (24). The broad r-apo(a) bands in Fig. 2 result from over-

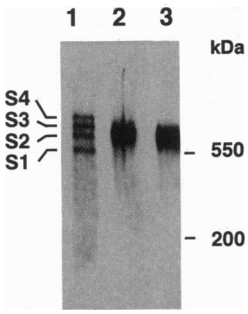

FIG. 2. Immunoblot analysis of wild-type r-apo(a) from HepG2 cells. After transfection of plasmid pCMV-A18 into HepG2 cells, equivalent aliquots of culture medium (lane 2) and of the lipoprotein fraction (lane 3) obtained by density ultracentrifugation were analyzed by reducing SDS/PAGE immunoblotting with apo(a)-specific monoclonal antibody 1A2. For size determination, apo(a) phenotyping standard containing isoforms S1, S2, S3, and S4 was included (lane 1). Positions of apoB (550 kDa) and myosin (200 kDa) are indicated.

loading the corresponding lanes. With less protein loaded to the gel, the r-apo(a) bands become much sharper and migrate to a position between isoforms S1 and S2 (data not shown).

To investigate how much of the pCMV-A18-encoded apo(a) expressed in HepG2 cells was associated with lipoproteins, culture medium conditioned by transfected cells was fractionated by ultracentrifugation. Equivalent aliquots of unfractionated culture medium and lipoprotein fraction were analyzed by immunoblotting. As shown in Fig. 2 (lanes 2 and 3), there was almost no difference in the intensity of anti-apo(a)-reactive protein in both samples, indicating that the majority of r-apo(a) produced by pCMV-A18-transfected HepG2 cells was present in the lipoprotein fraction.

Lp(a) Particle Assembly Is Affected by Mutations of Cys^{4057}. By means of site-directed mutagenesis we exchanged the TGT codon encoding Cys^{4057} in pCMV-A18 to either TCT (serine) or GGT (glycine). The resulting mutant plasmids were designated pCMV-A18-4057^{Ser} and pCMV-A18-4057^{Gly}, respectively.

Wild-type and mutant apo(a) plasmids were transiently expressed in HepG2 cells. Forty-eight hours after transfection, apo(a) entities in the culture medium were characterized by three methods: (*i*) SDS/PAGE performed under reducing and nonreducing conditions was combined with immunoblotting using antibodies specific for either apo(a) or apoB. The results are shown in Fig. 3. Under reducing conditions (Fig. 3*A*), wild-type and mutant r-apo(a) migrated to the same position corresponding to an apparent molecular mass of 550 kDa. When analyzed under nonreducing conditions, wild-type r-apo(a) exists almost exclusively as a high molecular weight complex with apoB. In contrast, both mutant r-apo(a) migrated as uncomplexed glycoproteins.

(*ii*) To analyze whether the mutant r-apo(a) associate with apoB under nondenaturing conditions, culture supernatants of transfected HepG2 cells expressing either wild-type or mutant or truncated apo(a) were fractionated by KBr density-gradient centrifugation. A truncated apo(a) variant lacking the C-terminal kringles 32 to kringle V and the protease domain was obtained by transfection of plasmid pCMV-A18 Δ5-P in which the corresponding cDNA sequences have been substituted by a stop codon. Gradient fractions were analyzed with respect to their apo(a) and apoB contents by ELISA. Wild-type r-apo(a) produced by HepG2 cells was found predominantly in lipoprotein particles with a density between LDL and HDL (high density lipoprotein), as described for Lp(a) particles in human plasma (Fig. 4*A*). Both mutant r-apo(a) as well as the truncated apo(a) variant occur

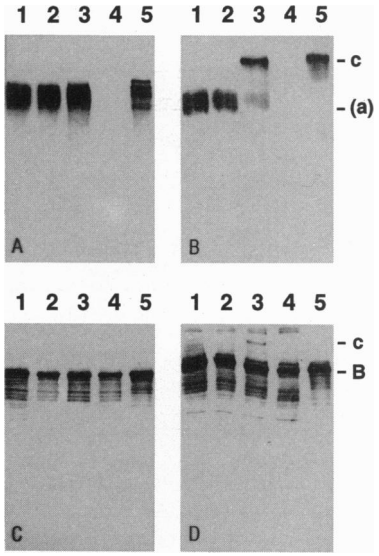

FIG. 3. Immunoblot analysis of wild-type and mutant r-apo(a) expressed by HepG2 cells. Culture supernatants from transfected HepG2 cells were analyzed by SDS/PAGE under reducing (*A* and *C*) or nonreducing (*B* and *D*) conditions. Lanes: 1, pCMV-A18-4057^{Gly}; 2, pCMV-A18-4057^{Ser}; 3, pCMV-A18 (wild type); 4, untransfected cells; 5, apo(a) phenotyping standard. Immunodetection was performed with apo(a)-specific monoclonal antibody 1A2 (*A* and *B*) or with a polyclonal antibody directed against human apoB (*C* and *D*). Positions of free r-apo(a), apoB, and of the r-Lp(a) complex are indicated [(a), B, and c, respectively)]. Note the presence of r-Lp(a) complex in lane 3 of *B* and *D*.

predominantly as free glycoproteins in the bottom fractions of the gradients (Fig. 4 *B–D*).

(*iii*) High-salt conditions during density-gradient centrifugation might prevent the detection of low-affinity interactions between mutant r-apo(a) and apoB-containing lipoproteins. Therefore, we combined apo(a)- and apoB-specific reagents in sandwich ELISAs to study the interaction of r-apo(a) with apoB-containing lipoproteins under physiological salt conditions. Again, apo(a)–apoB complexes were identified only in the supernatant of HepG2 cells transfected with wild-type apo(a) plasmid (Table 1). No such complexes could be detected for either of the mutant apo(a) proteins.

DISCUSSION

In the present study, we have tested whether Cys^{4057} is required for Lp(a) particle assembly by expressing wild-type r-apo(a) and two different mutants thereof in HepG2 cells. The mutants have either a Cys → Ser or a Cys → Gly substitution at position 4057. Neither substitution should interfere with the folding of the penultimate kringle IV domain of apo(a) because serine and glycine are present in most other kringle IV domains of apo(a) at the position homologous to Cys^{4057} (7). The presence of Lp(a)-like particles in the media from cells transfected with wild-type apo(a) plasmid was demonstrated by three methods. The same methods did not reveal Lp(a) particle formation for either of the two mutants. This was not due to reduced expression of apo(a) or apoB, because cells transfected with either wild-type or mutant apo(a) plasmids expressed comparable amounts of both apo(a) and apoB as shown in several independent experiments (Table 1; unpublished results). Therefore, our results confirm the hypothesis that Cys^{4057} is covalently linked to apoB within the Lp(a) particle. It is unlikely that Cys^{4057} is specifically required for any type of interaction between apo(a) and apoB other than formation of a disulfide bridge. Our data are in agreement with sulfhydryl-

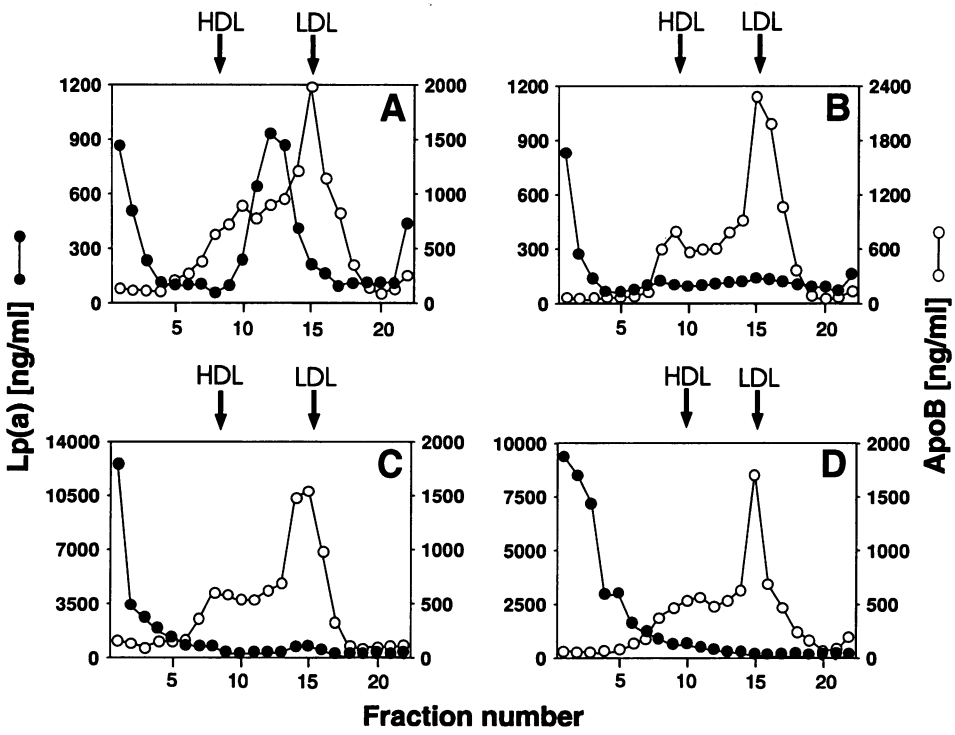

FIG. 4. ELISA analysis of apo(a) (●) and apoB (○) in density-gradient fractions from transfected HepG2 cells. HepG2 cells were transfected with pCMV-A18 (*A*), pCMV-A18 Δ5-P [truncated apo(a) lacking C-terminal domains from kringle 32 to the protease domain], (*B*) pCMV-A18-4057^{Gly} (*C*), and pCMV-A18-4057^{Ser} (*D*). Positions of LDL and HDL are indicated.

selective fluorescence labeling experiments and molecular modeling studies, which suggest a single disulfide bridge between apo(a) and apoB (25, 26) with Cys^{3734} of apoB covalently linked to apo(a) within the Lp(a) complex (26, 27).

Beyond the corroboration of the disulfide hypothesis, our data strongly suggest an essential role of Cys^{4057} for assembly of Lp(a) particles. In the HepG2 system, we were unable to detect any Lp(a)-like particles in the absence of Cys^{4057} even under physiological salt conditions. We cannot exclude the involvement of other, noncovalent interactions in the Lp(a) assembly. On the contrary, it is difficult to imagine that two of the largest proteins should interact exclusively by a single disulfide bridge. Molecular modeling and docking experiments with models of apo(a) kringle 36 and energy-minimized segments of apoB suggest complex interactions between the penultimate kringle IV repeat of apo(a) and the putatively surface-exposed segment 3732–3739 of apoB including multiple van der Waals contacts (26). Interactions between other apo(a) kringle domains and apoB might also contribute to the Lp(a) assembly. Recently, reconstitution experiments with apo(a) transgenic plasma and purified human LDL demonstrated rapid SDS-resistant association between r-apo(a) and LDL particles, which could be dissociated only by reductive treatment (28). A study with long-term cultured primary baboon hepatocytes also suggests that apo(a) and apoB can become disulfide bridged outside the cell (29). The inhibition of this association in the presence of the lysine analog ε-aminocaproic acid (EACA) suggests that interactions between lysine residues of apoB and lysine-binding apo(a) kringle domains might play an important role during Lp(a) assembly. *In vitro* association experiments with 293 cell-derived r-apo(a) and human plasma LDL (30) add further support to this hypothesis. In these experiments, noncovalent, EACA-sensitive interactions between purified r-apo(a) and LDL were demonstrated by sedimentation analysis.

Table 1. ELISA detection of r-Lp(a) particles (ng/ml) in the medium of transfected HepG2 cells

Detecting antibody	Capturing antibody: Sheep anti-human Lp(a)	Capturing antibody: Sheep anti-human apoB
Anti-apo(a)*		
Untransfected	< min	< min
pCMV-A18	305	100
pCMV-A18-4057^{Gly}	102	< min
pCMV-A18-4057^{Ser}	276	< min
Anti-apoB†		
Untransfected	< min	1930
pCMV-A18	234	1004
pCMV-A18-4057^{Gly}	< min	924
pCMV-A18-4057^{Ser}	< min	730

< min, Below detection level of 15 ng/ml.
*Standardized to Lp(a) reference standard (Immuno).
†Standardized to apoB in Lp(a)-free human LDL.

Based on our finding of the essential role of Cys^{4057} for Lp(a) assembly and the data mentioned above on the interference of EACA with this process, we propose a two-step model for Lp(a) assembly as shown in Fig. 5. According to this model, multiple noncovalent interactions, including interactions between lysine-binding kringles of apo(a) and lysine residues in apoB, serve to position Cys^{4057} of apo(a) close to Cys^{3734} of apoB in the first step. This EACA-sensitive step might be followed by formation of the interchain disulfide bridge in the second step.

The experiments described here emphasize the importance of the disulfide bridge between apoB and apo(a) but do not provide a clue to the site of Lp(a) assembly. In eukaryotic cells, intra- and interchain disulfide bridging of newly synthesized secretory proteins usually occurs under the oxidizing conditions within the endoplasmic reticulum (ER). The ER resident enzyme protein disulfide isomerase (PDI) plays an important role in disulfide bridge formation of many proteins (31). The rapid covalent association of r-apo(a) and human LDL in the plasma of apo(a) transgenic mice (28) indicates that PDI is not required for extracellular Lp(a)

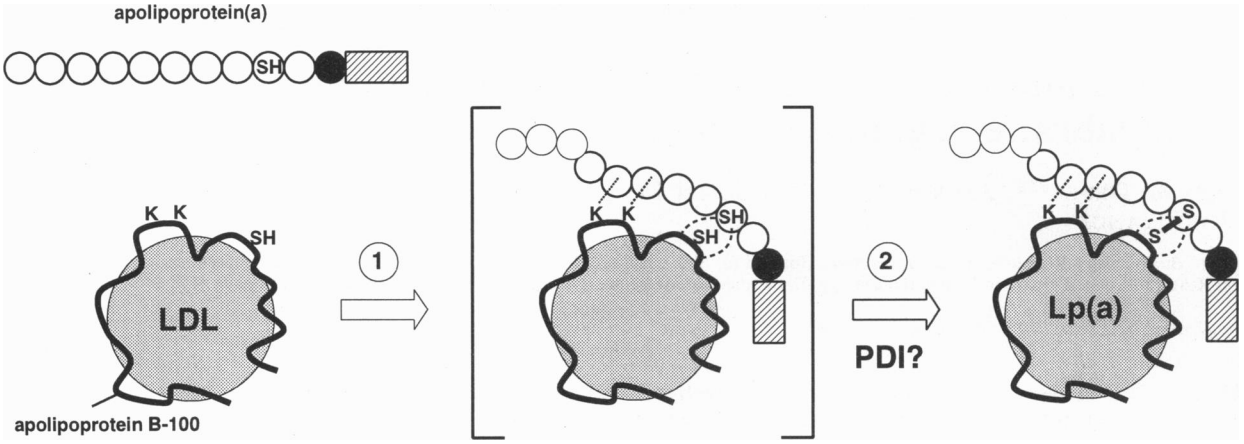

FIG. 5. Two-step model of Lp(a) assembly. Positioning of apo(a) Cys^{4057} close to a free cysteine (Cys^{3734}) in apoB might be achieved by multiple noncovalent (· · ·) interactions including kringle/lysine (K) interactions between apo(a) and apoB in the first step before both proteins become covalently linked by (PDI catalyzed?) formation of a disulfide bridge.

assembly. Whether *in vivo* Lp(a) assembly occurs in the hepatocyte and/or in the plasma remains to be resolved.

The recombinant HepG2 model described here represents a useful tool to study the biosynthesis of Lp(a) in an *in vitro* system. Stable variants of this model can now be used to identify inhibitors of Lp(a) biosynthesis. As plasma levels of Lp(a) are controlled by the apo(a) gene and regulated at the biosynthetic level (32–34), such compounds might be developed to a new class of antiatherosclerotic drugs that reduce the increased risk of premature atherosclerosis associated with high plasma Lp(a) levels.

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