Apolipoprotein(a) (Lp(a))1 is formed by the association of apolipoprotein(a) (apo(a)) with apolipoprotein B (apoB) of low density lipoprotein (LDL). Although weaker non-covalent interactions may occur between apoB and apo(a), it is the presence of a disulfide bond that binds them covalently (1–4). A single cysteine of apo(a) (Cys-4057) has been identified in forming the in vitro covalent association of apo(a) with human LDL (5, 6). The introduction of a point mutation into the apo(a) molecule to remove Cys-4057 and replace it with either a serine or glycine was sufficient to prevent the formation of a tight association between LDL and apo(a). However, the cysteine of apoB involved in this association has not been unambiguously identified. Several studies (7–9) have suggested that Cys-3734 or possibly Cys-4190 of apoB could potentially form a disulfide bridge with apo(a). Many of these studies were based upon the use of fluorescent probes that bind to free cysteine residues of proteins. Fluorescently labeled tryptic peptides of apoB that contain Cys-3734 were absent in the apoB of Lp(a), suggesting that this cysteine was unavailable for binding to the fluorescent probe because of an association with apo(a) (8). Computer modeling also demonstrated that the peptide region around Cys-3734 was the most energetically favorable to associate with the amino acids surrounding Cys-4057 of apo(a) (8). Both of these approaches, however, lacked direct experimental evidence for the involvement of Cys-3734 in the binding of apoB to apo(a).

Studies utilizing a truncated form of apoB (apoB-90) expressed in transgenic mice suggested that one of the two cysteines in the deleted terminal 10% of the molecule, Cys-4190 or -4326, could be involved in binding to apo(a) (10). Another study, using a truncated apoB-94 peptide produced in cell culture, presented evidence not indicated by the fluorescent labeling studies and suggested that Cys-4326, the terminal cysteine of apoB, is involved in disulfide linkage with apo(a) (11). In both of these studies, deletion of the terminal 6 or 10% of the apoB peptide could not be excluded from inhibiting an association with an alternative cysteine such as Cys-3734. This is important because it has been demonstrated that the conformation of apoB may influence the binding characteristics of apo(a) to apoB on LDL. Individuals with lecithin:cholesterol acyltransferase deficiency have been demonstrated to lack plasma Lp(a) (12). The LDL of these individuals was found to be deficient in cholesterol, which suggested that interactions between apo(a) and apoB may be impacted upon by the composition and size of LDL mediating conformational changes in apoB. The low binding characteristics of very low density lipoprotein with apo(a) (13) may also be due to conformational changes. Truncated forms of apoB could adopt conformations inhibiting an association with apo(a) in a similar manner.

Ap(a) is not normally expressed in mice, and in human apo(a) transgenic mice, apo(a) is not covalently linked to murine apoB (14, 15). However, in mice that contain both transgenes for human apo(a) and apoB, apoB particles, similar to that observed in humans, are present in the plasma of the double transgenic animals (16, 17). In this study we have used transgenic mice to study the in vivo interactions of apo(a) and apoB. To determine which free cysteine of human apoB is involved in disulfide linkage to apo(a), we have employed site-directed mutagenesis to convert Cys-3734 and Cys-4326 to serines within separate apoB P1 phagemid genomic clones. The P1 clone of the human apoB gene was then used to prepare transgenic mice expressing the mutated protein. In combination with an apo(a)-expressing yeast artificial chromosome transgene (18), the formation of Lp(a) by the mutant apoB molecules in an in vivo setting was examined.
**EXPERIMENTAL PROCEDURES**

**Mutagenesis—** ApoB P1-phagemid (DMPC-HFF #L-0261G) DNA was prepared by alkaline lysis of the bacterial cells followed by phenol extraction and ethanol precipitation of the DNA. A 1.55-kilobase pair (kb) EcoRI fragment from apoB-P1 DNA, containing codon 3734 (19, 20), was subcloned into an M13 vector (M13B/M20, Boehringer Mannheim) and mutagenized by standard procedures (21, 22) with primer A. Primer B was used for sequencing of both the parental phage and the mutagenized phage using the Sequenase version 2.0 sequencing kit (U. S. Biochemical Corp.). For mutation of codon 4326, PCR with Pfu DNA polymerase (Stratagene) was used to amplify a 123-bp product from the P1 DNA with primers C and D. Primer C contained the PfuDNA polymerase (Stratagene) was used to amplify a 123-bp product and the mutagenized phage using the Sequenase version 2.0 sequencing primer A. Primer B was used for sequencing of both the parent alphage extraction and ethanol precipitation of the DNA. A 1.55-kilobase pair was prepared by alkaline lysis of the bacterial cells followed by phenol.

**Hin**

**Methylate**

**selected for removal by RARE digestion.**

**Hin**

**dIII sites left unprotected by two oligonucleotides tar-

**dIII and one of the two PvuI sites of pBluescript II SK**

**to form the plasmid pH. Sequencing of pH was carried out using the 5K primer (Stratagene). A 4.1-kbp Scal-PvuI fragment of the apoB gene, contiguous with the 160-bp PvuI-HindIII fragment and containing a single HindIII site, was subcloned to a separate PvuI-digested pBluescript plasmid to form pSP. The 448-bp PvuI fragment from pH was then subcloned into the single PvuI site of pSP to recreate a 4.1-kbp HindIII fragment suitable for replacement of the native 4.1-kbp HindIII fragment. RARE digestion of Plasmid DNA—RARE digestion of P1 DNA (25, 26) and the creation of the apoB-P1 containing the mutated codon 3734 was performed as described previously (27). However, for the replacement of codon 4326, a 4.1-kbp HindIII fragment of the apoB-P1 was selected for removal by RARE digestion. Alu methylase was used to methylate HindIII sites left unprotected by two oligonucleotides targeted to the HindIII sites surrounding codon 4326. The DNA was then digested with HindIII to specifically cut these sites protected from methylation and used directly for ligation of the mutated 4.1-kbp fragment along with the chloramphenicol resistance/sacB selectable markers (27). Plasmids containing the mutated fragment and the chloramphenicol resistance/sacB genes were selected on chloramphenicol-containing plates. A P1 plasmid was then isolated and characterized by restriction enzyme digestion. The chloramphenicol resistance/sacB genes were removed with selection on 5% sucrose (28), leaving the mutated 4.1-kbp fragment within the apoB gene.

**Production of Transgenic Mice—** To prepare the DNA for microinjection, P1 phagemid DNA that had been prepared as above was gently treated with GeneClean (Bio101) and microanalyzed on 0.25-μm type V filters (Millipore, Bedford, MA) against TE. Microinjection of FVB embryos was performed as described previously (29, 30). Transgenic founder animals were screened for expression of the transgene by enzyme-linked immunosorbent assay (ELISA) (16). The Ser-3734 apoB and Ser-4326 apoB transgenic founder animals were bred with apo(a) yeast artificial chromosome hemizygous transgenic mice (18) to obtain mice expressing both apo(a) and apoB proteins. Centrifugation and Immunoblotting—EDTA plasma was collected from the tail vein of non-fasted mice. The total lipoprotein fraction was prepared by adjusting 25 μl of plasma to a volume of 230 μl and a density of 1.21 g/ml with NaBr before centrifugation at 40,000 rpm for 18 h and at 10°C in the type 42.2 rotor. A volume of 25 μl was removed from the top of the tubes (d < 1.21 g/ml) and also from the bottoms (d > 1.21 g/ml). Plasma fractions of 5 μl were combined with 50 μl of 15% SDS, 6x urea, 5% 2-mercaptoethanol, 0.2 x Tris, pH 6.8, sample buffer, boiled for 5 min, and electrophoresed on precast SDS-polyacrylamide gels (4-12% acrylamide, Novex, San Diego, CA). The proteins were transferred to nitrocellulose and immuno-detected with a goat polyclonal anti-Lp(a) antibody (Biodesign, Kennebunkport, ME) for the detection of apo(a) (18) or a biotinylated human apoB specific monoclonal antibody for apoB (16).

**Oligonucleotides—** The oligonucleotides (5’-3’ used were: A, GT-CAGTTGTTCCGGATGACCC; B, CAAATGATGAAGTTCTCAGC; C, AAAGAAAGCACAAGTTGATCCAT; D, CAAATATTCTTCACG-GAAGGGC; E, AAGCCAAAGAGGTATTTAAAGGC; F, GGCTGATAATTCAATTATTCCTG; G, CTTCTAGGGAATTATTCCTCGG.

**RESULTS AND DISCUSSION**

**Vector Construction and Creation of Transgenic Mice—** The P1 phagemid genomic clone described here has previously been used in the production of apoB transgenic mice and contained 75 kb of human genomic sequence including the 43-kbp apoB gene with extensive 5’ and 3’ sequences (16). Manipulation of the intact phagemid to allow mutagenesis was not feasible due to the lack of conveniently located, infrequent, restriction enzyme sites. To achieve selective removal and reinsertion of small fragments of DNA from the phagemid we utilized RARE digestion. We have previously reported the use of this technique to remove a 1.55-kbp DNA fragment from the apoB-P1 and insertion of the mutated fragment (27). This technique involves protecting specific restriction enzyme sites by forming a triplex DNA complex over the region with a complementary oligonucleotide. Unprotected sites are methylated, followed by dissociation of the complex and inactivation of the methylase. The protected sites can then be specifically cut with the restriction enzyme. We have expanded the use of this technique to include the chloramphenicol resistance/sacB genes as positive and negative selection markers for replacement of the wild-type sequence with the mutated sequence.

**Mutagenesis of the 1.55-kbp EcoRI fragment containing codon 3734 was carried out in M13, whereas PCR mutagenesis was used for alteration of codon 4326 followed by subcloning of the product to a plasmid for sequencing.** The DNA sequences surrounding the mutated sites were determined and compared with that of the published sequence. For both codon 3734 and codon 4326, a single base change was introduced into the apoB sequence converting a thymidine to an adenosine and thus effectively converting a cysteine to a serine in the translated product. No other changes to the apoB gene sequence were detected.

Four Ser-3734 apoB and five Ser-4326 apoB founder mice expressing human apoB as determined by immuno-dot blot assay were created, but only two founders of each mutation type were bred with apo(a) transgenic mice to generate transgenic lines. PCR was used to confirm the presence of the mutated DNA sequences in the genomes of the transgenic mice (Fig. 1). Introduction of the point mutations and conversion of thymidine to adenosine within the apoB DNA sequence introduces TaqI and Ddel restriction enzyme sites for codon 3734 and codon 4326, respectively. No product was obtained from non-transgenic mouse genomic DNA in each case, demonstrating that both primer sets were specific for human DNA. Genomic DNA from apoB transgenic mice gave a 203-bp PCR product from the codon 3734 region and a 331-bp product from the codon 4326 region. However, the product from the Ser-3734 apoB genomic DNA was able to produce the TaqI digestion pattern confirming the presence of the mutated apoB in these mice. Similarly, the PCR product for the codon 4326 region was digested with Ddel in Ser-4326 apoB mice.
The size of human apoB found in plasma from the various transgenic strains was compared by immunoblot analysis (Fig. 2). The human protein found in both of the mutated apoB transgenic lines was identical in size to the human apoB from wild-type (wt) apoB transgenic mouse plasma. Both apoB-48 and apoB-100 bands were present demonstrating normal mRNA editing and synthesis of the peptide.

Association of Human ApoB with Apo(a)—Previous studies have demonstrated that the covalent association of apo(a) with apoB can withstand centrifugation during the separation of lipoproteins from other plasma proteins. Ultracentrifugation was used in this study to demonstrate the presence or absence of apo(a) within the lipoprotein fraction of plasma. Apo(a) was found associated with lipoproteins in plasma from wt apoB/apo(a) transgenic mice and Ser-3734 apoB/apo(a) transgenic mice. However, in apo(a) and Ser-4326 apoB/apo(a) mice, apo(a) was found only within the lipoprotein-free fraction of plasma and was not associated with lipoproteins (Fig. 3). These results clearly demonstrate that the single amino acid conversion of Cys-4326 to serine, in itself, was sufficient to prevent formation of Lp(a). In contrast, conversion of Cys-3734 to serine failed to prevent an association of apo(a) with apoB.

A significant proportion of apo(a) was found in the lipoprotein-free fraction of plasma from the wt apoB/apo(a) mice (Fig. 3). This was a consistent feature of this line and was attributed to the low concentration of human apoB in these animals compared with higher expressors. To ensure that adequate levels of apoB were available for maximum binding of apo(a), the plasma concentration of human apoB was assessed for those mice presented in Figs. 2 and 3 using a human apoB specific antibody. The wt apoB line contained 14 mg/dl compared with the Ser-3734 apoB and Ser-4326 apoB lines that contained 59 and 20 mg/dl, respectively. These data demonstrate that the lack of association between Ser-4326 apoB and apo(a) could not be attributed to low levels of circulating apoB in these mice.

ApoB contains 25 cysteine residues with at least 16 of these committed to intramolecular disulfide bonds. This leaves 9 potential cysteine residues available for intermolecular interactions (9). In the present study we chose to replace Cys-3734 and Cys-4326 of human apoB with serine, based upon previous reports suggesting that at least one of these cysteines is a likely candidate for the single disulfide linkage to apo(a). Our results clearly demonstrate that only Cys-4326 is required for the covalent interaction of human apo(a) and human apoB. This is in contrast to a recent study suggesting that 2 molecules of apo(a) are associated per single apoB molecule within the Lp(a) structure (31). The complete lack of detectable apo(a) within the lipoprotein fraction of the Ser-4326 apoB mice strongly implies that only one molecule of apo(a) is associated covalently with each apoB molecule. Furthermore, the possibility that Cys-3734 is responsible for binding one of the apo(a) molecules also seems unlikely. Non-reduced Lp(a) from Ser-3734 apoB/apo(a) mice demonstrated identical migration compared with Lp(a) from wt apoB/apo(a) mice on SDS-polyacrylamide gels (data not shown), suggesting an equal number of apo(a) molecules bound in each case. However, it is not inconceivable that Lp(a) could require the binding of an initial apo(a) molecule at Cys-4326 to expose alternative binding sites for a second apo(a) molecule.

The large size of the apoB gene has made it difficult to study structural features of this protein. However, with the advent of new technologies such as RARE digestion of DNA, site-specific mutagenesis, and manipulation of large genomic clones can be achieved. Here, we have used this approach to demonstrate that Cys-4326 of human apoB, and not Cys-3734, is required for the covalent, disulfide linkage between human apoB and apo(a) in vivo. Furthermore, the transgenic mice developed in these studies may prove useful for the analysis of early events leading to the formation of the disulfide bond. Inhibition of this process could provide new approaches to reducing plasma levels of Lp(a).

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