Structural features of apolipoprotein B synthetic peptides that inhibit lipoprotein(a) assembly

Rebecca J. Sharp,* Matthew A. Perugini,† Santica M. Marcovina,‡ and Sally P. A. McCormick[1,*]

Department of Biochemistry,* University of Otago, Dunedin, New Zealand; Department of Biochemistry and Molecular Biology,† University of Melbourne, Victoria, Australia; and Department of Medicine, Northwest Lipid Research Laboratories,‡ University of Washington, Seattle, WA 98103

Abstract Lipoprotein(a) [Lp(a)] is assembled via an initial noncovalent interaction between apolipoprotein B100 (apoB) and apolipoprotein(a) [apo(a)] that facilitates the formation of a disulfide bond between the two proteins. We previously reported that a lysine-rich, α-helical peptide spanning human apoB amino acids 4372–4392 was an effective inhibitor of Lp(a) assembly in vitro. To identify the important structural features required for inhibitory action, new variants of the apoB$_{4372\text{-}4392}$ peptide were investigated. Introduction of a central leucine to proline substitution abolished the α-helical structure of the peptide and disrupted apo(a) binding and inhibition of Lp(a) formation. Substitution of hydrophobic residues in the apoB$_{4372\text{-}4392}$ peptide disrupted apo(a) binding and inhibition of Lp(a) assembly without disrupting the α-helical structure. Substitution of all four lysine residues in the peptide with arginine decreased the IC$_{50}$ from 40 μM to 5 μM. Complexing of the arginine-substituted peptide to dimyristoylphosphatidylcholine improved its activity further, yielding an IC$_{50}$ of 1 μM. We conclude that the α-helical structure of apoB$_{4372\text{-}4392}$, in combination with hydrophobic residues at the lipid/water interface, is crucial for its interaction with apo(a). Furthermore, the interaction of apoB$_{4372\text{-}4392}$ with apo(a) is not lysine specific, because substitutions with arginine result in a more effective inhibitor.—Sharp, R. J., M. A. Perugini, S. M. Marcovina, and S. P. A. McCormick. **Structural features of apolipoprotein B synthetic peptides that inhibit lipoprotein(a) assembly. *J. Lipid Res*. 2004.** 45: **2227–2234.**

Supplementary key words apolipoprotein(a) • DMPC vesicles • noncovalent interaction • peptide inhibitor

Lipoprotein(a) [Lp(a)] is an atherogenic lipoprotein comprised of apolipoprotein(a) [apo(a)] attached to the apolipoprotein B100 (apoB) of a LDL (1). Large clinical trials have identified high plasma levels of Lp(a) as a risk factor for developing heart disease (2–4). The deposition of Lp(a) in vascular lesions has been well documented in humans (5, 6) and in animal models (7). Unfortunately, Lp(a) has proven resistant to traditional lipid-lowering therapies (8, 9), except high doses of niacin (10). Estrogen dramatically reduces Lp(a) levels (11), but a safe and effective agent for lowering Lp(a) levels has yet to be developed.

The majority of evidence suggests that Lp(a) assembly occurs in circulation (12) after secretion of apo(a) from the liver and metabolism of VLDL to LDL in the circulation. Lp(a) assembly is thought to be a two-step process (13, 14) with noncovalent interactions between apo(a) and apoB preceding the formation of the disulfide bond between apo(a) Cys4057 (13, 15) and apoB Cys4326 (16, 17). The two-step model of Lp(a) assembly was confirmed in a study of a human apoB100 mutant lacking Cys4326, which still showed binding to apo(a), despite being unable to form the disulfide bond (18).

Recent research has focused on characterizing the apoB residues involved in the noncovalent interaction with apo(a). Lysine residues on apoB have been implicated in the noncovalent interaction with apo(a), because lysine analogues disrupt Lp(a) assembly in vitro (19–21) and deletion of lysine-binding sites in apo(a) kringle IV domains interfere with Lp(a) formation (22–24). A number of apoB sequences that noncovalently bind apo(a) have been reported. Becker et al. (25) identified an apoB lysine residue in the N terminus, apoBLys680, that mediates the noncovalent binding of an apoB18 fragment to apo(a); in their study, a synthetic peptide spanning the Lys680 residue was shown to inhibit Lp(a) formation at similar concentrations to lysine analogues.

Sequences in the carboxyl terminus of apoB have also been implicated in the noncovalent interaction with apo(a).

Manuscript received 28 April 2004 and in revised form 12 July 2004 and in re-revised form 30 August 2004.

Published, JLR Papers in Press, September 16, 2004.
DOI 10.1194/jlr.M400163-JLR200

Abbreviations: apo(a), apolipoprotein(a); apoB, apolipoprotein B100; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; Lp(a), lipoprotein(a).

[1] To whom correspondence should be addressed.
e-mail: sally.mccormick@stonebow.otago.ac.nz

We recently reported that a synthetic peptide spanning apoB amino acids 4372–4392, bound apo(a) and inhibited Lp(a) assembly in vitro (26). Note that the $apoB_{4372\text{-}4392}$ peptide was 250-fold more effective than the lysine analogue, ε-amino-*n*-caproic acid, at inhibiting Lp(a) assembly (26). This work suggested that the apoB 4372–4392 sequence constitutes an apo(a) binding site that is important for Lp(a) assembly. The peptide sequence is highly conserved between species of apoB known to interact with apo(a) (27) and contains four highly conserved lysine residues surrounded by clusters of hydrophobic residues. Circular dichroism (CD) spectroscopy studies showed that $apoB_{4372\text{-}4392}$ is α-helical (26). Binding of the peptide to dimyristoylphosphatidylcholine (DMPC) increased its α-helical content and improved its capacity to inhibit Lp(a) assembly (26). To elucidate the key structural features responsible for the inhibitory properties of the $apoB_{4372\text{-}4392}$ peptide, a series of apoB peptides based on the apoB 4372–4392 sequence were designed and tested for their ability to bind apo(a) and inhibit Lp(a) assembly.

EXPERIMENTAL PROCEDURES

Peptides

Five new synthetic apoB peptides based on the human apo B4372–4392 sequence were synthesized by Chiron Mimotopes using an Applied Biosystems model 430A synthesizer. Peptides were purified by HPLC and stock solutions solubilized in Milli-Q water at a concentration of 1 mM, except for $apoB_{4372\text{-}92ARG}$, which was solubilized at 100 μM. Peptides were dissolved in 10 mM Tris, pH 7.4, for CD spectroscopy studies and Milli-Q water for inhibitory studies. **Table 1** gives the amino acid sequences and associated characteristics of the new series of peptides. The importance of the α-helical structure for activity was tested with the $apoB_{L4383P}$ peptide, which contains a central leucine to proline substitution that was expected to perturb the α-helical structure. The importance of the N- and C-terminal lysine residues was tested with the $apoB_{K4372A}$ and $apoB_{K4392A}$ peptides, which contain lysine to alanine substitutions at Lys4372 and Lys4392, respectively. The importance of a cluster of four conserved hydrophobic residues surrounding the Lys4392 residue was tested in the $apoB_{4372\text{-}92HYDRO}$ peptide by substitution of these residues with alanines. To test whether the inhibitory capacity of the $apoB_{4372\text{-}4392}$ peptide is specific to lysines in the sequence, $apoB_{4372\text{-}92ARG}$ was created, in which all four lysine residues were substituted with arginine. A murine sequence spanning apoB residues 4372–4392 (KYYEIEENMVELIKTLLVSFR) was tested in Lp(a) formation assays. The murine sequence has conservation of two of the four lysine residues and a conservative substitution of the lysine at position 4392 to arginine.

Mice

Human apo(a) transgenic mice (19) were obtained from Dr. Robert Hammer (University of Texas Southwestern Medical Center, Dallas, TX) and human apoB transgenic mice (28) were provided by Dr. Stephen Young (Gladstone Institute of Cardiovascular Disease, San Francisco, CA). Wild-type mouse plasma was obtained from nontransgenic littermates.

Preparation of phospholipid vesicles

Small unilamellar vesicles of DMPC (Sigma-Aldrich, St. Louis, MO) were prepared using a method similar to that described by New (29). The DMPC was suspended in 10 mM Tris-HCl, pH 7.4, and sonicated using a 9.5 mm probe (Soniprep 150, MSE Scientific Instruments, Sussex, England) at 20 MHz for 10 × 60 s with 30 s breaks in between. The temperature was maintained at 30°C, above the phase transition temperature of DMPC (~24°C), using a jacketed vial with a circulating water bath (30). The vesicles were centrifuged at 2,250 × *g* for 5 min to pellet titanium originating from the sonicator probe. Phospholipid concentrations were determined at 20°C using an enzymatic phospholipid assay kit (Roche, Australia).

CD spectroscopy

The CD spectra of the apoB peptides in the absence and presence of DMPC were recorded at 25°C on an Aviv 62DS CD spectrophotometer using a 1 mm pathlength quartz cuvette. Spectra were collected between the wavelengths of 195 nm and 250 nm, with a step size of 0.5 nm and a slit bandwidth of 1.5 nm. Signal averaging time was 1 s, and ellipticities were reported as mean residue ellipticity ([θ]) in $°cm^2dmol^{-1}$. The concentration of each peptide used in the CD studies (150 μg/ml) was determined by absorption spectroscopy on a Cary 5 UV/Vis spectrophotometer at 280 nm. The peptides have a molar absorption or extinction coefficient ε of 2,980 M^{-1} cm^{-1} at 280 nm, except for $apoB_{4372\text{-}92HYDRO,}$ for which ε is 1,490 M^{-1} cm^{-1} at 280 nm (31). Freshly prepared DMPC vesicles (3 mM) were added to aliquots of the peptides at a molar ratio of 50:1 DMPC-peptide to form discoidal complexes, which were characterized as previously described (26). The α-helical content of each apoB peptide in the absence and presence of saturating amounts of DMPC was calculated from the molar ellipticity at 222 nm according to equation 1 by Aggerbeck et al. (32).

TABLE 1. Characteristics of the $ApoB_{4372\text{-}4392}$ peptide series

Peptide name	Amino acid sequence[a]	Purity (%)	M_r	% α-Helix[b]	
				−DMPC	+DMPC
$ApoB_{4372\text{-}4392}$	KYYELEEKIVSLIKNLLVALK	86	2,507	68	88
$ApoB_{L4383P}$	KYYELEEKIVS**p**IKNLLVALK	95	2,491	6	1
$ApoB_{K4372A}$	**a**YYELEEKIVSLIKNLLVALK	89	2,540	49	63
$ApoB_{K4392A}$	KYYELEEKIVSLIKNLLVAL**a**	95	2,540	66	87
$ApoB_{4372\text{-}92HYDRO}$	KY**a**ELEEKI**a**SLIKNL**aa**ALK	90	2,317	72	94
$ApoB_{4372\text{-}92ARG}$	**r**YYELEE**r**IVSLI**r**NLLVAL**r**	88	2,619	57	100

DMPC, dimyristoylphosphatidylcholine; M_r, relative molecular mass.

[a] Residues altered from the original $apoB_{4372\text{-}4392}$ peptide are shown in lowercase boldface type.

[b] The % α-helix was calculated using equation 1 and the $[\theta]_{222nm}$ obtained from the CD spectra; the average error was found to be ±2% for repeated scans of the same peptide.

$$\% \ \alpha\text{-helix} = -([\theta]_{222nm} + 2340)/303 \qquad (Eq.\ 1)$$

Apo(a) binding studies

Each peptide was evaluated for its ability to bind apo(a) using a solid-phase enzyme-linked ligand sorbent assay (26). Briefly, 96-well polystyrene microtiter plates (Costar®, Corning Inc., Corning, NY) were coated with 5 μg per well of each apoB peptide. Apo(a) transgenic mouse plasma, and wild-type mouse plasma (negative control) were diluted 1 in 10 and 50 μl aliquots and incubated on the peptide-coated plates for 90 min at room temperature to allow binding of apo(a) to the peptides. Bound apo(a) was detected with the HRP-labeled MAb-a5 antibody (33), washed, and then developed with substrate solution [*O*-phenylenediamine dihydrochloride and hydrogen peroxide (Sigma-Aldrich). The level of background binding was determined from the wild-type plasma sample and this was subtracted from the apo(a) binding curves.

In vitro Lp(a) formation assays

The peptides were tested for their ability to act as inhibitors of Lp(a) assembly as described by Sharp et al. (26). Plasma (1 μl) from an apo(a) transgenic mouse was incubated with plasma (2 μl) from a human apoB transgenic mouse for 3 h at 37°C in the presence of increasing amounts (1–400 μM) of each apoB peptide. The amount of Lp(a) formed in each incubation was assessed after aliquots were size-fractionated on SDS/4% polyacrylamide gels under nonreducing conditions and the separated proteins transferred to nitrocellulose for Western blotting with the HRP-labeled MAb-a5 antibody. Bands were visualized using the ECL detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). The amounts of Lp(a) formed in the incubations were quantified by an Lp(a)-specific ELISA, as detailed by Sharp et al. (26). Background absorbance was determined from incubations containing human apoB only and this was subtracted from all data points. The percent Lp(a) formed in each incubation was calculated relative to the incubation containing no inhibitor.

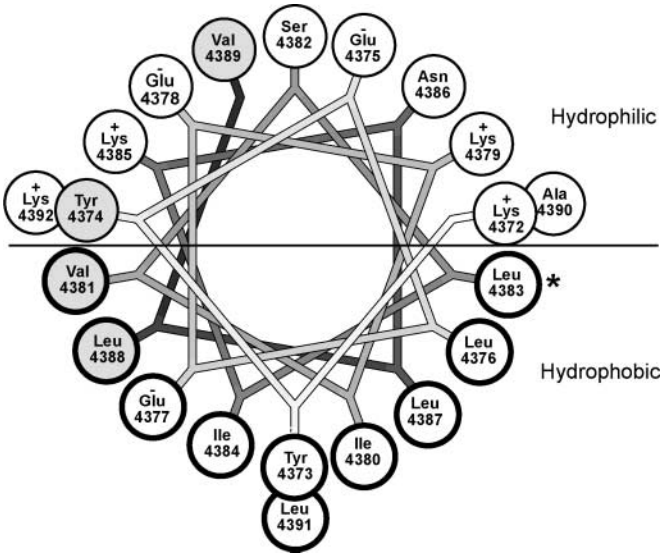

Fig. 1. Helical wheel diagram of the apolipoprotein B100 $apoB_{4372\text{-}4392}$ peptide. A helical wheel diagram of the predicted amphipathic helix formed by apoB residues 4372–4392 was produced using the WHEEL program (33). Within the helix, the side chains of the four lysine residues are found at the lipid/aqueous interface projecting into the aqueous phase; the side chains of the hydrophobic residues (in boldface) are predicted to be buried in the lipid phase. The hydrophobic residues (shaded in gray) were substituted with alanine in the $apoB_{4372\text{-}92HYDRO}$ peptide. The Leu4383 residue (marked with an asterisk) was targeted for substitution with proline in $apoB_{L4383P}$. The four lysine residues on either side of the lipid/water interface were targeted for substitution with arginines in $apoB_{4372\text{-}92ARG}$. The Lys4372 was targeted for substitution with alanine in $apoB_{K4372A}$ and Lys4392 was targeted for substitution in $apoB_{K4392A}$.

RESULTS

The WHEEL program (34) was used to produce a helical wheel diagram of the $apoB_{4372\text{-}4392}$ peptide (**Fig. 1**). Amino acids targeted for substitution in the new series of peptides are highlighted in Fig. 1. The secondary structures of the five human apoB peptides were determined by CD spectroscopy both in the absence and in the presence of DMPC and each was compared with the control $apoB_{4372\text{-}4392}$ peptide (**Fig. 2**). Using equation 1, the percent α-helical structure for each peptide was calculated from the ellipticity value at 222 nm and the data are reported in Table 1. The spectra for the control $apoB_{4372\text{-}4392}$ peptide (Fig. 2A) showed the characteristic double minima at 208 and 222 nm indicative of α-helical structure. The α-helicity of $apoB_{4372\text{-}4392}$ was further induced by the binding of the peptide to DMPC, as previously reported (26). As predicted, the proline-substituted peptide, $apoB_{L4383P}$, had lost all secondary structure and adopted largely random structure, as indicated by a single minimum at 205 nm (Fig. 2B). Furthermore, the addition of DMPC had little effect on the structure of this peptide (Fig. 2B). Substitution of the N-terminal lysine residue to alanine resulted in a 19% decrease in α helical content in the $apoB_{K4372A}$ peptide compared with $apoB_{4372\text{-}4392}$ (Fig. 2C). The addition of DMPC increased the helical content of the $apoB_{K4372A}$ peptide by 14%; however, the DMPC-complexed peptide was 25% less helical than $apoB_{4372\text{-}4392}$ in the presence of the same concentration of phospholipid. Substitution of the C-terminal lysine residue for alanine did not affect α-helical content; $apoB_{K4392A}$ showed a secondary structure similar to $apoB_{4372\text{-}4392}$ both in the absence and in the presence of DMPC (Fig. 2D). The hydrophobic replacement peptide, $apoB_{4372\text{-}92HYDRO}$, was slightly more helical than the original peptide, both in the absence (72% helical) and in the presence (94% helical) of DMPC (Fig. 2E). The $apoB_{4372\text{-}92ARG}$ peptide showed α-helical structure (Fig. 2F), with the binding of DMPC significantly increasing its α-helical content from 57% to 100%.

The ability of each new human apoB peptide to bind apo(a) was assessed using a solid-phase binding assay (**Fig. 3**). The original $apoB_{4372\text{-}4392}$ peptide had previously been shown to bind apo(a) in a saturable manner (26). Both the $apoB_{K4372A}$ and $apoB_{K4392A}$ peptides showed minimal binding to apo(a) (Fig. 3). The $apoB_{L4383P}$ and $apoB_{4372\text{-}92HYDRO}$ peptides did not demonstrate any apo(a) binding beyond background levels. The $apoB_{4372\text{-}92ARG}$ peptide was the only peptide shown to effectively bind apo(a) from transgenic mouse plasma. $ApoB_{4372\text{-}92ARG}$ bound apo(a) to a saturable level in a manner similar to that seen for the original $apoB_{4372\text{-}4392}$ peptide (26).

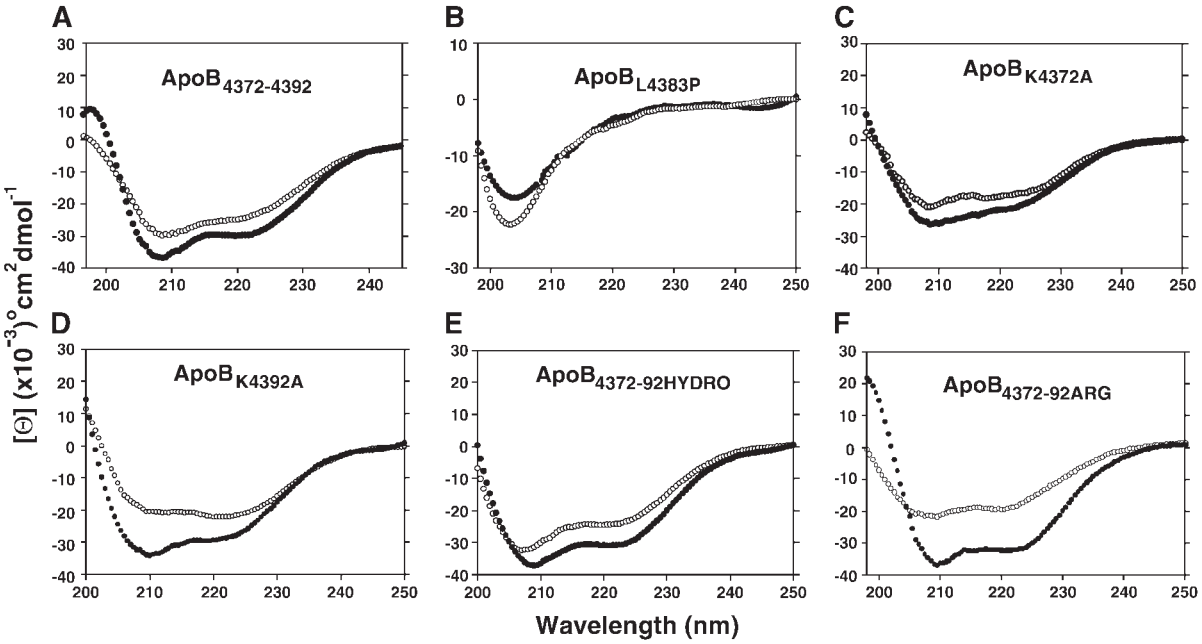

Fig. 2. Circular dichroism (CD) spectra of the apoB$_{4372\text{-}92}$ peptide variants. A: apoB$_{4372\text{-}4392}$. B: apoB$_{L4383P}$. C: apoB$_{k4372A}$. D: apoB$_{K4379A}$. E: apoB$_{4372\text{-}92HYDRO}$. F: apoB$_{4372\text{-}92ARG}$. The CD spectra of the variant apoB peptides (150 μg/ml, dissolved in 10 mM Tris, pH 7.4) in the presence (solid symbols) and absence (open symbols) of dimyristoylphosphatidylcholine (DMPC) vesicles are shown compared with the original apoB$_{4372\text{-}4392}$ peptide. Spectra were recorded on an Aviv62DS spectrophotometer at 25°C using a step size of 0.5 nm and signal averaging time of 1 s. The mean residue ellipticity ([θ]) is plotted as a function of wavelength (nm) for each peptide.

The capacity of each apoB peptide in Table 1 to inhibit Lp(a) assembly in vitro was investigated. Increasing amounts of each peptide were incubated with fixed amounts of apo(a) and human apoB in an Lp(a) formation assay. The amount of Lp(a) formed was visualized by Western blot analysis (**Fig. 4A–F**). The inhibitory effect of the apoB$_{4372\text{-}4392}$ control peptide is shown in Fig. 4A. Compared with the control (Fig. 4A), the apoB$_{L4383P}$ peptide (Fig. 4B) had little effect on Lp(a) assembly in vitro, suggesting that the loss in α-helical structure had significantly reduced its ability to inhibit Lp(a) assembly. The apoB$_{K4372A}$ peptide (Fig. 4C) showed only a slight disruption to Lp(a) assembly, and apoB$_{K4392A}$ (Fig. 4D) showed very little effect. The apoB $_{4372\text{-}92HYDRO}$ peptide also showed no effect on Lp(a) assembly, despite having retained its α-helical structure (Fig. 4E). The apoB$_{4372\text{-}92ARG}$ peptide proved to be a highly effective inhibitor of Lp(a) assembly. In the incubations containing apoB$_{4372\text{-}92ARG}$, Lp(a) levels decreased with increasing amounts of the peptide, and a corresponding increase in free apo(a) was seen (Fig. 4F). Indeed, the apoB$_{4372\text{-}92ARG}$ peptide was more effective than the original apoB$_{4372\text{-}4392}$ peptide as an inhibitor, showing complete inhibition of Lp(a) assembly at a concentration of 25 μM (compared with 100 μM for apoB$_{4372\text{-}4392}$).

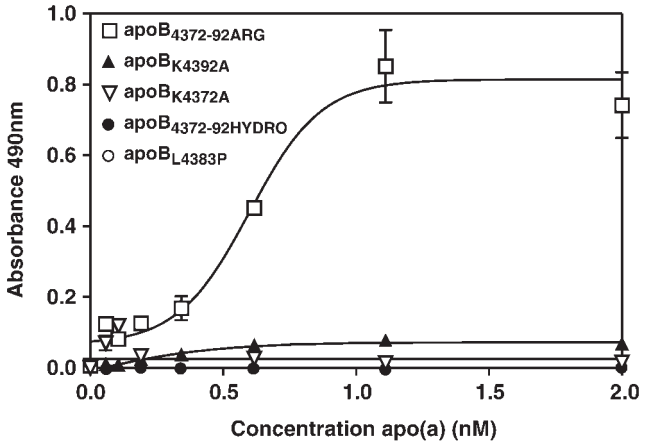

Fig. 3. Binding of the apoB$_{4372\text{-}92}$ peptides to apo(a). The binding capacity of each apoB peptide was measured using a solid-phase enzyme-linked ligand sorbent assay assay. Peptides were immobilized on microtiter plates and incubated with increasing amounts of apo(a) contained in diluted apo(a) transgenic mouse plasma. Bound apo(a) was detected with the MAb-a5 antibody. Data points represent the average of the absorbance values obtained from quadruplicate incubations. Error bars indicate the standard deviation.

In addition to Western blot analyses, the amount of Lp(a) formed in each incubation was quantified by an Lp(a) ELISA and the results were used to determine IC$_{50}$ values for each of the peptides (**Fig. 5**). The original apoB$_{4372\text{-}4392}$ peptide showed an IC$_{50}$ value of 40 μM, as previously reported (26). The apoB$_{L4383P}$ peptide had little inhibitory capacity, with an IC$_{50}$ value $>$ 400 μM. The hydrophobic replacement peptide, apoB$_{4372\text{-}92HYDRO}$, also showed very little inhibitory capacity, with an IC$_{50}$ $>$ 400 μM, whereas both apoB $_{K4372A}$ and apoB$_{K4392A}$ showed similar IC$_{50}$ values of approximately 100 μM. By contrast, the arginine-substituted peptide, apoB$_{4372\text{-}92ARG}$, was the most potent inhibitor of Lp(a) formation, with an IC$_{50}$ of 5 μM, 8-fold more effective than the apoB$_{4372\text{-}4392}$ peptide. The inhibi-

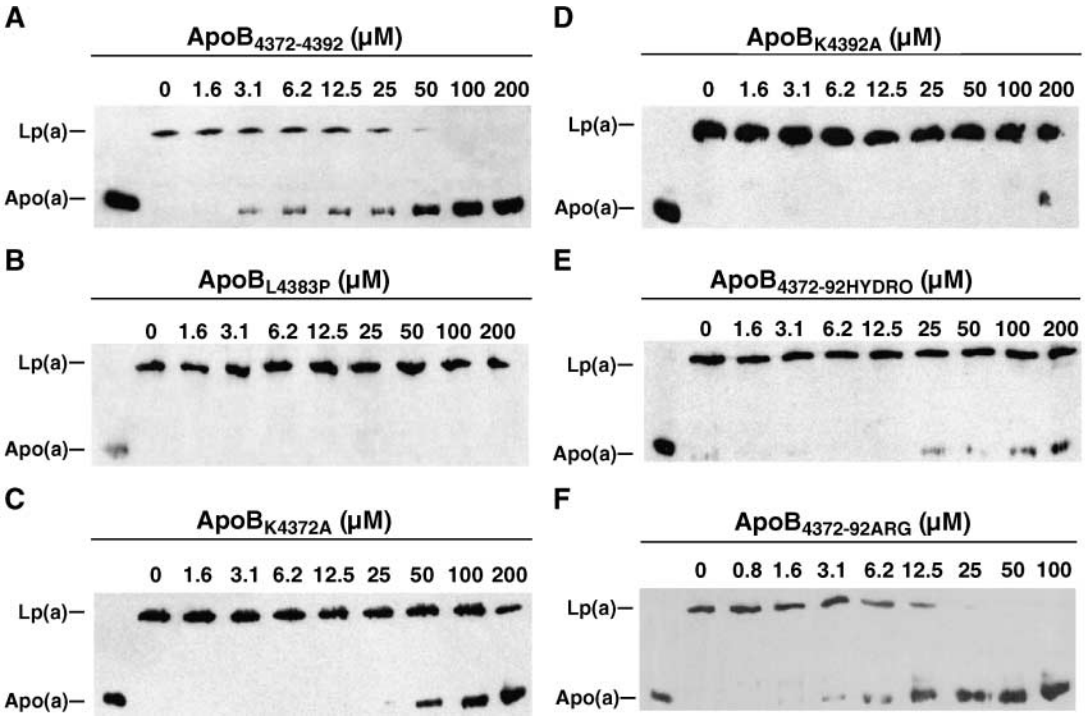

Fig. 4. Inhibition of lipoprotein(a) [Lp(a)] formation by apoB$_{4372\text{-}92}$ peptides. A: apoB$_{4372\text{-}4392}$. B: apoB$_{L4383P}$. C: apoB$_{k4372A}$. D: apoB$_{K4379A}$. E: apoB$_{4372\text{-}92HYDRO}$. F: apoB$_{4372\text{-}92ARG}$. Increasing amounts of each apoB peptide were added to incubations containing human apo(a) transgenic mouse plasma and human apoB transgenic mouse plasma. The original apoB$_{4372\text{-}4392}$ peptide was included as a control. The amount of Lp(a) formed in each incubation was assessed by separation of the incubation mix on SDS/4% polyacrylamide gels under nonreducing conditions and Western blot analysis with the MAb-a5 antibody.

tory capacity of a murine peptide spanning apoB 4372–4392, apoB$_{4372\text{-}92MURINE}$, which shows conservation of two of the four lysines in the human sequence and an arginine at position 4392, was also tested. ApoB$_{4372\text{-}92MURINE}$ was found to inhibit Lp(a) similarly to the human peptide, with an IC_{50} value of 45μM.

As apoB$_{4372\text{-}92ARG}$ proved to be the only peptide in the new series effective at inhibiting Lp(a) assembly, further studies were performed to investigate the inhibitory capacity of this peptide in the presence of phospholipid. The addition of DMPC to the apoB$_{4372\text{-}4392}$ peptide had previously been shown to improve the effectiveness of the peptide as an inhibitor of Lp(a) assembly (26). Similarly, we show that apoB$_{4372\text{-}92ARG}$ in the presence of DMPC im-

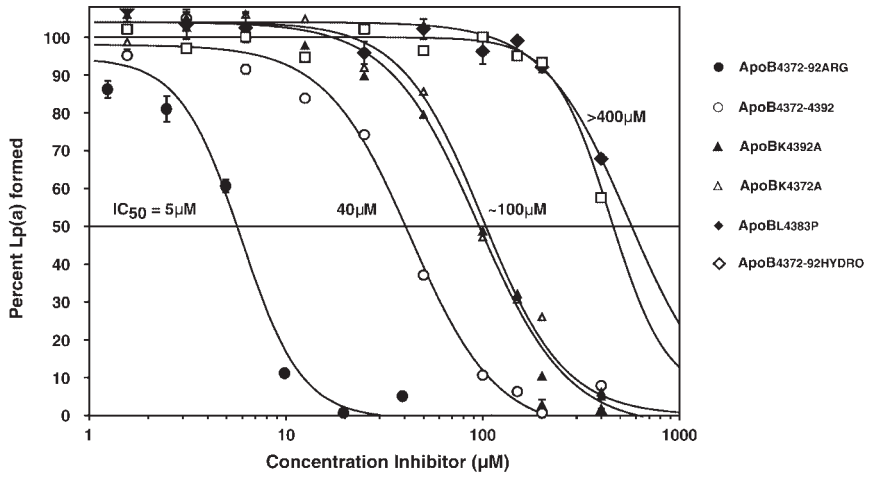

Fig. 5. Inhibitory capacity of the apoB$_{4372\text{-}92}$ peptides. Increasing amounts of each apoB peptide were added to incubations containing human apo(a) transgenic mouse plasma and human apoB transgenic mouse plasma. The apoB$_{4372\text{-}4392}$ peptide was included as a control. The amount of Lp(a) formed in each incubation was quantified using an Lp(a)-specific sandwich ELISA. Each incubation was measured in quadruplicate and the average value was expressed as a percentage of Lp(a) formed relative to the incubation containing no inhibitor. Error bars indicate the standard deviation. The IC_{50} values for each of the peptides are shown.

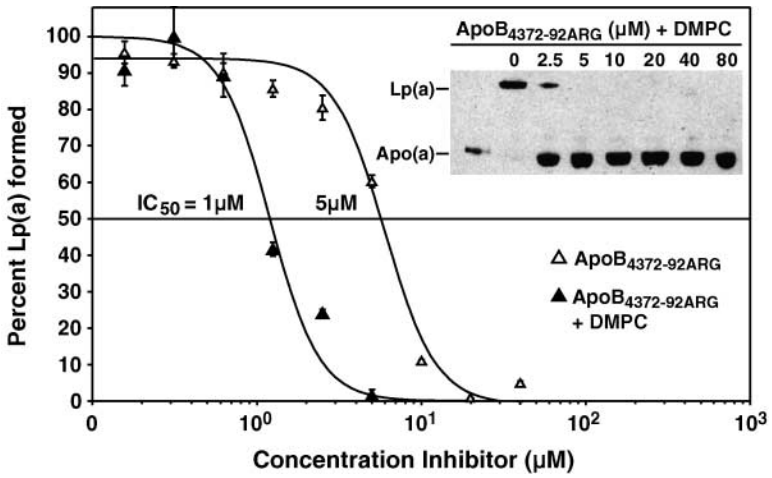

Fig. 6. Inhibitory capacity of apoB$_{4372\text{-}92ARG}$ in the presence and absence of DMPC. Increasing amounts of the apoB$_{4372\text{-}92ARG}$ peptide, in the presence or absence of saturating amounts of DMPC, were added to incubations containing human apo(a) transgenic mouse plasma and human apoB transgenic mouse plasma. The amount of Lp(a) formed in each incubation was quantified using an Lp(a)-specific sandwich ELISA. Each incubation was measured in quadruplicate and the average value was expressed as a percentage of Lp(a) formed relative to the incubation containing no inhibitor. The IC$_{50}$ values are shown. Error bars indicate the standard deviation. The inset shows a Western blot analysis of the Lp(a) formed in incubations containing DMPC-complexed apoB$_{4372\text{-}92ARG}$.

proved its inhibitory capacity by 5-fold, shifting the IC$_{50}$ value from 5 μM to 1 μM (**Fig. 6**). This is supported by Western blot analyses (Fig. 6, inset) showing that the DMPC-bound apoB$_{4372\text{-}92ARG}$ peptide caused complete inhibition of Lp(a) assembly at a concentration of 5 μM. Complexing of the less effective apoB $_{K4372A}$ and apoB$_{K4392A}$ peptides with DMPC showed a similar decrease in IC$_{50}$ to that seen with the apoB$_{4372\text{-}4392}$ and apoB$_{4372\text{-}92ARG}$ peptides (data not shown), suggesting that complexing to DMPC has a general stabilizing effect on peptide structure that results in a more effective inhibitor.

DISCUSSION

Although elevated levels of Lp(a) have been identified as an independent risk factor for developing vascular disease, there is currently no safe and effective Lp(a)-lowering therapy available. An understanding of the molecular interactions between apo(a) and apoB that initiate Lp(a) formation may lead to the design of novel Lp(a)-lowering agents. Previous research has established that apo(a) lysine-binding domains most likely interact with lysine residues on apoB to facilitate Lp(a) formation. A recent study indicates that the lysine-binding domains in apo(a) kringle IV$_7$ and IV$_8$ are the two most likely candidates to be involved in Lp(a) assembly (35). The apo(a) lysine-binding domains consist of an anionic center followed by a hydrophobic trough involving four hydrophobic residues. Lysine analogues interact with the pocket via the amino group of their side chain, making an ion-pair interaction with the anionic center; the aliphatic backbone interacts with the hydrophobic trough (36, 37).

We are interested in defining the apoB residues involved in the noncovalent interaction with apo(a). The current study aimed to identify the key structural features of a synthetic apoB peptide spanning amino acids 4372–4392, which was recently reported to bind apo(a) and effectively inhibit Lp(a) assembly in vitro (26). The apoB 4372–4392 sequence is predicted to lie within a class A amphipathic helix with lipid-binding capacity (38). Indeed, CD spectroscopy studies confirmed that the apoB$_{4372\text{-}4392}$ peptide is α-helical in nature and binds phospholipid in a saturable manner (26). The importance of the α-helical structure for the inhibitory action of apoB$_{4372\text{-}4392}$ was tested in this study by replacing the central leucine at position 4383 with proline to create the apoB$_{L4372P}$ peptide. CD spectroscopy of apoB$_{L4383P}$ showed a complete loss of α-helix

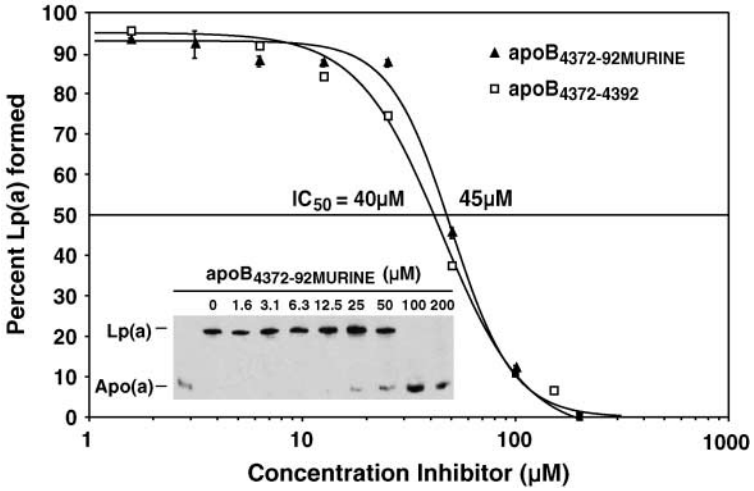

Fig. 7. Inhibitory capacity of apoB$_{4372\text{-}92MURINE}$ compared with apoB$_{4372\text{-}4392}$. Increasing amounts of the apoB$_{4372\text{-}92MURINE}$ peptide were added to incubations containing human apo(a) transgenic mouse plasma and human apoB transgenic mouse plasma. The amount of Lp(a) formed in each incubation was quantified using an Lp(a)-specific sandwich ELISA. Each incubation was measured in quadruplicate and the average value was expressed as a percentage of Lp(a) formed relative to the incubation containing no inhibitor. The IC$_{50}$ values are shown. Error bars indicate the standard deviation. The inset shows a Western blot analysis of the Lp(a) formed in incubations with the apoB$_{4372\text{-}92MURINE}$ peptide.

with the L4383P substitution, which converted the peptide into a random coil structure (Fig. 2B). As a result, apo(a) binding studies showed that the peptide had lost the capacity to bind apo(a) (Fig. 3). Not surprisingly, the $apoB_{L4383P}$ had also lost the ability to inhibit Lp(a) assembly showing an $IC_{50} > 400$ μM. These data support the notion that the helical nature of the peptide is vital for its inhibitory capacity.

We also tested the importance of individual lysine residues in the apoB 4372–4392 sequence on Lp(a) assembly inhibition. We speculated that a lysine residue in close proximity to hydrophobic residues at the lipid/water interface could be important (Fig. 1). The Lys4372 and Lys4392 residues are highly conserved in species capable of binding apo(a) (27) and both lie close to hydrophobic residues at the lipid/aqueous interface. Relative to the control, CD spectroscopy studies showed a substantial reduction in the propensity for $apoB_{K4372A}$ to adopt an α-helical structure both in the absence and in the presence of DMPC (Fig. 2). In addition, only minimal binding of the $apoB_{K4372A}$ peptide to apo(a) was detected (Fig. 3); this was accompanied by a significant loss in the inhibition of Lp(a) assembly (Fig. 4). The $apoB_{K4372A}$ peptide had an IC_{50} of 100 μM (Fig. 5), which is 2.5-fold higher than the original $apoB_{4372\text{-}4392}$ peptide. As $apoB_{K4372A}$ had lost some α-helical structure (Fig. 2C), it is possible that this may account for its loss of activity. Substitution of the Lys4392 residue had no effect on secondary structure, but resulted in a similar loss of binding and inhibitory capacity to the $apoB_{K4372A}$ peptide, with an IC_{50} of 100 μM. As the secondary structure of $apoB_{K4392A}$ was unaffected, it is most likely the loss of positive charge at this position accounted for its reduced activity. We therefore propose that Lys4392 is a likely candidate for binding to the anionic lysine binding pocket of apo(a). Because the $apoB_{K4392A}$ peptide still shows inhibitory activity, however, other residues besides Lys4392 must be important in binding apo(a).

The importance of a cluster of hydrophobic residues adjacent to the Lys4392 residue was tested by creating a peptide, $apoB_{4372\text{-}92HYDRO}$, in which four hydrophobic residues located on one face of the helix were replaced with alanines (Fig. 1; Table 1). Note that the $apoB_{4372\text{-}92HYDRO}$ peptide showed a complete loss of apo(a) binding and inhibitory capacity, with an IC_{50} value > 400 μM. Because the secondary structure of this peptide has remained α-helical (Fig. 2E), the loss in activity is likely due to the loss of hydrophobic residues, indicating that both the primary and secondary structure of the $apoB_{4372\text{-}4392}$ peptide are important for its inhibitory activity. We conclude that the substituted hydrophobic residues form part of the apo(a) binding motif in the $apoB_{4372\text{-}4392}$ peptide. One or more of these hydrophobic residues presumably help to anchor the peptide into the binding pocket along with the Lys4392 residue.

Finally, to test whether the interaction of the $apoB_{4372\text{-}4392}$ peptide with apo(a) was specific to lysine residues, we replaced all four lysine residues in the $apoB_{4372\text{-}4392}$ peptide with arginines to create $apoB_{4372\text{-}92ARG}$. CD spectroscopy studies of $apoB_{4372\text{-}92ARG}$ showed the peptide to be α-helical, with a significant increase in helical content in the presence of phospholipid (Fig. 2F). Apo(a) binding studies showed that $apoB_{4372\text{-}92ARG}$ specifically bound to apo(a) (Fig. 3). The ELISA analysis determined an IC_{50} value for $apoB_{4372\text{-}92ARG}$ of 5 μM (Fig. 5) which is 8-fold more effective than the original $apoB_{4372\text{-}4392}$ peptide at inhibiting Lp(a) assembly. Furthermore, addition of the $apoB_{4372\text{-}92ARG}$ peptide to DMPC increased the inhibitory capacity 5-fold ($IC_{50} = 1$ μM), resulting in a highly effective inhibitor. The results with the $apoB_{4372\text{-}92ARG}$ peptide suggest that the apo(a) lysine binding domains may have a stronger affinity for arginines than lysines. This is consistent with data showing that the addition of arginine can inhibit recombinant apo(a) derivatives from binding native LDL particles in vitro (39). We therefore propose that the guanidinium side chain of arginine may fit more tightly into the anionic binding pocket of apo(a). Note that the murine sequence contains an arginine at position 4392. A peptide spanning mouse apoB 4372–4392 was tested and found to inhibit Lp(a) formation, with an IC_{50} similar to the human $apoB_{4372\text{-}4392}$ peptide (**Fig. 7**). Future studies investigating the importance of individual lysine to arginine substitutions in the context of the human apoB 4372–4392 sequence may help pinpoint the key lysine residue important for the interaction with the apo(a) lysine-binding pocket.

In summary, this study has determined several structural features that are responsible for the inhibitory action of the $apoB_{4372\text{-}4392}$ peptide on Lp(a) assembly. First, we show that the α-helical nature of this sequence is critical for inhibition, as demonstrated with the L4383P peptide, which shows a loss of helical structure coinciding with a loss in activity. This is also supported by data showing that the addition of phospholipid, which stabilizes the helical structure of the $apoB_{4372\text{-}4392}$ peptide, enhances the inhibition of Lp(a) assembly (26). Second, we show using the $apoB_{K4392A}$ and $apoB_{4372\text{-}92HYDRO}$ peptides that a positive charge at position 4392, along with hydrophobic residues at the interface of the amphipathic helix, is important for the interaction with apo(a). Finally, we show that substitution of lysines for arginines in the sequence increases the inhibitory capacity of the peptide, suggesting a tighter fit of arginine in the anionic apo(a) binding cavity. The $apoB_{4372\text{-}4392}$ peptide has proved to be an excellent model for studying the nature of the noncovalent interactions between apoB and apo(a) in Lp(a) assembly and thus provides scope for the design of novel inhibitors of Lp(a) assembly.

The authors thank Dr. Stephen Young for the human apoB mice and Dr. Robert Hammer for the human apo(a) transgenic mice. This work was supported by grants to S.P.A.M. from the Marsden Fund (Contract UOO804) and the National Heart Foundation (Grant 852).

REFERENCES

1. Utermann, G. 1989. The mysteries of lipoprotein(a). *Science.* **246:** 904–910.

2. Berg, K., G. Dahlen, B. Christophersen, T. Cook, J. Kjekshus, and T. Pedersen. 1997. Lp(a) lipoprotein level predicts survival and major coronary events in the Scandinavian Simvastatin Survival Study. *Clin. Genet.* **52:** 254–261.
3. Seman, L. J., C. DeLuca, J. L. Jenner, L. A. Cupples, J. R. McNamara, P. W. Wilson, W. P. Castelli, J. M. Ordovas, and E. J. Schaefer. 1999. Lipoprotein(a)-cholesterol and coronary heart disease in the Framingham Heart Study. *Clin. Chem.* **45:** 1039–1046.
4. Luc, G., J. M. Bard, D. Arveiler, J. Ferrieres, A. Evans, P. Amouyel, J. C. Fruchart, and P. Ducimetiere. 2002. Lipoprotein (a) as a predictor of coronary heart disease: the PRIME Study. *Atherosclerosis.* **163:** 377–384.
5. Rath, M., A. Niendorf, T. Reblin, M. Dietel, H. J. Krebber, and U. Beisiegel. 1989. Detection and quantification of lipoprotein(a) in the arterial wall of 107 coronary bypass patients. *Arteriosclerosis.* **9:** 579–592.
6. Dahlen, G. H., J. R. Guyton, M. Attar, J. A. Farmer, J. A. Kautz, and A. M. Gotto, Jr. 1986. Association of levels of lipoprotein Lp(a), plasma lipids, and other lipoproteins with coronary artery disease documented by angiography. *Circulation.* **74:** 758–765.
7. Sun, H., H. Unoki, X. Wang, J. Liang, T. Ichikawa, Y. Arai, M. Shiomi, S. M. Marcovina, T. Watanabe, and J. Fan. 2002. Lipoprotein(a) enhances advanced atherosclerosis and vascular calcification in WHHL transgenic rabbits expressing human apolipoprotein(a). *J. Biol. Chem.* **277:** 47486–47492.
8. Dobs, A. S., M. Prasad, A. Goldberg, M. Guccione, and D. R. Hoover. 1995. Changes in serum lipoprotein(a) in hyperlipidemic subjects undergoing long-term treatment with lipid-lowering drugs. *Cardiovasc. Drugs Ther.* **9:** 677–684.
9. Cobbaert, C., J. W. Jukema, A. H. Zwinderman, A. J. Withagen, J. Lindemans, and A. V. Bruschke. 1997. Modulation of lipoprotein(a) atherogenicity by high density lipoprotein cholesterol levels in middle-aged men with symptomatic coronary artery disease and normal to moderately elevated serum cholesterol. Regression Growth Evaluation Statin Study (REGRESS) Study Group. *J. Am. Coll. Cardiol.* **30:** 1491–1499.
10. Crouse III, J. R. 1996. New developments in the use of niacin for treatment of hyperlipidemia: new considerations in the use of an old drug. *Coron. Artery Dis.* **7:** 321–326.
11. Shewmon, D. A., J. L. Stock, C. J. Rosen, K. M. Heiniluoma, M. M. Hogue, A. Morrison, E. M. Doyle, T. Ukena, V. Weale, and S. Baker. 1994. Tamoxifen and estrogen lower circulating lipoprotein(a) concentrations in healthy postmenopausal women. *Arterioscler. Thromb.* **14:** 1586–1593.
12. Dieplinger, H., and G. Utermann. 1999. The seventh myth of lipoprotein(a): where and how is it assembled? *Curr. Opin. Lipidol.* **10:** 275–283.
13. Brunner, C., H. G. Kraft, G. Utermann, and H. J. Muller. 1993. Cys4057 of apolipoprotein(a) is essential for lipoprotein(a) assembly. *Proc. Natl. Acad. Sci. USA.* **90:** 11643–11647.
14. Trieu, V. N., and W. J. McConathy. 1995. A two-step model for lipoprotein(a) formation. *J. Biol. Chem.* **270:** 15471–15474.
15. Koschinsky, M. L., G. P. Cote, B. Gabel, and Y. Y. van der Hoek. 1993. Identification of the cysteine residue in apolipoprotein(a) that mediates extracellular coupling with apolipoprotein B-100. *J. Biol. Chem.* **268:** 19819–19825.
16. McCormick, S. P., J. K. Ng, S. Taylor, L. M. Flynn, R. E. Hammer, and S. G. Young. 1995. Mutagenesis of the human apolipoprotein B gene in a yeast artificial chromosome reveals the site of attachment for apolipoprotein(a). *Proc. Natl. Acad. Sci. USA.* **92:** 10147–10151.
17. Callow, M. J., and E. M. Rubin. 1995. Site-specific mutagenesis demonstrates that cysteine 4326 of apolipoprotein B is required for covalent linkage with apolipoprotein (a) in vivo. *J. Biol. Chem.* **270:** 23914–23917.
18. Cheesman, E. J., R. J. Sharp, C. H. Zlot, C. Y. Liu, S. Taylor, S. M. Marcovina, S. G. Young, and S. P. McCormick. 2000. An analysis of the interaction between mouse apolipoprotein B100 and apolipoprotein(a). *J. Biol. Chem.* **275:** 28195–28200.
19. Chiesa, G., H. H. Hobbs, M. L. Koschinsky, R. M. Lawn, S. D. Maika, and R. E. Hammer. 1992. Reconstitution of lipoprotein(a) by infusion of human low density lipoprotein into transgenic mice expressing human apolipoprotein(a). *J. Biol. Chem.* **267:** 24369–24374.
20. Frank, S., S. Durovic, and G. M. Kostner. 1994. Structural requirements of apo-a for the lipoprotein-a assembly. *Biochem. J.* **304:** 27–30.
21. Frank, S., S. Durovic, K. Kostner, and G. M. Kostner. 1995. Inhibitors for the in vitro assembly of Lp(a). *Arterioscler. Thromb. Vasc. Biol.* **15:** 1774–1780.
22. Ernst, A., M. Helmhold, C. Brunner, A. Petho-Schramm, V. W. Armstrong, and H. J. Muller. 1995. Identification of two functionally distinct lysine-binding sites in kringle 37 and in kringles 32–36 of human apolipoprotein(a). *J. Biol. Chem.* **270:** 6227–6234.
23. Frank, S., S. Durovic, and G. M. Kostner. 1996. The assembly of lipoprotein Lp(a). *Eur. J. Clin. Invest.* **26:** 109–114.
24. Gabel, B. R., L. F. May, S. M. Marcovina, and M. L. Koschinsky. 1996. Lipoprotein(a) assembly. Quantitative assessment of the role of apo(a) kringle IV types 2–10 in particle formation. *Arterioscler. Thromb. Vasc. Biol.* **16:** 1559–1567.
25. Becker, L., R. S. McLeod, S. M. Marcovina, Z. Yao, and M. L. Koschinsky. 2001. Identification of a critical lysine residue in apolipoprotein B-100 that mediates noncovalent interaction with apolipoprotein(a). *J. Biol. Chem.* **276:** 36155–36162.
26. Sharp, R. J., M. A. Perugini, S. M. Marcovina, and S. P. McCormick. 2003. A synthetic peptide that inhibits lipoprotein(a) assembly. *Arterioscler. Thromb. Vasc. Biol.* **23:** 502–507.
27. Liu, C. Y., R. Broadhurst, S. M. Marcovina, and S. P. McCormick. 2004. Mutation of lysine residues in apolipoprotein B-100 causes defective lipoprotein[a] formation. *J. Lipid Res.* **45:** 63–70.
28. Nielsen, L. B., S. P. McCormick, V. Pierotti, C. Tam, M. D. Gunn, H. Shizuya, and S. G. Young. 1997. Human apolipoprotein B transgenic mice generated with 207- and 145-kilobase pair bacterial artificial chromosomes. Evidence that a distant 5′′-element confers appropriate transgene expression in the intestine. *J. Biol. Chem.* **272:** 29752–29758.
29. New, R. R. C. (1990) Preparation of Liposomes *In* Liposomes: A Practical Approach. Oxford University Press, Oxford. 33–104.
30. New, R. R. C. (1990) Introduction *In* Liposomes: A Practical Approach. Oxford University Press, Oxford. 1–32.
31. Pace, C. N., F. Vajdos, L. Fee, G. Grimsley, and T. Gray. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4:** 2411–2423.
32. Aggerbeck, L. P., J. Wetterau, K. H. Weisgraber, C. S. Wu, and F. T. Lindgren. 1988. Human apolipoprotein E3 in aqueous solution. II. Properties of the amino- and carboxyl-terminal domains. *J. Biol. Chem.* **263:** 6249–6258.
33. Marcovina, S. M., J. J. Albers, B. Gabel, M. L. Koschinsky, and V. P. Gaur. 1995. Effect of the number of apolipoprotein(a) kringle 4 domains on immunochemical measurements of lipoprotein(a). *Clin. Chem.* **41:** 246255.
34. Jones, M. K., G. M. Anantharamaiah, and J. P. Segrest. 1992. Computer programs to identify and classify amphipathic alpha helical domains. *J. Lipid Res.* **33:** 287–296.
35. Becker, L., P. M. Cook, T. G. Wright, and M. L. Koschinsky. 2004. Quantitative evaluation of the contribution of weak lysine-binding sites present within apolipoprotein(a) kringle IV types 6–8 to lipoprotein(a) assembly. *J. Biol. Chem.* **279:** 2679–2688.
36. Maderegger, B., W. Bermel, A. Hrzenjak, G. M. Kostner, and H. Sterk. 2002. Solution structure of human apolipoprotein(a) kringle IV type 6. *Biochemistry.* **41:** 660–668.
37. Ye, Q., M. N. Rahman, M. L. Koschinsky, and Z. Jia. 2001. High-resolution crystal structure of apolipoprotein(a) kringle IV type 7: insights into ligand binding. *Protein Sci.* **10:** 1124–1129.
38. Segrest, J. P., M. K. Jones, V. K. Mishra, V. Pierotti, S. H. Young, J. Boren, T. L. Innerarity, and N. Dashti. 1998. Apolipoprotein B-100: conservation of lipid-associating amphipathic secondary structural motifs in nine species of vertebrates. *J. Lipid Res.* **39:** 85–102.
39. Gabel, B. R., and M. L. Koschinsky. 1998. Sequences within apolipoprotein(a) kringle IV types 6–8 bind directly to low-density lipoprotein and mediate noncovalent association of apolipoprotein(a) with apolipoprotein B-100. *Biochemistry.* **37:** 7892–7898.