Mutation of lysine residues in apolipoprotein B-100 causes defective lipoprotein[a] formation

Catherine Y. Y. Liu, Ric Broadhurst, Santica M. Marcovina, and Sally P. A. McCormick

Department of Biochemistry, University of Otago, Dunedin, New Zealand; AgResearch Ruakura, Hamilton, New Zealand; and Department of Medicine, Northwest Lipid Research Laboratories, University of Washington, Seattle, WA 98103

Abstract Lipoprotein[a] (Lp[a]) is assembled by a two-step process involving an initial lysine-dependent binding between apolipoprotein B-100 (apoB-100) and apolipoprotein[a] (apo[a]) that facilitates the formation of a disulphide bond between apoB-100 Cys4,326 and apo[a] Cys4,057. Previous studies of transgenic mice expressing apoB-95 (4,330 amino acids) and apoB-97 (4,397 amino acids) have shown that apoB-100 amino acids 4,330–4,397 are important for the initial binding to apo[a]. Furthermore, a lysine-rich peptide spanning apoB-100 amino acids 4,372–4,392 has recently been shown to bind apo[a] and inhibit Lp[a] assembly in vitro. This suggests that a putative apo[a] binding site exists in the apoB-4,372–4,392 region. The aim of our study was to establish whether the apoB-4,372–4,392 sequence was important for Lp[a] assembly in the context of the full-length apoB-100. Transgenic mice were created that expressed a mutant human apoB-100, apoB-100K4,372→S4, which in which all four lysine residues in the 4,372–4,392 sequence were mutated to serines. The apoB-100K4,372→S4 mutant showed a reduced capacity to form Lp[a] in vitro compared with wild-type human apoB-100. Double transgenic mice expressing both apoB-100K4,372→S4 and apo[a] contained significant amounts of free apo[a] in the plasma, indicating a less-efficient assembly of Lp[a] in vivo. Taken together, these results clearly show that the apoB-4,372–4,392 sequence plays a role in Lp[a] assembly.

Supplementary key words apolipoprotein[a] • assembly • coronary heart disease • lysine • site-directed mutagenesis • transgenic mice • yeast artificial chromosome

Large clinical studies have identified high plasma levels of lipoprotein[a] (Lp[a]) as an independent risk factor for developing coronary heart disease (1–3). Lp[a] is structurally similar to LDL but contains an additional plasminogen-like protein, apolipoprotein[a] (apo[a]), that is covalently linked to apolipoprotein B-100 (apoB-100) (4).

The assembly of Lp[a] is a two-step process (5, 6) initiated by the noncovalent binding of apo[a] to apoB-100 and completed by the formation of a disulphide bond between apo[a] Cys4,057 (5, 7) and apoB-100 Cys4,326 (8, 9). This two-step model is supported by studies of a mutant apoB-100 lacking Cys4,326 which, despite being unable to form the disulphide bond, still binds apo[a] (10). The initial binding step is thought to be dependent on lysine residues, because deletion of apo[a] lysine binding domains interferes with Lp[a] formation (11–13). Furthermore, lysine analogs inhibit Lp[a] formation (14–16). These studies suggest that one or more apoB lysine residues interact with one or more of the lysine binding domains of apo[a] to facilitate Lp[a] assembly.

Recent studies have focused on characterizing the apoB sequences involved in the initial binding to apo[a]. Experiments with a series of truncated apoB proteins expressed in cell culture have identified an N-terminal region between apoB15 and apoB18 that is important for apo[a] binding (17). A subsequent study identified an apoB lysine residue within this region (apoBLys680) that was required for the binding of the apoB18 molecule to apo[a] (18). The importance of the apoBLys680 residue for binding to apo[a] in the context of a full-length apoB-100, however, remains to be shown.

Carboxyl-terminal sequences of apoB have been implicated in apo[a] binding by a study of two truncated apoB proteins expressed in transgenic mice, apoB95 (4,330 amino acids), and apoB-97 (4,397 amino acids) (19). In that study, apoB-97 formed Lp[a] with an efficiency similar to that of full-length apoB-100, whereas apoB95 formed Lp[a] very poorly, suggesting that the region between apoB-100 amino acids 4,330 and 4,397 is important for apo[a] binding. A comparison of the apoB-4,330–4,397 amino acid sequence of human and mouse reveals blocks

Abbreviations: apo[a], apolipoprotein[a]; apoB-100, apolipoprotein B-100; FPLC, fast protein liquid chromatography; Lp[a], lipoprotein[a]; YAC, yeast artificial chromosome.

1 To whom correspondence should be addressed.

e-mail: sally.mccormick@stonebow.otago.ac.nz

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of similarity (19, 20). Mouse apoB is of interest, because it is well documented that mouse apoB can associate noncovalently with apo[a] despite lacking the Cys4,326 residue (10, 21, 22). Interestingly, there are only five lysine residues within the human apoB-4,390–4,397 sequence. Four of these, Lys4,372, Lys4,379, Lys4,385, and Lys4,392, are clustered within a 21 amino acid region that is α-helical in structure (20). Recently, a synthetic peptide spanning the human apoB-4,372–4,392 sequence was shown to bind apo[a] and effectively inhibit Lp[a] assembly (20). Taken together, these observations have led us to hypothesize that the apoB-4,372–4,392 region constitutes a putative apo[a] binding site that is important for Lp[a] assembly. Furthermore, we speculate that one or more of the lysine residues in the apoB-4,372–4,392 sequence are important for apo[a] binding.

A line of human apoB-100 transgenic mice was generated that expressed a mutant apoB in which all four lysines in the apoB-4,372–4,392 sequence were mutated to serine residues. The ability of the mutant apoB-100 to form Lp[a] was evaluated both in vitro and in vivo.

MATERIALS AND METHODS

Generation of transgenic mice expressing a mutant human apoB-100

Four lysine-to-serine substitutions at human apoB-100 positions 4,372, 4,379, 4,385, and 4,392 were introduced into a 108 kb yeast artificial chromosome (YAC) spanning the apoB-100 gene using a previously reported “pop-in, pop-out” gene-targeting strategy (8). The point mutations were introduced into a yeast-integrating vector containing a 0.9 kb fragment of exon 29 spanning the intro-duced mutations was amplified by PCR from yeast DNA with the yeast markers as previously described (8). High-molecular-weight yeast transformants were selected using the uracil, tryptophan, and leucine auxotroph harboring the 108 kb human apoB-100 YAC. Pop-in and pop-out events were analyzed on a yeast clone (23), and the targeting event was analyzed on a 1% agarose gel. Two new restriction sites (underlined) were generated in association with the mutations; Bpu1102 in K → S5 and Clal in K → S4. An additional silent substitution (shown in lower case) was needed to create the Bpu1102 site. Clones containing the correct mutations were identified by DNA sequencing. The modified yeast integrating vector, apoB-100K → S4, was then linearized at a unique EcoRI site in exon 29 and transformed into yeast spheroplasts harboring the 108 kb human apoB-100 YAC. Pop-in and pop-out transformants were selected using the uracil, tryptophan, and lysine yeast markers as previously described (8). High-molecular-weight yeast DNA was prepared from selected pop-in and pop-out yeast clones (23), and the targeting event was analyzed on a 1% agarose pulsed-field gel. To confirm the incorporation of mutations, a 0.9 kb fragment of exon 29 spanning the introduced mutations was amplified by PCR from yeast DNA with the human apoB-specific primers 5SP2 (5′-GGGTTAGAAATCTCGTGTTCCT-3′) and 3SP1 (5′-GTTTTCACTGACGGACTCCT-3′). The PCR products were digested with the Bpu1102 and Clal restriction enzymes and analyzed on 1% agarose gels.

To generate transgenic mice expressing the human apoB-100K4 → S4 mutant, the apoB-100K4 → S4 YAC DNA was purified from pulsed-field gels, adjusted to a concentration of 1 ng/μl, and microinjected into FVB/N fertilized mouse eggs. Transgenic founders were identified by PCR of genomic DNA using the 5SP2 and 3SP1 PCR primers, followed by direct sequencing of the PCR product. Plasma from transgenic founders was tested for the presence of the mutant apoB by Western blotting using the human-apoB-specific monoclonal antibody, 1D1 (24).

Other transgenic mice used in this study were human apo[a] transgenic mice (14) obtained from Dr. Robert Hammer (University of Texas Southwestern Medical Center, Dallas, TX) and transgenic mice expressing human apoB-100 (25), human apoBCys4,326Gly (8), and human apoB-90 (26) obtained from Dr. Stephen Young (Gladdstone Institute of Cardiovascular Disease, San Francisco, CA). Human Lp[a] transgenic mice were generated as previously described (27) by breeding the human apo[a] transgenic mice with the human apoB-100 transgenic mice.

Characterization of human apoB-100K4 → S4 in transgenic mouse plasma

The ability of the apoB-100K4 → S4 protein to bind to multiple human apoB-specific monoclonal antibodies was examined by Western blot analysis. For these studies, we used the following antibodies: 1D1, which binds between apoB-100 amino acids 474 and 539 (24); MB47, which binds near amino acid 3,500 (28); Bso16, which binds between amino acids 4,157 and 4,189 (24); and 605, which binds between amino acids 4,235 and 4,536 (29). Plasma samples from transgenic mice that express human apoB-100 or human apoB-90 were used as controls. To evaluate the distribution of the mutant apoB-100 among the plasma lipoproteins, apoB-100K4 → S4 transgenic mouse plasma was size fractionated by fast protein liquid chromatography (FPLC) on a Superose 6 10/30 column (Pharmacia Biotech, Uppsala, Sweden) (22). The lipoprotein fractions were analyzed for cholesterol content by an enzymatic assay (Cholesterol CHOD-PAP, Boehringer Mannheim, Mannheim, Germany), and apoB content was analyzed by Western blot analysis with the 1D1 antibody.

Assessing the ability of the apoB-100K4 → S4 protein to form Lp[a]

The ability of apoB-100K4 → S4 to form Lp[a] was assessed using a modification of the assay described by McCormick et al. (26). Plasma (10 μl) from an apo[a] transgenic mouse was incubated with plasma samples from either human apoB-100 transgenic mice (20 μl) or human apoB-100K4 → S4 transgenic mice (34 μl) in 0.15 M NaCl in a total volume of 400 μl at 37°C for 2 h. Similar incubations containing apo[a] only and apo[a] with human apoBCys4,326Gly, a mutant apoB that noncovalently binds apo[a] but cannot make the covalent linkage, were included as negative controls. The amount of Lp[a] formed in each incubation was measured by an Lp[a]-specific sandwich ELISA (30) using MAb a6 as the capture antibody and horseradish peroxidase (HRP)-labeled 1D2 as the detection antibody (24). The amounts of human apoB in each incubation were measured using the same ELISA procedure, only in this case, the detection antibody was the human apoB-specific antibody, MB47 (28). A human serum control (Precipath®, Roche Diagnostics Corporation, Indianapolis, IN) was used as the apoB standard. All steps were performed at room temperature unless otherwise stated. A time course assay of Lp[a] formation was also performed on similar incubations in which samples (40 μl) were removed from the incubations at 10 min, 30 min, 60 min, 120 min, and 240 min, and were immediately snap frozen and stored at −80°C. The amount of Lp[a] in the incubations versus free apo[a] was visualized by Western blot analysis with the MAb a5-HRP antibody (30). The percent of apo[a] in the form of Lp[a] in each incubation was determined by densitometric analysis of the blot, where the density of the Lp[a] band was divided by the sum of the densities of the Lp[a] and free apo[a] bands.
**RESULTS**

**Generation of the mutant apoB-100K4→S4 YAC**

To test the importance of apoB-100 amino acids 4,372–4,392 for Lp[a] assembly, we generated a line of transgenic mice expressing a mutant apoB-100, apoB-100K4→S4, in which serines were substituted for the lysine residues at amino acid positions 4,372, 4,379, 4,385, and 4,392. The base substitutions required to effect the lysine-to-serine mutations were targeted into exon 29 of the apoB-100 gene-targeting vector coding for lysine-to-serine substitutions at amino acid positions 4,372, 4,379, 4,385, and 4,392 was linearized with EcoRI and transformed into spheroplasts made from yeast harboring a 108 kb human apoB-100 YAC. Correctly targeted URA3-positive pop-in clones and properly recombined URA3-negative pop-out clones were identified by pulsed-field gel and PCR analysis. B: Yeast DNA from clones that contained the 108 kb wild-type human apoB-100 YAC (Wt), the pop-in apoB-100K4→S4 YAC (PI), and the pop-out apoB-100K4→S4 YAC (PO) was separated on a 1% agarose pulsed-field gel alongside DNA markers (New England Biolabs, Beverly, MA). A 7 kb increase in YAC size seen in the PI clone was reversed by intrachromosomal recombination in the PO clone. C: A 0.9 kb DNA fragment of the human apoB gene spanning the mutated region was amplified from the genomic DNA of Wt, PI, and PO yeast clones. The amplified DNA fragments were digested with either Bpu1102I or Clal and analyzed on an ethidium bromide-stained 1% agarose gel alongside molecular weight marker XIII (Boehringer Mannheim).

Characterization of the apoB-100K4→S4 transgenic mice

The mutant YAC was purified from pulsed-field gels and microinjected into mouse zygotes to generate transgenic mice. Analysis of the genomic DNA from 27 founders by PCR and Bpu1102I and Clal restriction digestion identified two animals harboring the mutant human apoB transgene. Both founder animals expressed the full-length mutant human apoB-100 as detected by Western blots of mouse plasma using the anti-human-apoB-specific monoclonal antibody, 1D1. To verify that the introduced mutations had not had any major effect on the conformation of the mutant apoB, four different human apoB-specific monoclonal antibodies were tested for their ability to bind the mutant apoB (Fig. 2A). The apoB-100K4→S4 mutant bound all antibodies tested, including the 605 antibody that binds an epitope in the same region as the mutated site. The distribution of the mutant apoB among the plasma lipoproteins was assessed by FPLC fractionation of mouse plasma and Western blot analysis. The distribution of apoB-100K4→S4 was almost identical to that of wild-type apoB-100, with both proteins residing mainly in the LDL fractions (Fig. 2B).

**Lp[a] formation with the mutant apoB-100K4→S4 in vitro**

To assess the ability of the apoB-100K4→S4 mutant to bind human apo[a] and form Lp[a] in vitro, we incubated plasma from an apoB-100K4→S4 transgenic mouse with plasma from an apo[a] transgenic mouse. A Lp[a] ELISA revealed that the mutant apoB-100 was capable of
forming Lp[a], but the amount of Lp[a] formed in a 2 h incubation was less than that formed in a control incubation containing wild-type human apoB-100 (Fig. 3A). Both incubations contained similar amounts of human apoB, as measured by a human apoB-specific ELISA (Fig. 3B). A control incubation containing apo[a] and the human apoBCys4,326 mutant showed no Lp[a] formation, demonstrating that the Lp[a] ELISA assay only measures covalently bound apo[a]/apoB complexes. To further investigate Lp[a] formation with the apoB-100K4→S4 protein, we used a Western blot-based assay to assess the kinetics of Lp[a] formation with the mutant apoB-100 compared with human apoB-100 (Fig. 4). For these experiments, incubations containing apo[a] and equal amounts of human apoB-100K4→S4 or human apoB-100 from transgenic mouse plasma were stopped at 10 min, 30 min, 60 min, 120 min, and 240 min, and the amount of Lp[a] formed during the time period was assessed by Western blot analysis. Densitometric analysis of the Western blots showed that at each time point, a higher percentage of apo[a] was in the form of Lp[a] in the apoB-100 incubation compared with the apoB-100K4→S4 incubation. The difference was most striking at the 30 min time point where 82% of the total apo[a] had formed Lp[a] in the apoB-100 containing incubation compared with only 10% in the apoB-100K4→S4 incubation. These results indicate a clear difference in assembly kinetics between apoB-100 and apoB-100K4→S4, with the mutant apoB showing a much slower time course of Lp[a] formation.

Lp[a] formation with the mutant apoB-100K4→S4 in vivo

To test the ability of the apoB-100K4→S4 protein to form Lp[a] in vivo, we bred mice expressing the mutant apoB with apo[a] transgenic mice and assessed the amount of Lp[a] in the plasma of double transgenic offspring. Figure 5 shows the relative amounts of apo[a] and apoB in five offspring (lanes 1–5) resulting from a mating between an apoB-100K4→S4/apo[a] double transgenic mouse and a human apoB-100 transgenic mouse.
an apoB-100K\textsubscript{4→S4} transgenic mouse. There was some Lp[a] in the plasma of each apoB-100K\textsubscript{4→S4}/apo[a] animal; however, a significant amount of the apo[a] in all five animals was in the form of free apo[a]. The Lp[a] formed with the mutant apoB in vivo was reduced to free apo[a] under reducing conditions, indicating a covalent linkage with apo[a] (data not shown). These results were in contrast to that seen in a control Lp[a] mouse expressing apo[a] and wild-type human apoB-100 (far left lane). As previously reported (27), all of the apo[a] in the plasma of the Lp[a] mouse was covalently bound to apoB to form Lp[a] and there was no free apo[a] present. The actual levels of human apoB and apo[a] in the apoB-100K\textsubscript{4→S4}/apo[a] and Lp[a] mice are given in Fig. 5 and appear to be similar between animals.

**DISCUSSION**

The current study sought to define the structural features of apoB that facilitate its association with apo[a] to form Lp[a]. Previously, we demonstrated that apoB-100Cys\textsubscript{4,326} was the site of covalent attachment to apo[a] (8). It was obvious, however, even before these studies, that Lp[a] assembly relied on more than just two free cysteines. A widely accepted two-step model of Lp[a] assembly depicts a noncovalent association between apoB and apo[a] preceding the formation of the disulphide bond (5, 6). The exact amino acids required for the noncovalent association between apo[a] and apoB are not well characterized, although a growing body of evidence suggests that apoB lysine residues may be important (11–16).

Our original hypothesis was that the apoB sequences involved in the noncovalent binding to apo[a] would be in close proximity to the Cys\textsubscript{4,326} residue. Previous studies of two truncated apoB proteins suggested the presence of a putative apo[a] binding site in the region between apoB amino acids 4,330 and 4,397 (19). An alignment of the human apoB\textsubscript{4,330–4,397} region against pig, mouse, and rat apoB is shown in Fig. 6A. These other apoB species are of interest inasmuch as it has been shown that pig, mouse, and rat apoB is shown in Fig. 6A. These other apoB species are of interest inasmuch as it has been shown that pig, mouse, and rat apoB still bind apo[a] despite lacking the Cys\textsubscript{4,326} residue required for the disulphide link with apo[a] (10, 31). The alignment shows clusters of amino acids within the 4,330–4,397 region that are highly conserved. Also of note are four well-conserved lysine residues at apoB amino acids 4,372, 4,379, 4,385, and 4,392. All four lysine residues are contained within a sequence (amino acids 4,365–4,396) that is predicted to form a Class A amphipathic helix (10, 32) with lipid binding properties. Indeed, circular dichroism studies of a syn-
thetic apoB peptide spanning amino acids 4,372–4,392 show the sequence to be of α-helical structure and capable of interacting with lipid (20). Furthermore, the same study showed that the apoB 4,372–4,392 peptide was able to bind apo[a] and effectively inhibit Lp[a] formation (20). This led us to hypothesize that one or more of the four lysines in the 4,372–4,392 sequence were important for the interaction with apo[a].

To test the physiological importance of the apoB lysine residues at positions 4,372, 4,379, 4,385, and 4,392 for the interaction with apo[a], we generated a line of transgenic mice expressing a mutant apoB in which all four lysines were substituted for serines. Serine was selected, because its polar side chain would not be expected to perturb the local α-helical structure. Evaluation of the apoB-100K4→S4 protein by Western blot analysis with a panel of monoclonal antibodies showed no major conformational changes compared with wild-type apoB-100. In vitro experiments showed that the mutant apoB-100 was impaired in its ability to form Lp[a] compared with wild-type human apoB-100. The apoB-100K4→S4 protein showed a slower rate of Lp[a] formation than did the wild-type apoB-100. These results were reiterated in vivo in double transgenic mice expressing both the apoB-100K4→S4 protein and apo[a]. The double transgenic mice were capable of forming some Lp[a]; however, their plasma contained significant amounts of free apo[a], demonstrating that the mutant apoB was inefficient at associating with apo[a]. Our findings show that mutations in the apoB-4,372–4,392 sequence clearly disrupt Lp[a] assembly. Furthermore, we demonstrate that lysine residues in the sequence are important for the interaction with apo[a].

Figure 6B shows the predicted structure of the α-helix spanning the apoB amino acids 4,365–4,396 sequence.
The four lysine residues, Lys4,372, Lys4,379, Lys4,385, and Lys4,392, contained within the helix are located along the lipid-water interface. We hypothesize that one of the lysine side chains interacts with a kringle IV lysine binding pocket [possibly kringle IV₀ (33) or kringle IV₇ (34)] to facilitate the noncovalent binding to apo[a]. The Lys4,372 and Lys4,385 residues are the most highly conserved of the four residues. Interestingly, a variant of the apoB4,372–4,392 peptide containing a Lys4,372Ala substitution shows substantial loss of Lp[a] inhibitor activity (35), suggesting that Lys4,372 may be the lysine involved in the interaction with apo[a].

Although lysine residues in the apoB4,372–4,392 sequence are clearly important for binding to apo[a], other amino acids in the apoB4,372–4,392 sequence must play a role, because the apoB100K₄→S₄ mutant was still capable of forming some Lp[a]. Molecular modelling studies of the lysine binding pocket in apo[a] kringle IV₀ (33) and apo[a] kringle IV₇ (34) bound to lysine analogs show an anionic center that interacts with the amide group of the lysine side chain. Adjacent to the anionic center is a hydrophobic trough consisting of two tyrosine and two tryptophan residues that interact with the hydrocarbon backbone of the free lysine residue. In the physiological setting, where the lysine backbone is constrained as part of a polypeptide chain, it is possible that the hydrophobic trough in apo[a] is available for a hydrophobic interaction with apoB. The interaction of apo[a] kringle IV structures with hydrophobic residues such as phenylalanine, praline, and leucine has been predicted by computer modelling of these amino acids in the ligand binding domain of kringle IV₀ (36). Interestingly, there are several highly conserved hydrophobic residues within the apoB4,372–4,392 sequence (Fig. 6A), and mutation of hydrophobic residues in the apoB4,372–4,392 peptide does disrupt its ability to bind apo[a] (R. Sharp and S. McCormick, unpublished observations). It is possible that a hydrophobic interaction between the apoB100K₄→S₄ mutant and apo[a], combined with a weak interaction of the substituted serine with the anionic center, still allows for some Lp[a] formation.

An alternative explanation for the Lp[a] formation seen with the apoB100K₄→S₄ protein is that other apoB sequences remote from the 4,372–4,392 sequence are able to facilitate the binding of apoB to apo[a] to allow Lp[a] formation, albeit more slowly. A recent study by Becker et al. (18) of a truncated apoB, apoB18, identified a 25 amino acid sequence in the amino terminus of apoB (amino acids 680–704) that interacts with apo[a] in vitro. The apoB18/S680 was found to be important for the binding of apoB18 to apo[a], suggesting the presence of an N-terminal site that may also be necessary for the proper anchoring of apoB to apo[a] to facilitate Lp[a] formation.

Our study is the first to document defective Lp[a] assembly in vivo after the introduction of point mutations into a full-length apoB100 molecule. We have demonstrated that mutation of amino acids in the apoB4,372–4,392 sequence significantly impairs Lp[a] formation. Lysine residues in the sequence are important for the interaction, although it is likely that other amino acids in this sequence play a role in binding to apo[a]. Binding of the apoB4,372–4,392 sequence onto apo[a] kringle IV structures, combined with further mutagenesis studies of the 4,372–4,392 sequence, will be required to establish the exact amino acids in the 4,372–4,392 sequence involved in the binding to apo[a]. A more complete understanding of the protein sequences involved in Lp[a] assembly may ultimately lead to the design of novel inhibitors to prevent Lp[a] formation and hence provide a potential new class of Lp[a]-lowering agents.

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