The apolipoprotein B^{3304–3317} peptide as an inhibitor of the lipoprotein (a):apolipoprotein B-containing lipoprotein interaction

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Lipoprotein (a) [Lp(a)] is a risk factor for coronary artery disease. It is characterized by apolipoprotein (a) [apo(a)] disulphide linked to apolipoprotein B (apoB), by Cys⁴⁰⁵⁷ of apo(a) and possibly Cys³⁷³⁴ of apoB. We call this the covalent apo(a):apoB-Lp interaction, to distinguish it from the non-covalent Lp(a):apoB-Lp interaction, mediated by the proline-binding kringle-4-like domain(s) of Lp(a). The Lp(a):apoB-Lp interaction was inhibited by an apoB peptide spanning residues 3304–3317. This peptide was found by a computerized search for sites on apoB similar to the plasminogen's kringle-4-binding site of α_2 -antiplasmin. It probably constitutes part of the Lp(a)-

binding site on apoB because: (1) it corresponds to the α_2 -antiplasmin minimum binding domain for plasminogen's kringle-4; (2) the competitive nature of inhibition [$K_i = (1.5 \pm 0.7) \times 10^{-4}$ M, $n = 5$] suggested that it and apoB-Lp bound to Lp(a) by the same mechanism at the same site; and (3) it specifically bound Lp(a) and not apoB-Lp, and the bound Lp(a) was dissociated by inhibitors of the Lp(a):apoB-Lp interaction, 6-aminoheptanoic acid and L-proline. Inhibition was independent of its proline residue, suggesting that proline in the context of a peptide is not a ligand for the kringle(s) which mediated the binding of Lp(a) to apoB-Lp.

INTRODUCTION

Lipoprotein (a) [Lp(a)] is a risk factor for coronary artery disease [1–3]. It is a low-density lipoprotein (LDL) particle with a distinguishing glycoprotein, apolipoprotein (a) [apo(a)], covalently linked to apolipoprotein B (apoB) by a disulphide bond [4]. Apo(a) is highly homologous to plasminogen and its gene possibly arose by duplication of the plasminogen gene [5]. It has many repeats of kringle-4-like domains, one kringle-5-like domain, and a domain analogous to the protease domain of plasminogen but lacking the activation site [5]. Lp(a) assembly is mediated by Cys⁴⁰⁵⁷ of apo(a)'s kringle-36 and possibly Cys³⁷³⁴ of apoB [6–8]. We call this the covalent apo(a):apoB-Lp interaction, to distinguish it from the non-covalent Lp(a):apoB-Lp interaction which is the topic of this paper. ApoB-Lp is defined as apoB-containing lipoprotein, exclusive of Lp(a).

The Lp(a):apoB-Lp interaction was initially demonstrated by affinity chromatography, affinity electrophoresis and ligand blotting [9]. When Lp(a) and LDL are mixed together, they form an Lp(a):LDL complex which has an intermediate mobility between Lp(a) and LDL on agarose gel electrophoresis [9]. Lp(a), immobilized on nitrocellulose membrane or coupled to Sepharose-6B, binds apoB-Lp [9]. The nitrocellulose membrane system was exploited to quantify the Lp(a):apoB-Lp interaction [10]. The interaction exhibits a binding constant of 2×10^{-8} M and a stoichiometry of two apoB-Lp/one Lp(a) [10].

The kringle-4-like domain(s) of Lp(a) is responsible for the binding of Lp(a) to apoB-Lp as a synthetic kringle peptide inhibits the binding of apoB-Lp to Lp(a) ($K_i = 1.9 \times 10^{-4}$ M) [11]. In comparison with plasminogen kringle-4, the aspartic acid residue critical for lysine binding is replaced by valine in most of Lp(a)'s kringle-4-like domains, conferring affinity to L-proline rather than L-lysine [11]. An analogue of lysine, 6-aminoheptanoic

acid, can adopt a conformation similar to that of L-proline and can also inhibit the binding of apoB-Lp to Lp(a) [11].

The Lp(a):apoB-Lp interaction has also been observed in other systems. Immunoprecipitation by anti-apo(a) brought down both Lp(a) and apoB-Lp non-covalently associated with Lp(a) [12]. The accumulation of Lp(a), in the triacylglycerol-rich apoB-Lp fraction of hypertriacylglycerolaemic patients, is mimicked by the addition of purified Lp(a) to the affected plasma due to the binding of Lp(a) to the triacylglycerol-rich apoB-Lp [10,13,14]. Lp(a), bound or immobilized on LDL-receptor-negative fibroblasts, mediates the binding of additional apoB-Lp; the effect is inhibited by L-proline [15]. Similarly, Lp(a), bound or immobilized on HepG2 cells, causes a greater than 2-fold increase in LDL binding [16].

In this paper, we report our studies of an apoB peptide which inhibits the Lp(a):apoB-Lp interaction. This peptide was found during a computerized search for an apoB equivalent to the α_2 -antiplasmin-binding domain for plasminogen's kringle-4. The possibility that this peptide constitutes the Lp(a)-binding site on apoB is suggested.

MATERIALS AND METHODS

Materials

BSA (essentially fatty acid- and globulin-free), peroxidase substrate, antibodies to sheep IgG, 6-aminoheptanoic acid, D-proline and L-proline were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.; Tween 20 was from J. T. Baker Chemical Company, Phillipsburg, NJ, U.S.A.; prestained molecular-mass standards were from Gibco-BRL, Gaithersburg, MD, U.S.A.; streptavidin-biotinylated peroxidase complex from Amersham Corporation, Arlington Heights, IL, U.S.A.; and nitrocellulose

Abbreviations used: Lp(a), lipoprotein (a); apo(a), apolipoprotein (a); apoB, apolipoprotein B; apoB-Lp, apoB-containing lipoprotein exclusive of Lp(a); LDL, low-density lipoprotein ($\rho = 1.006$ – 1.063 g/ml); LDL₂, subclass of low-density lipoprotein ($\rho = 1.030$ – 1.063 g/ml); HDL₂, subclass of high-density lipoprotein ($\rho = 1.063$ – 1.12 g/ml); VLDL, very-low-density lipoprotein ($\rho < 1.006$); NaSCN, sodium thiocyanate.

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The bars, two dots, and one dot indicate identical, similar, and weakly similar residues. The percentage similarity of each site is indicated on the right-hand column. For each pair, the T-11 sequence is listed on top and the apoB sequence is listed on bottom.

SITE 1

	B1		B2	
3284	RNLKLSLPDFKELCTISHIFIPAMGNITYDFSFKS			3318
			: : :	: :
427	GDKLFGPDLK.....LVPPMEEDYPQFGSPK			452

SITE 2

	B1		B2	
3700	ADKFIIPGLKLNLDNSVLVMPFTHVVPETDLOVPSCKL			3736
	: : : :		: : :	: : :
427	GDKLFGPDLK.....VPPMEEDYPQFGSPK			452

Figure 2 Putative Lp(a)-binding site(s) on apoB

The two sites exhibiting the greatest similarity to the T-11 peptide are shown. The bars, two dots, and one dots indicate identical, similar, and weakly similar residues. The underlined residues indicate the four peptides tested in the lower panel for inhibition of binding. The lower panel shows 150 ng of Lp(a) immobilized per dot and reacted with 1.2×10^{-8} M biotinylated LDL₂ in the presence of 8.5×10^{-4} M inhibitors [B1, B2 (apoB³³⁰⁴⁻³³¹⁷), B3, and B4]. C indicates the control with no inhibitor added.

RESULTS

Inhibition of Lp(a):apoB-Lp interaction by apoB³³⁰⁴⁻³³¹⁷ peptide

The T-11 tryptic peptide (G⁴²⁷D⁴²⁸K⁴²⁹L⁴³⁰F⁴³¹G⁴³²P⁴³³D⁴³⁴L⁴³⁵K⁴³⁶L⁴³⁷V⁴³⁸P⁴³⁹P⁴⁴⁰M⁴⁴¹E⁴⁴²E⁴⁴³D⁴⁴⁴Y⁴⁴⁵-P⁴⁴⁶Q⁴⁴⁷F⁴⁴⁸G⁴⁴⁹S⁴⁵⁰P⁴⁵¹K⁴⁵²) of α_2 -antiplasmin has an affinity for plasminogen's kringle-4 with a K_D of 0.7–29 μ M; the right half of which is the minimum binding site and has a K_D of 250 μ M [23]. The human apoB amino acid sequence was searched for sequences similar to the T-11 sequence. The results are shown in Figures 1 and 2. The two sites shown in Figure 2 were chosen for further analyses, since they have the highest similarity to the T-11 sequence. Site 1 (residues 3284–3318) and site 2 (residues 3700–3733) exhibit 76% and 65% sequence similarity to the T-11 sequence respectively (Figure 2). Alignment at site 1 and site 2 forced a gap which breaks the T-11 sequence into half, the right half corresponds to its minimum binding site.

Synthetic peptides spanning residues 3285–3296 (B1), 3304–3317 (B2), 3700–3711 (B3), and 3719–3733 (B4), were tested for inhibition of the Lp(a):apoB-Lp interaction. As shown in Figure 2, peptide B2 (apoB³³⁰⁴⁻³³¹⁷) completely inhibited the binding of biotinylated LDL₂ to immobilized Lp(a). The apoB³³⁰⁴⁻³³¹⁷ peptide also inhibited the binding of LDL₂ to recombinant apo(a) (data not shown). These data suggested that site 1 is the Lp(a)-binding site on apoB. This interpretation is consistent with the competitive nature of inhibition by the apoB³³⁰⁴⁻³³¹⁷ peptide. In the presence of increasing amounts of apoB³³⁰⁴⁻³³¹⁷ peptide, the apparent K_D decreased while maximum binding (B_{max}) was not affected (Figure 3). The K_I was determined to be $(1.5 \pm 0.7) \times 10^{-4}$ M of apoB³³⁰⁴⁻³³¹⁷ peptide ($n = 5$). K_I was determined graphically by plotting the apparent K_D values versus the inhibitor concentrations; the x-axis intercept is equal to $-K_I$ (Figure 3) [10,11].

Binding of Lp(a) to the apoB³³⁰⁴⁻³³¹⁷ peptide

When Lp(a)-positive plasma samples were passed over the

Figure 3 Competitive inhibition of apoB-Lp binding to Lp(a) by apoB³³⁰⁴⁻³³¹⁷ peptide

(a) The binding isotherm was determined in the presence of apoB³³⁰⁴⁻³³¹⁷ peptide at the concentration of 0 ($\square$, solid line), 0.89×10^{-4} M ($\triangle$, dashed line), 1.18×10^{-4} M ($\diamond$, dashed line), and 1.58×10^{-4} M ($\circ$, dotted line). The amount of Lp(a) used per dot was 150 ng. The curves were fitted to the data using the equation $\text{apoB-LDL}_2 \text{ bound} = (B_{max} \times [\text{LDL}_2]) / (K_D + [\text{LDL}_2])$ with the GraphPad computer program. (b) The apparent K_D values were plotted versus the concentration of apoB³³⁰⁴⁻³³¹⁷ peptide used. The x-axis intercept equals $-K_I$.

apoB³³⁰⁴⁻³³¹⁷-Sepharose column, Lp(a) was bound and was eluted with 0.1 M 6-aminohexanoic acid (Figure 4). This was the case with or without prior passage over L-lysine-Sepharose to remove proteins with affinity for amino acids [especially L-lysine which also has an affinity for Lp(a)]. The double immunodiffusion reactions to apo(a) and apoB of all 6-aminohexanoic acid fractions exhibited a line of identity, indicating that apo(a) and apoB reside on the same particle, i.e. Lp(a) (Figures 4b and 4c). And upon immunoblotting, Lp(a)-positive plasma exhibited two apoB reactivities corresponding to apoB and apoB linked to apo(a) (Figure 5, lane 1); while the 6-aminohexanoic acid eluate exhibited primarily one reactivity corresponding to apoB linked to apo(a) (Figure 5, lanes 3 and 4). This showed that only Lp(a), and not apoB-Lp, was bound by the column. Little or no Lp(a) remained after 6-aminohexanoic acid elution, as no Lp(a) was detected with the subsequent 3 M NaSCN wash (Figure 5, lane 2).

The binding of Lp(a) to the column was specific because of four facts. First, Lp(a) did not bind to a column constructed

Figure 4 Double immunodiffusion analyses of proteins bound to apoB³³⁰⁴⁻³³¹⁷-Sepharose

Lp(a)-positive plasma samples were passed over the column and the bound material eluted with 0.1 M 6-aminohexanoic acid. The eluate, centre wells, was reacted with the following antisera in clockwise order from the top: (a) apoA-I, apo(a), apoA-II, apoA-IV, apoC-III, apoC-II, apoC-I, and apoD; (b) apoE, apo(a), apoB, plasminogen, fibrinogen, fibronectin, cholesterol ester transfer protein, and apoH. (c) A mixture containing only LDL and Lp(a) was passed over the apoB³³⁰⁴⁻³³¹⁷-Sepharose column and eluted with 0.1 M 6-aminohexanoic acid. The unretained material (U) and the retained material (R) were reacted against anti-apo(a) and anti-apoB.

Figure 5 Immunoblot analysis of apoB³³⁰⁴⁻³³¹⁷-Sepharose column fractions

Protein (1 μ g) from the 6-aminohexanoic acid eluate (lanes 3 and 4), the corresponding 3 M NaSCN eluate (lane 2), and 1 μ l of the starting plasma (lane 1) were separated on a 2.5–10% SDS/PAGE. The separated proteins were immunoblotted with anti-apoB (lanes 1–3) and anti-apo(a) (lane 4). The arrows indicate the location of apoB and apoB linked to apo(a).

using an unrelated peptide (Asp-Pro-Gly-Val-Arg-Trp-Glu-Tyr-Cys-Asn-Leu-Thr-Glu) (results not shown). Secondly, L-proline, which is less effective than 6-aminohexanoic acid in inhibiting the

Table 1 Inhibition of binding by various peptides

B2 and its variants were used at 5×10^{-4} M. The dipeptides and tripeptide were used at 0.1 M. Other experimental details are as in Figure 6. The results are given as means $\pm$ S.D. ($n = 3$).

Inhibitors	Inhibition (%)
B2	99.7 ± 0.6
B2 (Lys $\rightarrow$ Gly)	73.3 ± 19.8
B2 (Pro $\rightarrow$ Gly)	91.5 ± 9.6
Gly-Tyr	13.8 ± 4.4
Gly-Pro-Ala	13.8 ± 4.4
Gly-Pro	53.7 ± 5.5

Figure 6 Inhibition of Lp(a):apoB-Lp interaction by L-proline and D-proline

Lp(a) (158 ng/dot) was immobilized and reacted with 2.34×10^{-8} M biotinylated LDL₂ in the presence of L-proline (●) or D-proline (○). The percentage inhibition was calculated as the ratio of LDL₂ bound in the presence of the inhibitor and LDL₂ bound in the absence of the inhibitor, multiplied by 100. The data were compiled from three separate experiments.

Lp(a):apoB-Lp interaction, was also less effective in eluting Lp(a) from the apoB³³⁰⁴⁻³³¹⁷ column (results not shown). Thirdly, no other apolipoproteins, including apoB-Lp, were bound to the column, as determined by double immunodiffusion analyses (Figures 4a and 4b) or immunoblotting (Figure 5). Fourthly, binding was independent of other plasma proteins as only Lp(a) was bound when a mixture of purified LDL and Lp(a) was passed over the column (Figure 4c).

The proline of apoB³³⁰⁴⁻³³¹⁷ is not essential

Since lysine is probably blocked during coupling of the peptide to Sepharose, the effective binding of Lp(a) to the immobilized peptide suggested that lysine was not essential for binding. Consequently, replacement of lysine by glycine did not significantly reduce its inhibitory activity in comparison with the control peptide ($P = 0.083$) (Table 1). Surprisingly, replacement of proline by glycine also did not reduce its inhibitory activity ($P = 0.216$; Table 1). This suggested that proline, in the context of a peptide, is not a ligand for the kringle(s) which mediates the binding of Lp(a) to apoB-Lp. Consequently, the tripeptide Gly-Pro-Ala was ineffective as an inhibitor; and the dipeptide Gly-Pro was inhibitory only at a concentration of 0.1 M ($P < 0.001$, in comparison with the control peptide, Gly-Tyr) (Table 1).

However, like lysine and plasminogen [24], inhibition by free proline was characterized by stereospecificity; L-proline was more effective than D-proline (Figure 6). Furthermore, the K_i of L-proline for Lp(a) (Figure 6) is equivalent to the K_i of L-lysine for plasminogen [25] (30 mM versus 25–27 mM). Thus free L-proline is probably a ligand for the kringle(s) which mediates the binding of Lp(a) to apoB-Lp.

DISCUSSION

We have shown that the binding of Lp(a) to apoB-Lp is inhibited by an apoB peptide spanning residues 3304–3317. It is likely that this peptide is part of the Lp(a)-binding site on apoB for three reasons. First, the peptide corresponds to the α_2 -antiplasmin minimum binding domain for plasminogen's kringle-4 which is highly homologous to Lp(a)'s kringle-4-like domains. Secondly, the competitive nature of inhibition suggested that the peptide and apoB-Lp bound to Lp(a) by the same mechanism at the same site(s). Thirdly, the peptide specifically bound Lp(a) and not apoB-Lp; and the bound Lp(a) was dissociated by inhibitors of the Lp(a):apoB-Lp interaction, i.e. 6-aminohexanoic acid and L-proline.

The Lp(a):apoB-Lp interaction is both hydrophobic and ionic; however, the hydrophobic forces predominated because the interaction is demonstrable at a high salt concentration (> 2 M NaCl), while no interaction is demonstrable at low salt concentration (< 0.08 M NaCl). Therefore, the binding site for Lp(a) was predicted to be an exposed hydrophobic domain on apoB [10]. Site 1 is probably hydrophobic and exposed on the basis of its theoretical hydrophobicity index and antigenicity index. In contrast, site 2 may not be exposed based on its low antigenicity index. Close to site 1 are three glycosylation sites (residues 3309, 3331, and 3384) [26]. Glycosylation should insure the surface exposure of site 1 and might contribute sterically to the binding reaction. In this regard, it has been shown by protease digestion, using 12 different proteases, that there is only one highly exposed region in LDL and very-low-density lipoproteins (VLDL) (residues 3180–3280) [27]. Site 1 is in close proximity to this exposed region and should be available for the binding between apoB-100 of both VLDL and LDL to Lp(a) as shown previously [28]. Therefore, site 1 is probably the Lp(a)-binding site on apoB. The fact that site 2, which corresponds to the apoB domain involved in the covalent apo(a):apoB-Lp interaction, was also detected by the computerized search suggested that this apo(a):apoB-Lp interaction could also be analogous to the plasminogen: α_2 -antiplasmin interaction.

Despite the fact that site 1 was found using the α_2 -antiplasmin minimum binding site for plasminogen kringle-4, we would like to point out that site 1 is similar to, but not the same as, the T-11 peptide of α_2 -antiplasmin, as reflected in the low degree of similarity between the two domains. Purified α_2 -antiplasmin was not able to inhibit the binding of LDL to immobilized Lp(a) even when added at a concentration of 0.5 mg/ml (7.6×10^{-6} M). Furthermore, apoB-Lp binding to Lp(a) is lipid dependent, delipidized apoB refolded according to the method of Kamanna et al. [29] did not bind to Lp(a) (V. N. Trieu, unpublished work); and α_2 -antiplasmin binding to plasminogen is not lipid dependent.

The apoB^{3304–3317} column has allowed us to remove Lp(a) from the plasma compartment rapidly and efficiently. The minimum peptide concentration of this column is 4.5×10^{-3} M which is 30 times the K_i of the peptide and 5 times the concentration of the peptide which completely inhibited apoB-Lp binding to Lp(a) at any apoB-Lp concentrations. Therefore, it is not unexpected that we could remove most of the Lp(a) from 2 ml of plasma despite

the presence of apoB-Lp in the plasma. This observation offers an opportunity to develop a new and rapid Lp(a) assay, i.e. Lp(a) from plasma could be immobilized on to a peptide-containing support and its cholesterol determined directly or after dissociation of Lp(a) from the matrix.

The formation of Lp(a):apoB-Lp complex could be of importance physiologically. It has been shown to result in the enhancement of apoB-Lp binding to LDL-receptor-negative fibroblasts and enhanced uptake of both Lp(a) and LDL in LDL-receptor-positive HepG2 cells [15,16]. The piggybacking effect of Lp(a) could explain why Lp(a) was catabolized more rapidly in transgenic mice overexpressing LDL receptor although it has little affinity for LDL receptor [30]. As there is also a distinct receptor on macrophages [31,32], the binding of Lp(a) to apoB-Lp should result in a similar enhancement of apoB-Lp uptake and therefore foam-cell formation which is the first step in atherosclerosis. Furthermore, as Lp(a) has an affinity for both the extracellular matrix and apoB, it could enhance the binding of apoB to the vascular wall. Normal cellular responses to the bound LDL could result in the formation of atherosclerotic lesions.

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