An Analysis of the Interaction between Mouse Apolipoprotein B100 and Apolipoprotein(a)*

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SUMMARY

The assembly of lipoprotein(a) [Lp(a)] involves an initial noncovalent interaction between apolipoprotein (apo) B100 and apo(a), followed by the formation of a disulfide bond between apoB100 cysteine-4326 and apo(a) cysteine-4057. The structural features of apoB100 that are required for its noncovalent interaction with apo(a) have not been fully defined. To analyze that initial interaction, we tested whether apo(a) could bind noncovalently to two apoB proteins that lack cysteine-4326—mouse apoB100 and human apoB100-Cys4326Gly. Our experiments demonstrated that both mouse apoB and the human apoB100-Cys4326Gly bind noncovalently to apo(a). We next sought to gain insights into the apoB amino acid sequences required for the interaction between apoB100 and apo(a). Previous studies of truncated human apoB proteins indicated that the carboxyl terminus of human apoB100 (amino acids 4330–4397) is important for Lp(a) assembly. To determine if the carboxyl terminus of mouse apoB100 can interact with apo(a), transgenic mice were produced with a mutant human apoB gene construct in which human apoB100 amino acids 4279–4536 were replaced with the corresponding mouse apoB100 sequences, and tyrosine-4326 was changed to a cysteine. The mutant apoB100 bound to apo(a) and formed bona fide disulfide-linked Lp(a), but Lp(a) assembly was less efficient than with wild-type human apoB100. The fact that Lp(a) assembly was less efficient with the mouse apoB sequences provides additional support for the notion that sequences in the carboxyl-terminus of apoB100 are important for Lp(a) assembly.
Lipoprotein(a) [Lp(a)] (1) is formed by the disulfide linkage of apoB100 on a low density lipoprotein (LDL) particle to apo(a), a plasminogen-like glycoprotein (2). Lp(a) is found only in a relatively small group of mammals that synthesize apo(a), notably humans, old-world monkeys, and the hedgehog (3-5). Most mammals cannot synthesize apo(a) and therefore cannot produce Lp(a). In addition, many mammals (e.g., mouse, rat, and pig) synthesize an apoB100 molecule that cannot form a disulfide linkage with human apo(a) (6). The development and analysis of human apo(a) transgenic mice illustrated the latter point (7). The mouse apoB100 in the plasma of apo(a) transgenic mice did not form a covalent linkage with apo(a), and biochemical assays suggested that the apo(a) circulated free of the apoB-containing lipoproteins in the plasma (7).

During the past five years, we have sought to define the structural features of apoB100 that are important for Lp(a) assembly. In 1995, we produced human apoB transgenic mice expressing a mutant form of human apoB in which cysteine-4326 was replaced by a glycine (human apoB100-Cys4326Gly) (8). The mutant human apoB could not form Lp(a) in in vitro assays of Lp(a) assembly or in vivo in the plasma of transgenic mice, establishing that cysteine-4326 was crucial for the disulfide linkage with apo(a). Callow and collaborators (9) made similar observations. Of note, cysteine-4326 is not present in the apoB100 of mouse, rat, or pig (8), likely explaining why LDL from those species do not form a disulfide linkage with human apo(a).

The identification of human apoB cysteine-4326 as the critical residue for Lp(a) formation represented a significant advance in understanding Lp(a) assembly. However, it seemed obvious that apoB’s association with apo(a) must rely on more than a single free cysteine. A two-step model for Lp(a) assembly has been proposed (10, 11) whereby certain domains within the apoB molecule initially associate with apo(a) in a noncovalent fashion, bringing the molecules into close approximation so that the disulfide bond can form.

The nature of the initial noncovalent interaction between apoB and apo(a) and the structural features of apoB that are involved in that interaction are incompletely understood. However, several
clues exist. First, lysine residues within apoB are likely to be important for the association with apo(a). Lysine analogues such as ε-aminocaproic acid block the binding of apoB to apo(a) (7), and the deletion of lysine-binding domains from apo(a) interferes with the assembly of Lp(a) (12-14).

Second, recent studies by McCormick et al. (15) have suggested that amino acid residues 4330–4397 are important for apoB’s interaction with apo(a). In these studies, two truncated human apoB proteins (apoB95 and apoB97) were tested for their ability to interact with apo(a) and form Lp(a). ApoB95 (4330 amino acids) bound to apo(a) very slowly and inefficiently, whereas apoB97 (4397 amino acids) bound to apo(a) almost as well as the full-length apoB100. Interestingly, the region spanning amino acids 4330–4397 contains multiple lysines, including a cluster of four between residues 4372 and 4392.

Some of the sequences downstream from cysteine-4326 are highly conserved between human and mouse apoB (15). If these sequences were important for apoB’s initial interaction with apo(a), one might predict that apoB proteins lacking cysteine-4326 (e.g., mouse apoB) would retain the ability to bind noncovalently to apo(a), despite being unable to form bona fide Lp(a). The possibility that nonhuman LDLs might conceivably bind to apo(a) was raised by the work of Trieu and McConathy (6), who reported that fully assembled human Lp(a) was capable of binding to several nonhuman LDLs.

The current study was designed to further characterize the noncovalent interaction between apoB100 and apo(a). Our first goal was to document the existence of a noncovalent association between apo(a) and apoB by analyzing the capacity of apo(a) to stably associate with two apoBs that lack cysteine-4326—mouse apoB100 and human apoB100-Cys4326Gly. A second goal was to test whether the carboxyl-terminal sequences of mouse apoB100 would support the formation of bona fide, disulfide-linked Lp(a) if the mouse apoB100 sequences were supplied with a cysteine at residue 4326.
MATERIALS AND METHODS

Transgenic Mice—Human apo(a) transgenic mice (7) were obtained from Drs. Helen H. Hobbs and Robert Hammer (University of Texas Southwestern Medical Center, Dallas, TX). We also used transgenic mice expressing wild-type human apoB100 (16, 17), human apoB90 (4084 amino acids) (18), human apoB95 (4030 amino acids) (15), and a mutant human apoB100 in which cysteine-4326 was replaced with glycine (apoB100-Cys4326Gly) (8). All mice had a mixed genetic background. They were housed in a barrier facility and fed a chow diet containing 4.5% fat (Ralston Purina, St. Louis, MO).

Assessing Whether Apo(a) Is Associated with the ApoB-containing Lipoproteins in the Plasma of Apo(a) Transgenic Mice—Lipoproteins in the plasma (200 µl) of apo(a) transgenic mice and human apoB100-Cys4326Gly/apo(a) transgenic mice were size-fractionated by fast protein liquid chromatography (FPLC) on a Superose 6 HR 10/30 column (Pharmacia Biotech, Piscataway, NJ) (16). The cholesterol content of each fraction was measured with an enzymatic kit (TC Cholesterol, Boehringer Mannheim). The apo(a) and apoB content of each fraction was assessed by Western blots of sodium dodecyl sulfate (SDS)/4% polyacrylamide gels with the apo(a)—specific monoclonal antibody IgG-a5 (19) and a rabbit antiserum against mouse apoB (20). Antibody binding was detected with an enhanced chemiluminescence kit (Amersham, Piscataway, NJ). FPLC fractionation studies were also used to assess the distribution of apo(a), mouse apoB, and human apoB within the plasma of human apoB100-Cys4326Gly/apo(a) transgenic mice. In those experiments, human apoB was detected with the human apoB—specific monoclonal antibody, 1D1 (21). In parallel, FPLC was used to determine the distribution of free apo(a) in the $d > 1.21$ g/ml fraction of apo(a)/apoB100-Cys4326Gly transgenic mouse plasma. As a control, we assessed the elution profile of ferritin, a plasma protein with a molecular mass (440 kDa) similar to that of the apo(a) isoform expressed in the apo(a) transgenic mice (7).

Assessing the Ability of Mouse ApoB100 and Human ApoB100-Cys4326Gly to Inhibit the Covalent Linkage of Apo(a) and Wild-type Human ApoB100—We assessed the capacity of mouse
apoB100 and human apoB100-Cys4326Gly to inhibit the formation of a disulfide bond between wild-type human apoB100 and apo(a). Plasma was obtained from wild-type mice and human apoB100-Cys4326Gly transgenic mice. Mouse apoB in the plasma of the human apoB100-Cys4326Gly mice was removed by immunoprecipitation with a mouse apoB–specific antiserum (20). Next, LDL (d = 1.006–1.063 g/ml) were prepared from both plasma samples by sequential density ultracentrifugation (22). The LDL samples were dialyzed against 0.15 M NaCl containing 1 mM EDTA. The apolipoprotein content of the sample was assessed by SDS/polyacrylamide gel electrophoresis, and the protein concentration was measured with a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The ability of the LDL samples to compete with wild-type human apoB for noncovalent binding to apo(a) was assessed in an in vitro assay of Lp(a) assembly (18).

Plasma (1.0 µl) from an apo(a) transgenic mouse (7) and plasma (2.5 µl) from a transgenic mouse expressing wild-type human apoB100 (17) were incubated for 3 h with increasing amounts of mouse LDL or human apoB100-Cys4326Gly LDL (0–2 µg). Aliquots from these mixtures were size-fractionated on SDS/4% polyacrylamide gels under nonreducing conditions and analyzed by Western blotting with the apo(a)–specific monoclonal antibody IgG-a5 (19) conjugated to horseradish peroxidase.

Generation of Transgenic Mice Expressing a Human/Mouse Hybrid ApoB100—We previously reported a “pop-in, pop-out” gene-targeting system for introducing mutations into a 100-kilobase (kb) yeast artificial chromosome (YAC) spanning the human apoB gene (8). Using that system, we generated a human apoB gene construct in which the sequences encoding human apoB100 amino acids 4279–4536 were replaced with the corresponding mouse apoB sequences. The human/mouse hybrid gene-targeting vector was constructed with the overlap extension polymerase chain reaction (PCR) procedure (23). A 0.6-kb PCR fragment from exon 29 of the human apoB gene (encoding human apoB100 amino acids 4078–4278) was amplified with a human apoB exon 29 primer, HM1 (5′-GAGAAGAAATCTAGAGAACAATGCTG-3′; XbaI site underlined) and a human/mouse hybrid primer, HM2 (5′-cttcatatgtctaaacGAATTGTAAA
AGATCTTTGAAGATTACG-3’). The mouse sequence in HM2 is shown in lower case, and a BgII site (to facilitate the formation of the human/mouse hybrid) is underlined. A 1.0-kb PCR fragment from mouse exon 29 (encoding mouse apoB amino acids 4279–4536) was amplified with HM3 (5’-CGTAATCTTCAAGATCTTTTACAATTcgttagacatcataag-3’; mouse sequence lower case, BgII site underlined) and a mouse-specific exon 29 primer, HM4 (5’-CAAATAATCCTATCTAGATATATCCC-3’; XbaI site underlined). The two PCR products were annealed, and a 1.6-kb chimeric human/mouse hybrid fragment was amplified with HM1 and HM4. That fragment was cleaved with XbaI and cloned into the polylinker XbaI site of pRS406 (Stratagene, La Jolla CA). Next, site-directed mutagenesis with the oligonucleotide 5’-AAGAGAAGACGCATGCTTTGTCCTCGGTGAG-3’ was used to change tyrosine-4326 in the mouse sequence to cysteine and to create a new SphI site (underlined). Mutagenesis was confirmed by DNA sequencing.

The human/mouse hybrid gene-targeting vector was linearized at the unique EcoRI site in exon 29 and introduced into yeast spheroplasts containing a YAC spanning the human apoB gene (8). Transformants were selected on plates containing uracil and later on plates lacking uracil, tryptophan, and lysine. To confirm that the gene targeting was successful, a 304-base pair (bp) fragment from exon 29 of the mouse apoB gene was amplified with the mouse apoB-specific primers M1 (5’–CCTAAAGGACAATGAATC–3’) and M2 (5’–GACATCCCTAAAGGAAAC–3’) and the PCR product digested with SphI.

The human/mouse hybrid YAC DNA fragment was purified from a pulsed-field agarose gel (24), adjusted to a concentration of 3.0 ng/µl, and microinjected into F2 C57BL/6 × SJL zygotes. Transgenic founder mice were identified by Southern blot analysis of tail DNA and by a monoclonal antibody-based enzyme-linked immunoassay (18) for human apoB. In addition, Western blots of SDS/polyacrylamide gels were used to detect the human/mouse hybrid apoB100. For these studies, we used several human apoB–specific monoclonal antibodies: 1D1 (binds between human apoB amino acid residues 474 and 539) (21), MB47 (binds near human apoB100 residue 3500) (25), MB43 (binds between residues 4027 and 4081 (26), BSol2 (binds between residues 4235 and 4355
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(21), BSol22 (binds between residues 4521 and 4536) (21), and 605 (binds between residues 4235 and 4536) (27).

Assessing the Ability of the Human/Mouse Hybrid ApoB100 to Form Lp(a)—The ability of the human/mouse hybrid apoB to covalently bind apo(a) in vitro was analyzed with a Western blot assay (18). Briefly, plasma from a human apo(a) transgenic mouse (1.5 µl) was mixed with plasma (2.0 µl) from a transgenic mouse expressing wild-type human apoB100 (16), human apoB100-Cys4326Gly (8), human apoB95 (15), or the human/mouse hybrid apoB100. The mixtures were then brought to a total volume of 20 µl with 0.15 M NaCl and incubated for 3 h at 37 °C. Samples were size-fractionated on an SDS/4% polyacrylamide gel under nonreducing conditions, and a Western blot was performed with horsesradish peroxidase–labeled IgG-a5.

In separate experiments, we compared the time course of Lp(a) formation with wild-type human apoB100, human apoB95, and the human/mouse hybrid apoB100. In these experiments, the total volume of the incubations was 40 µl, including 3 µl of human apo(a) transgenic mouse plasma and variable amounts (3.0–9.0 µl) of human apoB transgenic mouse plasma. Each incubation mixture contained exactly the same amount of human apoB or human/mouse hybrid apoB, as judged by a monoclonal antibody–based radioimmunoassay and Western blot analyses. In the time-course experiments, 5.0-µl aliquots of the incubation mixtures were removed after 10, 30, 60, 120, and 240 min and added to 1% SDS nonreducing sample buffer. These samples were immediately heated to 65 °C for 10 min and then stored at –20 °C and analyzed by SDS/polyacrylamide gel electrophoresis and Western blotting with antibody IgG-a5.

Lp(a) Formation in Transgenic Mice Expressing Both the Human/Mouse Hybrid ApoB100 and Apo(a) Transgenes—The human/mouse hybrid apoB100 transgenic mice were bred with human apo(a) transgenic mice. Offspring that expressed only the human/mouse hybrid apoB100 transgene, only the apo(a) transgene, or both transgenes were identified with Western blots. To assess the amount of apo(a) covalently linked to the human/mouse hybrid apoB, plasma samples
were size-fractionated on an SDS/4% polyacrylamide gel under nonreducing conditions and a Western blot performed with IgG-a5.
RESULTS

Distribution of Human Apo(a) in the Lipoprotein Fractions of Apo(a) Transgenic Mice and Apo(a)/Human ApoB-Cys4326Gly Transgenic Mice—To test whether apo(a) in the plasma of apo(a) transgenic mice is associated with apoB, we examined the distribution of apo(a) in the different lipoprotein fractions with gel-filtration chromatography. The fractionation was successful, as judged by the characteristic distribution of cholesterol in the very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) fractions (Figs. 1A and B). As expected, most of the mouse apoB in the apo(a) transgenic mouse plasma was in the LDL (Fig. 1D). Of note, a large fraction of the apo(a) was also located in the LDL fractions, with some trailing into the HDL fractions. This strongly suggested that a significant fraction of the apo(a) in the plasma was associated with the mouse apoB-containing lipoproteins.

To exclude the possibility that free apo(a) and LDL particles normally elute into the same fractions despite large differences in molecular weights, we performed FPLC-fractionation experiments with free apo(a). Free apo(a) was prepared from the plasma of human apo(a)/apoB100-Cys4326Gly transgenic mice by density ultracentrifugation. Density ultracentrifugation disrupts the noncovalent interaction between apo(a) and apoB100-Cys4326Gly, releasing apo(a) which can be recovered in the lipoprotein-depleted plasma (i.e., \(d > 1.21\) g/ml fraction). In contrast to the results with whole plasma, the apo(a) in the \(d > 1.21\) g/ml fraction (Fig. 1C) eluted almost exclusively into the HDL fractions (i.e., HDL particles and free apo(a) have a similar size, ~500 kDa). Control experiments revealed that ferritin, a protein with a similar molecular weight, also eluted in the HDL fractions (data not shown). Thus, the presence of apo(a) in the LDL fraction of mouse plasma indicates that apo(a) must be associated with the apoB-containing lipoproteins. Fractionation of the lipoproteins in the plasma of the apo(a)/human apoB100-Cys4326Gly transgenic mice (Fig. 1E) showed that human apoB100, mouse apoB100, and apo(a) were located almost exclusively in the LDL fractions, again indicating that the apo(a) was associated with the apoB-containing lipoproteins.
To further document a noncovalent interaction between apo(a) and mouse apoB, we compared the abilities of mouse apoB and human apoB100-Cys4326Gly to compete with wild-type human apoB100 for binding to apo(a) (Fig. 2). Increasing amounts of either human apoB100-Cys4326Gly (Fig. 2A) or mouse apoB (Fig. 2B) were incubated with fixed amounts of apo(a) and wild-type human apoB100. Western blots of SDS/polyacrylamide gels showed that when apo(a) and wild-type human apoB were incubated together, all of the apo(a) became covalently linked to the human apoB to form Lp(a). This in vitro assembly of Lp(a) was inhibited by both human apoB100-Cys4326Gly and mouse apoB as indicated by the appearance of free apo(a) in the incubations containing 0.25 µg of human apoB100-Cys4326Gly or 0.25 µg of mouse apoB. Moreover, Lp(a) formation was completely inhibited by the addition of 0.5 µg of either apoB. These data suggest that the noncovalent interaction between apo(a) and mouse apoB (or human apoB100-Cys4326Gly) prevents the initial noncovalent interaction between apo(a) and wild-type human apoB100 and thereby prevents the subsequent formation of the disulfide bond. These results also indicate that mouse apoB and human apoB100-Cys4326Gly are approximately equal in their ability to inhibit Lp(a) formation.

Further Analysis of the ApoB Sequences That Are Important for the Efficient Assembly of Lp(a)—To test whether the carboxyl terminus of mouse apoB can interact with apo(a) and contribute to Lp(a) formation, we produced transgenic mice that expressed a human/mouse hybrid apoB100 construct in which amino acids 1–4278 were specified by human apoB sequences and amino acids 4279–4536 by mouse apoB sequences (Fig. 3A). In addition, tyrosine-4326 in the mouse apoB sequence was changed to cysteine. To achieve this goal, we constructed a gene-targeting vector designed to introduce mouse apoB sequences into the human apoB gene of a 108-kilobase YAC (Fig. 3B). Successful gene-targeting events were confirmed by pulsed-field gel and PCR analysis.

YAC DNA was purified from pulsed-field gels and microinjected into F2 C57BL/6 × SJL fertilized mouse eggs, generating three founders. The human/mouse hybrid apoB100 was identical
in size to wild-type human apoB100 (Fig. 4A). As expected, human apoB–specific monoclonal antibodies with epitopes in the extreme carboxyl terminus of human apoB100 (antibodies BSol2, 605, and BSol22) did not bind to the human/mouse hybrid apoB100 (Fig. 4B-D).

To assess whether lipoproteins containing the human/mouse hybrid apoB100 could bind apo(a) and form Lp(a), we used an in vitro assay of Lp(a) formation. Plasma from a human/mouse hybrid apoB100 transgenic mouse was incubated with the plasma from an apo(a) transgenic mouse, and Lp(a) formation was assessed by Western blot analysis. The human/mouse hybrid apoB100 formed Lp(a) (Fig. 5), but the amount was less than that formed in a control incubation containing wild-type human apoB100. As expected, human apoB100-Cys4326Gly did not form Lp(a).

To compare the efficiency of Lp(a) formation with wild-type human apoB100 and the human/mouse hybrid apoB100, we assessed the time course of Lp(a) assembly with the in vitro Lp(a) assembly assay (Fig. 6). In incubations containing wild-type human apoB100, most of the apo(a) was covalently bound to the apoB at 10 min, and Lp(a) assembly was complete by 30 min. In contrast, when an equal amount of the human/mouse hybrid apoB100 was included in the incubation, very little Lp(a) formation could even be detected until 60 min, with the amount of Lp(a) gradually increasing at 120 and 240 min. A similar result was obtained with the human apoB95. Once again, no Lp(a) was detected with human apoB100-Cys4326Gly.

To further assess the efficiency of Lp(a) assembly with the human/mouse hybrid apoB100, we assessed the amount of Lp(a) in the plasma of transgenic mice expressing both the human/mouse hybrid apoB100 and apo(a) (Fig. 7). Significant amounts of Lp(a) were found in the plasma of these mice, again showing that the hybrid apoB100 could form Lp(a). However, small amounts of free apo(a) were also detectable in the plasma.
DISCUSSION

The assembly of Lp(a) is proposed to be a two-step process, the first step being the noncovalent association between apo(a) and apoB100, and the second step being the formation of a disulfide bond between apo(a) cysteine-4057 and apoB100 cysteine-4326 (8, 10). One of the goals of the current study was to provide experimental evidence for the initial noncovalent interaction between apo(a) and apoB100. ApoB100-Cys4326Gly and mouse apoB100 are ideal vehicles for studying the noncovalent interaction since both lack cysteine-4326 and thus cannot form the disulfide linkage. Our experiments have provided two lines of evidence for a noncovalent interaction between apoB and apo(a). First, size-fractionation of the plasma lipoproteins from apo(a) transgenic mice revealed that the most of the apo(a) was in the same fractions that contained LDL. When FPLC fractionation experiments were performed with the plasma of apoB100-Cys4326Gly/apo(a) transgenic mice, this finding was even more striking, likely reflecting the higher plasma levels of apoB in those animals. Second, by virtue of their ability to bind to apo(a), both purified mouse LDL and apoB100-Cys4326Gly LDL were competitive inhibitors of disulfide bond formation between wild-type human apoB100 and apo(a). Together, these results provide experimental evidence for the existence of the initial noncovalent step of Lp(a) assembly.

Our studies challenge a widely held view—that the apo(a) in the plasma of apo(a) transgenic mice is not associated with the lipoproteins. The notion that apo(a) is “free” in the plasma of the apo(a) transgenic mice originated with the initial characterization of those animals (7) and has been further bolstered by subsequent studies (8). This conclusion stemmed from two types of experiments. First, when the plasma of apo(a) transgenic mice is subjected to ultracentrifugation in high concentrations of salt, none of the apo(a) floats with the lipoproteins (7). Second, when subjected to polyacrylamide gel electrophoresis in the presence or absence of SDS, none of the apo(a) migrates with mouse apoB100 (7, 8). It appears that density ultracentrifugation and polyacrylamide gel electrophoresis effectively strip apo(a) from the apoB-containing lipoproteins and therefore cannot detect a noncovalent association between apo(a) and apoB100. Milder
techniques, such as the FPLC fractionation studies used in this study, preserve the noncovalent association.

The existence of a strong noncovalent interaction between apo(a) and mouse apoB helps to make sense of several earlier observations. Lawn and co-workers (28) noted the co-localization of apo(a) and mouse apoB in atherosclerotic lesions in apo(a) transgenic mice. This co-localization likely reflects the retention of apo(a)–apoB complexes within the arterial wall. Also, Hobbs and colleagues (29) reported that the plasma levels of apo(a) in human apo(a) transgenic mice were doubled by LDL receptor deficiency, suggesting that a defect in the removal of apoB100-containing lipoproteins also affected the removal of apo(a). This linkage of apo(a) and apoB100 metabolism is quite consistent with our demonstration that apo(a) and apoB100 exist as a complex in the plasma. Our findings may also make sense of the observation that mouse LDL can bind human Lp(a) (6), although it remains somewhat unclear whether the association of mouse LDL and Lp(a) is identical to that of mouse LDL and apo(a).

Another aim of our study was to further elucidate the apoB sequences important for the noncovalent binding to apo(a). Our finding that apo(a) binds noncovalently to mouse apoB100, together with our previous work (15) implicating the carboxyl terminus of human apoB in apo(a) binding, naturally evoked a simple question: Would the carboxyl terminus of mouse apoB100 support the formation of bona fide disulfide-linked Lp(a) if the critical cysteine were inserted into the mouse apoB sequences? To address that issue, we used gene targeting to insert the carboxyl terminus of mouse apoB100 into a full-length human apoB YAC clone, and then used the YAC DNA to produce transgenic mice expressing the hybrid human/mouse apoB100. The in vitro experiments revealed that the hybrid apoB100 was quite capable of forming Lp(a), but with significantly reduced efficiency. The in vivo experiments yielded consistent results. Transgenic mice expressing both apo(a) and the human/mouse hybrid apoB contained small amounts of free apo(a) in their plasma. Previously, we showed that in transgenic mice expressing
both apo(a) and wild-type human apoB100, all of the apo(a) was covalently linked to apoB100 and none was free (16).

The lower efficiency of Lp(a) assembly with the “mouse apoB carboxyl terminus,” than with the “human apoB carboxyl terminus” suggests that subtle changes in the apoB sequence or conformation in this region affects the efficiency of Lp(a) assembly. Previously we reported that human apoB97 but not apoB95 formed Lp(a) efficiently (15), emphasizing that the ~75 amino acids downstream from residue 4326 are likely important in apoB’s initial association with apo(a). The sequences downstream from the carboxyl terminus of apoB95 are fairly well conserved in several mammalian species and are predicted to contain several amphipathic α-helices with multiple lysine residues (30). For example, one of the predicted α-helices (residues 4365–4396) contains lysines at positions 4372, 4379, 4385, and 4392. We have recently shown that a synthetic apoB peptide spanning amino acids 4372–4392 is a potent inhibitor of Lp(a) assembly in vitro (31). Interestingly, two of the four lysines between amino acids 4372 and 4392 are missing from the mouse apoB sequence. In future studies, it would be of interest to delete one or more of those lysines from the human sequence and assess the efficiency of Lp(a) assembly.

Our studies to date support the hypothesis that nearby carboxyl-terminal sequences mediate both the noncovalent and covalent interactions of apoB and apo(a). This hypothesis is attractive in that it falls in line with Ockham’s razor. However, the current studies cannot exclude the possibility that remote sequences might also contribute to the noncovalent association between apo(a) and apoB. For example, Trieu and McConathy (32) proposed that amino acids 3304–3317 might be important for the noncovalent interaction between apo(a) and apoB, while Gabel et al. (33) suggested that sequences at the amino terminus of apoB could play a role in the binding of apo(a). We believe that more experiments will be required for a complete understanding of the apoB sequences governing the assembly of Lp(a). Gene targeting in YACs, followed by transgenic mouse expression studies, is an approach that is well-suited for mutating many different regions of the
apoB molecule. This approach could potentially be used to define additional sequences that are important for Lp(a) assembly.
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FOOTNOTES

1 The abbreviations used are: Lp(a), lipoprotein(a); apoB, apolipoprotein B; apo(a), apolipoprotein(a); LDL, low density lipoprotein; YAC, yeast artificial chromosome; kb, kilobase; PCR, polymerase chain reaction; bp, base pair; FPLC, fast protein liquid chromatography.
FIGURE LEGENDS

FIG. 1. Distribution of cholesterol, apo(a), and apoB in the plasma of transgenic mice. Distribution of cholesterol in the plasma of an apo(a) transgenic mouse (A) and an apo(a)/human apoB100-Cys4326Gly transgenic mouse (B). Lipoproteins in plasma samples (200 µl) were size-fractionated on an FPLC column, and the cholesterol content of each fraction was measured. Fractions 14–18 represent VLDL-sized lipoproteins; 19–25, LDL-sized lipoproteins; 26–32, HDL-sized lipoproteins. C, The distribution of apo(a) in the d > 1.21 g/ml fraction from apo(a)/human apoB100-Cys4326Gly transgenic mouse plasma. D, Distribution of apo(a) and mouse apoB within the plasma of apo(a) transgenic mice. E, Distribution of apo(a), human apoB, and mouse apoB within the plasma of apo(a)/human apoB100-Cys4326Gly transgenic mice. The first lane of each Western blot represents a 4 µl sample of unfractionated plasma.

FIG. 2. Inhibition of Lp(a) formation by human apoB100-Cys4326Gly and mouse apoB. Plasma samples from an apo(a) transgenic mouse (1 µl) and a human apoB transgenic mouse (2.5 µl) were mixed and incubated for 3 h with increasing amounts of purified human apoB100-Cys4326Gly LDL (A) or purified mouse LDL (B). Lipoproteins in the mixtures were then size-fractionated on SDS/4% polyacrylamide gels under nonreducing conditions. Western blots were performed with an apo(a)–specific antibody, IgG-a5.

FIG. 3. Strategy for inserting the carboxyl-terminal portion of the mouse apoB gene, along with a Tyr4326Cys mutation, into a YAC spanning the human apoB gene. A, Overlapping exon 29 apoB gene fragments were amplified from P1 clones spanning the human and mouse apoB genes; amino acids encoded by the PCR products are noted. Annealing PCR was used to create a chimeric human/mouse DNA fragment encoding human apoB amino acids 4078–4278 and mouse amino acids 4279–4536. The chimeric PCR fragment was cloned into the XbaI site of the yeast integrating vector pRS406, and site-directed mutagenesis was used to change tyrosine-
4326 to a cysteine (this mutation introduced a new SphI site). B, YAC gene-targeting strategy for generating the human/mouse (H-M) hybrid apoB100 construct. The gene-targeting vector was linearized at an exon 29 EcoRI site and transfected into yeast harboring a 108-kb human apoB YAC. Integration of the vector into the YAC produced a 115-kb YAC in which human apoB100 amino acids 4279–4536 were replaced with the corresponding mouse apoB100 sequences and tyrosine-4326 was changed to cysteine. Arrowheads indicate the location of the PCR primers used to characterize the gene-targeting event.

**FIG. 4.** Western blot analysis of the human/mouse (H-M) hybrid apoB100 in the plasma of transgenic mice. The plasma proteins from transgenic mice expressing wild-type human apoB100, apoB90, or the human/mouse hybrid apoB100 were size-fractionated on an SDS/4% polyacrylamide gel under reducing conditions. The separated proteins were then transferred to a nitrocellulose membrane for Western blots with four different human apoB–specific monoclonal antibodies: A, MB47, B, Bsol2, C, 605, and D, Bsol22.

**FIG. 5.** An in vitro assay of Lp(a) assembly with the plasma from a transgenic mouse expressing the human/mouse hybrid apoB100. Plasma from an apo(a) transgenic mouse was mixed with plasma samples from transgenic mice expressing wild-type human apoB100, human apoB95, human/mouse hybrid apoB100, or human apoB100-Cys4326Gly. After a 3-h incubation, the amount of Lp(a) in the mixtures was assessed by Western blots of SDS/4% polyacrylamide gels with IgG-a5.

**FIG. 6.** Time course of Lp(a) assembly with wild-type human apoB100, apoB95, apoB100-Cys4326Gly, and the human/mouse hybrid apoB100. Plasma from an apo(a) transgenic mouse was mixed with plasma samples from transgenic mice expressing wild-type human apoB100, human apoB95, human/mouse hybrid apoB100, or human apoB100-Cys4326Gly.
The amount of Lp(a) formed in the incubation mixtures at different time points was assessed by Western blots with antibody IgG-a5.

**FIG. 7. Western blots showing Lp(a) in the plasma of mice expressing both apo(a) and the human/mouse hybrid apoB100.** Human apo(a) transgenic mice (A) were mated with transgenic mice that expressed the human/mouse hybrid apoB100 (B). Plasma from the parents and their offspring (1–7) were analyzed by Western blots with a human apoB–specific monoclonal antibody (1D1) and an apo(a)–specific monoclonal antibody (IgG-a5). The plasma samples for the 1D1 Western blot were reduced with 2% 2-mercaptoethanol; the plasma samples for the IgG-a5 Western blot were electrophoresed under nonreducing conditions.
A

Annealing PCR

XbaI

a.a. 4078  Human  a.a. 4278

XbaI

a.a. 4078  Human/Mouse Hybrid  a.a. 4536

a.a. 4279  Mouse

a.a. 4326

XbaI

B

H/M Hybrid Apo-B100 YAC

TRP1

Apo-B100 YAC

B, BamHI; X, XbaI; E, EcoRI; S, SphI

H–M Hybrid Apo-B100 YAC

TRP1

URAs

LYS2

“Pop-in”
10-Minute Incubation

Lp(a) —
Apo(a) —

30-Minute Incubation

Lp(a) —
Apo(a) —

60-Minute Incubation

Lp(a) —
Apo(a) —

120-Minute Incubation

Lp(a) —
Apo(a) —

240-Minute Incubation

Lp(a) —
Apo(a) —

ApoB
ApoB95
ApoB100
ApoB100Cys4326Gly
Apo(e)
ApoB100/H-M Hybrid
An analysis of the interaction between mouse apolipoprotein B100 and apolipoprotein(a)
Emma J. Cheesman, Rebecca J. Sharp, Constance H. Zlot, Catherine Y-Y Liu, Stacy Taylor, Santica M. Marcovina, Stephen G. Young and Sally P. A. McCormick

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