Expression of apolipoprotein(a) kringle IV type 9 in Escherichia coli: demonstration of a specific interaction between kringle IV type 9 and apolipoproteinB-100

Mona Rahman, Zongchao Jia, Brent R. Gabel, Santica M. Marcovina and Marlys L. Koschinsky

Department of Biochemistry, Queen’s University, Kingston, Ontario, K7L 3N6, Canada and Department of Medicine, Northwest Lipid Research Laboratories, University of Washington, Seattle, WA, USA

To whom correspondence should be addressed

A number of studies have provided evidence that lipoprotein(a) [Lp(a)] assembly is a two-step process in which initial non-covalent interactions between apolipoprotein(a) [apo(a)] and apolipoproteinB-100 (apoB-100) precede specific disulfide bond formation. We have designed a construct encoding apo(a) kringle IV type 9 (KIV9) in which the unpaired cysteine at position 67 in this kringle is replaced with a tyrosine. The single kringle was expressed in bacteria and purified to homogeneity from cell homogenates. The purified derivative (designated KIV9ΔCys) was assessed for its ability to bind to purified human LDL. This interaction was detected either by ELISA using immobilized LDL or by column chromatography in which LDL binding to KIV9ΔCys immobilized on Ni2+-Sepharose was determined. In both cases, the interaction of KIV9ΔCys and LDL was observed. Further, we demonstrated that the binding interaction was sensitive to the addition of amino acids including lysine, the lysine analogue e-aminocaproic acid, arginine, phenylalanine and proline, with arginine and lysine having the greatest inhibitory effect. Binding of KIV9ΔCys to an immobilized apoB peptide spanning residues 3732–3745 of apoB was also demonstrated by ELISA. As was the case for LDL, this binding interaction was sensitive to the addition of arginine and lysine. Computer modeling of KIV9 demonstrated an excellent fit with residues 3732–3738 (PSCKLDF) of the apoB peptide. The modeling predicts the presence of overlapping lysine and phenylalanine-binding pockets in KIV9 which explains the inhibitory effects of lysine, arginine and phenylalanine which were observed in the binding assays. In summary, this study represents the first demonstration that KIV9 can interact directly with LDL through non-covalent interactions which may contribute to the first step of Lp(a) formation.

Keywords: apolipoprotein(a)/apolipoproteinB-100/kringle IV/apolipoprotein(a)/bacterial expression

Introduction

Lipoprotein(a) [Lp(a)] has become a major focus of research interest since elevated plasma levels of this lipoprotein have been correlated with an increased risk of the development of a variety of atherosclerotic disorders [reviewed by Durrington (1995) and Koschinsky and Marcovina (1997)]. Marked inherited variability has been observed in Lp(a) levels, which vary over 1000-fold in the human population. Lp(a) is clearly distinguishable from low-density lipoprotein (LDL) by the presence of the glycoprotein moiety apolipoprotein(a) [apo(a)], which confers the unique structural and functional properties attributed to the Lp(a) particle. Human apo(a) consists of tandem repeats of a sequence that is highly similar to plasminogen kringle IV, followed by sequences which are homologous to the kringle V and protease domains of plasminogen (McLean et al., 1987). Apo(a) isoforms contain 10 classes of kringle IV sequences (based on amino acid sequence); the kringle IV type 2 domain (KIV2) is present in variable numbers of copies, which forms the basis of apo(a) isoform size heterogeneity (Lackner et al., 1993; van der Hoeck et al., 1993).

In the Lp(a) particle, apo(a) is covalently linked to apolipoproteinB-100 (apoB-100) moiety of LDL by a single disulfide bond that involves Cys4057 present in apo(a) kringle IV type 9 (KIV9) (Brunner et al., 1993; Koschinsky et al., 1993). Recently, studies utilizing site-directed mutagenesis have identified Cys4326 in apoB-100 as the residue that is involved in a disulfide linkage with apo(a) in the context of the Lp(a) particle (Callow and Rubin, 1995; McCormick et al., 1995). Interestingly, the latter observations are in conflict with earlier biochemical and biophysical studies which suggest a role for Cys3734 in mediating disulfide linkage with apo(a) (Guevara et al., 1993a, 1996).

It is generally accepted that the process of Lp(a) assembly occurs in two steps, in which an initial non-covalent interaction between apo(a) and apoB-100 precedes specific disulfide bond formation. It has been demonstrated that the first step of Lp(a) assembly in vitro can be inhibited by the addition of lysine, lysine analogues and proline (Frank et al., 1995; Gabel et al., 1996). More recently, we have shown that the initial non-covalent interaction of apo(a) and apoB-100 can also be inhibited by phenylalanine (Gabel and Koschinsky, 1998) and arginine (Koschinsky et al., 1997; Gabel and Koschinsky, 1998). We have hypothesized that these amino acids may interact directly with apo(a) kringle IV types 6–8 (Gabel and Koschinsky, 1998), which have been shown to be important for Lp(a) assembly and which are masked in the context of the Lp(a) particle (Gabel et al., 1996).

Our most recent data suggest that in addition to apo(a) kringle IV types 6–8, apo(a) kringle IV type 9 may also contribute non-covalent contacts with apoB-100 in the first step of Lp(a) assembly (Gabel and Koschinsky, 1998). In the present study, in order to examine the role of apo(a) kringle IV type 9 in mediating non-covalent interactions with apoB-100, we expressed a variant of the apo(a) kringle IV type 9 sequence lacking the free cysteine (i.e. KIV9ΔCys) in Escherichia coli. We demonstrate that purified KIV9ΔCys binds to both LDL and to a peptide of apoB spanning residues 3732–3745 of the molecule (apoB3732–3745). Interestingly, the interaction of KIV9ΔCys with either LDL or apoB3732–3745 can be inhibited by amino acids which have also been shown to inhibit the first step of Lp(a) assembly in vitro.
and centrifuged at 20 000 g as described above for 15 min. The pellet was resuspended in 20 ml of binding buffer containing 6 M guanidine–HCl, 0.5% Triton X-100, 5 mM DTT and incubated with rocking for 2–3 days at 4°C to allow solubilization. The solubilized protein was then refolded by diluting 5-fold with binding buffer and incubating at 4°C overnight in the presence of oxidized and reduced glutathione (1.25 mM of each). Remaining insoluble debris was removed by centrifugation at 39 000 g for 20 min at 4°C prior to passage of the solubilized protein over Ni\(^{2+}\)-Sepharose (see below).

Purification of KIV\(_9\)ΔCys

His-Bind Resin (Novagen) was washed, charged with 50 mM NiSO\(_4\) and equilibrated in binding buffer. The resin was added to the refolded crude protein and incubated overnight at 4°C with rocking to allow adsorption of the protein. Following centrifugation at 1000 g, the majority of the supernatant containing unbound protein was removed by aspiration and the resin was washed in a 2.5 ml column. The purification of KIV\(_9\)ΔCys over the His-Bind Resin column was performed in the presence of reduced and oxidized glutathione (1.25 mM final concentration of each). The column was washed with six column volumes of binding buffer followed by six column volumes of wash buffer (20 mM Tris–HCl pH 7.9, 0.5 M NaCl, 60 mM imidazole). Specifically bound protein was eluted from the column with 20 mM Tris–HCl (pH 7.9) containing 0.5 M NaCl and 1 M imidazole (elution buffer). Protein-containing fractions in this buffer were pooled and applied to a Bio-Gel P60 (Bio-Rad; <300 ml) column and the column was developed in elution buffer by gravity at a flow-rate of ~1.5 ml/h at 4°C. Fractions containing KIV\(_9\)ΔCys were pooled and dialyzed against 1 g of 20 mM Tris–HCl, pH 7.9) for 1–2 days (minimum of four changes of buffer). The protein was then incubated in the presence of oxidized and reduced glutathione (1.25 mM each) for 2 days to ensure complete protein refolding. The purified protein was aliquoted, flash-frozen in liquid nitrogen and stored at −70°C prior to use.

Circular dichroism (CD) analysis on the purified kringle was performed by the Lipid and Lipoprotein Research Group at the University of Alberta, Edmonton, Canada, using a Jasco J-720 spectropolarimeter (Jasco, Easton, MD) interfaced to an Epson Equity 380/25 computer.

Removal of the His-tag from KIV\(_9\)ΔCys

For some experiments, the His-tag was removed from KIV\(_9\)ΔCys as follows. Total protein extract from induced cells was bound to the Ni\(^{2+}\)–Sepharose column and washed with binding and wash buffers as described above. The column was then equilibrated with 4–5 volumes of Factor Xa buffer (50 mM Tris, pH 8.0, containing 100 mM NaCl and 1 mM CaCl\(_2\)) and the resin was resuspended in 1.67 ml of this buffer. The liganded protein was incubated with 1 µM purified Factor Xa (a generous gift from Dr Michael Nesheim, Department of Biochemistry, Queen’s University) at room temperature overnight with constant agitation. To prevent non-specific binding of the digested protein, the buffer was made up to 0.5 M NaCl and 5 mM imidazole and the resin was incubated for an additional 1 h. Digested protein (i.e. KIV\(_9\)ΔCys lacking the His-tag) was collected in the column flow-through. The column was then washed with 12 volumes of binding buffer followed by 7 volumes of wash buffer to elute digested KIV\(_9\)ΔCys completely. Protein-containing samples were pooled and concentrated using a Centriprep concentrator and subjected to size-exclusion chromatography and refolding as described.

### Materials and methods

**Construction of KIV\(_9\)-pET16b and KIV\(_9\)ΔCys-pET16b expression plasmids**

Sequences corresponding to the KIV\(_9\) domain of apo(a) were amplified from a plasmid encoding this sequence using primers 1A and 1B (see Table I). Both primers contain Ndel sites at their 5’ ends resulting in a PCR product flanked by Ndel sites. The 400 bp PCR fragment was made blunt-ended and cloned into the EcoRV site of pBluescript SK+ (Stratagene) for DNA sequence analysis. Upon digestion with NotI, the resulting 379 bp fragment was cloned into the pET16b plasmid (Novagen) which had also been digested with Ndel and treated with calf intestinal phosphatase (Boehringer Mannheim; 0.05 units/pmol ends). The resulting plasmid was designated ΔCys-pET16b.

A PCR-based mutagenesis strategy was employed to replace the unpaired cysteine at amino acid position 67 of the KIV\(_9\) sequence with a tyrosine. The KIV\(_9\)-pET16b plasmid was used as a template for two separate PCR amplifications using primer pairs 2A and 2B and 3A and 3B (see Table I). Primers 2A and 2B resulted in the amplification of a 352 bp fragment (Fragment 1), while primers 3A and 3B resulted in the amplification of a 169 bp fragment (Fragment 2). Initially, both Fragments 1 and 2 were made blunt-ended and cloned into the EcoRV site in pBluescript SK+ (Stratagene) for DNA sequence analysis. Fragment 1 in pBluescript was digested with Xhol and BstWI and Fragment 2 in pBluescript was digested with BstWI and Xhol. These fragments were then ligated into Xhol/ Xhol-digested pET16b in a three-part ligation. The resultant construct was designated ΔCys-pET16b. This construct was used to transform the *E.coli* strain BL21 (DE3) (Novagen) for protein expression.

**Expression of KIV\(_9\)ΔCys in *E.coli***

Two liters of LB medium containing 50 µg/ml ampicillin were inoculated with 20 ml of an overnight culture of the bacterial strain BL21 (DE3) transformed with the ΔCys-pET16b plasmid and incubated with shaking at 37°C until the cultures reached an OD\(_{600}\) of ~0.6. Protein expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. The cultures were grown for an additional 3 h after induction and subsequently stored at 4°C overnight. Cells were harvested by centrifugation at 5000 g for 10 min at 4°C. The resulting cell pellets were resuspended in a total of 40 ml of binding buffer (20 mM Tris–HCl, pH 7.9, 5 mM imidazole, 0.5 M NaCl) and lysed by sonication. The lysate was centrifuged at 20 000 g for 20 min at 4°C. The resulting insoluble fraction was subsequently resuspended in 20 ml of binding buffer, sonicated to release trapped proteins and centrifuged at 20 000 g as described above for 15 min. The pellet was resuspended in 20 ml of binding buffer containing 6 M guanidine–HCl, 0.5% Triton X-100, 5 mM DTT and incubated with rocking for 2–3 days at 4°C to allow solubilization. The solubilized protein was then refolded by diluting 5-fold with binding buffer and incubating at 4°C overnight in the presence of oxidized and reduced glutathione (1.25 mM of each). Remaining insoluble debris was removed by centrifugation at 39 000 g for 20 min at 4°C prior to passage of the solubilized protein over Ni\(^{2+}\)-Sepharose (see below).

### Table I. Sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
</tr>
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<tbody>
<tr>
<td>1A (33mer)</td>
<td>5'-GGG CTG ACA TAT GCC ACC ACC TGA GAA AAG CCC-3'</td>
</tr>
<tr>
<td>1B (30mer)</td>
<td>5'-GGG CTG ACA TAT GGT TGG GCC ATG GCG GCC-3'</td>
</tr>
<tr>
<td>2A (17mer)</td>
<td>5'-CAG GTA CGG ATC GGT TG-3'</td>
</tr>
<tr>
<td>2B (21mer)</td>
<td>5'-TCC CCT GTA GAA ATA ATT TGG-3'</td>
</tr>
<tr>
<td>3A (20mer)</td>
<td>5'-CGA TCC GTA CGT GAG GTG GG-3'</td>
</tr>
<tr>
<td>3B (20mer)</td>
<td>5'-GCC GGA TCC TCG AGC ATA TG-3'</td>
</tr>
</tbody>
</table>

*Restriction enzyme cleavage sites are underlined.*
Expression of apo(a) kringle IV type 9 in E.coli

Results

**Expression of apo(a) KIV\(_a\)Cys in E.coli**

In order to assess whether the KIV type 9 sequence of apo(a) contributes non-covalent interactions with LDL in the first step of Lp(a) particle assembly, we engineered a construct in the vector pET16b which encodes apo(a) kringle IV type 9 and which contains a mutation of the free cysteine at position 67 in the kringle to a tyrosine (KIV\(_a\)Cys-pET16b) (Figure 1A).
We expressed this single kringle in *E. coli* and purified KIV$_{\Delta\text{Cys}}$ protein from the bacterial pellet by denaturation and refolding, followed by chromatography over Ni$^{2+}$-Sepharose and gel filtration. The resulting purified protein was analyzed by SDS–PAGE using a 15% gel followed by silver staining. The protein (including the 22 amino acids at the N-terminus corresponding to the His-tag) had an apparent molecular mass of ~18 kDa and migrated as a single band under both non-reducing and reducing conditions (Figure 1B). The slight shift in mobility between the non-reduced and reduced samples (Figure 1B) has also been observed for single kringles expressed in mammalian cells (Sangrar *et al.*, 1993) and probably corresponds to the unfolding of the kringle under reducing conditions which causes a decrease in mobility in SDS–PAGE.

In order to determine that purified KIV$_{\Delta\text{Cys}}$ was completely refolded and that its native structure was preserved, circular dichroism measurements were carried out in order to assess secondary structural content of the kringle. Similarly to what has been reported previously for other kringle structures such as plasminogen kringle 4 (Castellino *et al.*, 1989) and tissue-type plasminogen activator (tPA) kringle 2 (Cleary *et al.*, 1989), we found that KIV$_{\Delta\text{Cys}}$ contains a high $\alpha$-helical content (data not shown), thereby suggesting that KIV$_{\Delta\text{Cys}}$ is properly refolded. This conclusion is also supported by the lack of higher molecular mass species observed on non-reducing SDS–PAGE which would correspond to multimers of the kringle linked by inappropriate intermolecular disulfide bonds (Figure 1B).

**Analysis of the binding of LDL to a KIV$_{\Delta\text{Cys}}$-affinity column**

In order to determine whether the KIV$_{\Delta\text{Cys}}$ protein could bind to LDL, we utilized the N-terminal His-tag on the protein to create a KIV$_{\Delta\text{Cys}}$-affinity column by immobilizing the purified single kringle on Ni$^{2+}$-Sepharose. Purified human LDL was subsequently incubated with the KIV$_{\Delta\text{Cys}}$-Sepharose resin in order to assess binding. All buffers applied to the column contained an optimal imidazole concentration of 20 mM, which was found to be necessary to prevent non-specific association of LDL with the liganded resin without preventing specific binding of KIV$_{\Delta\text{Cys}}$ to the resin (data not shown). Unbound LDL was removed from the columns by washing with 20 mM Tris–HCl (pH 7.9) containing 20 mM imidazole and 0.15 M NaCl. At this low salt concentration, the His-Bind Resin may act as an ion-exchange resin. To avoid this and also to rule out a salt-specific component of the LDL–KIV$_{\Delta\text{Cys}}$ interaction, a salt wash (containing 0.5 M NaCl) was included. As shown in Figure 2A, no LDL was eluted from the column by the 0.5 M NaCl wash. In order to confirm that KIV$_{\Delta\text{Cys}}$ was not removed from the column during the washing steps, column fractions from these steps were analyzed by SDS–PAGE and silver-staining; no KIV$_{\Delta\text{Cys}}$ was detected in these fractions (data not shown). Bound LDL was eluted...
by stripping the column with a buffer containing EDTA to remove the KIV\(_9\)ΔCys (Figure 2A).

Once we had established that LDL could bind specifically to KIV\(_9\)ΔCys in this system, binding analyses were performed as described above, but in the presence of various amino acids in order to identify interactive residues in apo(a) kringle IV type 9 and LDL which may mediate their interaction. For these studies, parallel experiments were performed using separate KIV\(_9\)ΔCys columns. LDL was applied to the columns and after the columns had been washed with loading buffer and salt wash buffer, the appropriate amino acid was applied to the respective columns in order to test its ability to compete with LDL for binding to KIV\(_9\)ΔCys. Western blot analyses demonstrated that LDL could be eluted from the columns by the lysine analogue ε-ACA, phenylalanine and proline (Figure 2B–D) but not by glycine (Figure 2A). Lysine and arginine were also assessed for their ability to remove LDL from the KIV\(_9\)ΔCys columns. However, these amino acids were observed to remove KIV\(_9\)ΔCys from the column, resulting in co-elution of LDL (data not shown). As such, no conclusions could be made regarding the ability of these amino acids to elute LDL from the columns. However, the results obtained with ε-ACA suggest that the non-covalent interaction of apo(a) kringle IV type 9 and LDL may involve a lysine-specific component.

ELISA analysis of the binding of KIV\(_9\)ΔCys either to immobilized LDL or to immobilized apoB\(_{3732-3745}\) peptide

In order to confirm the results obtained using the KIV\(_9\) Cys-Sepharose column, we also utilized an ELISA system in order to assess the ability of KIV\(_9\)ΔCys to bind to immobilized LDL. In these studies, increasing amounts of KIV\(_9\)ΔCys were added to LDL-coated microtitration plates. Bound KIV\(_9\)ΔCys was detected using either the monoclonal antibody mAb 40 [specific for apo(a) kringle IV type 9] or an anti-apo(a) polyclonal antibody raised in rabbits; binding was detected using both antibodies (Figure 3A). Assays were also performed in which increasing concentrations of LDL were added to microtitration plates which had been coated with KIV\(_9\)ΔCys or with KIV\(_9\)ΔCys-His (in which the His-tag had been removed by digestion with Factor Xa) (see Figure 1A). LDL bound in an identical fashion to either KIV\(_9\)ΔCys or KIV\(_9\)ΔCys-His, suggesting that the His-tag is not involved in the interaction between LDL and KIV\(_9\)ΔCys (Figure 3B).

We also compared the ability of a variety of amino acids to inhibit the binding of KIV\(_9\)ΔCys to immobilized LDL in the ELISA system. Similarly to what was observed in the column analysis (Figure 2), inhibition of the LDL–KIV\(_9\)ΔCys interaction was observed with phenylalanine and proline in the ELISA system (~23 and 30% apparent inhibition at 5 µM KIV\(_9\)ΔCys, respectively; Figure 3C), while the addition of glycine resulted in <5% inhibition (Figure 3C). However, in the ELISA, in contrast to the affinity chromatographic results described above, the addition of ε-ACA had a relatively small effect (~18% apparent inhibition) on the KIV\(_9\)ΔCys–LDL interaction. Significant inhibition of binding was observed using lysine and arginine (~67 and 76% apparent inhibition at 5 µM KIV\(_9\)ΔCys, respectively; Figure 3C).

ELISA was also performed to determine whether KIV\(_9\)ΔCys could bind to the immobilized synthetic apoB peptide apoB\(_{3732-3745}\) (Figure 4A). Bound KIV\(_9\)ΔCys was detected using either the apo(a) monoclonal antibody mAb/40 or an anti-apo(a) polyclonal antibody raised in rabbits. Although binding of KIV\(_9\)ΔCys to the immobilized peptide was observed using both antibodies, the sensitivity was much greater using the polyclonal antibody. As such, this antibody was utilized for competition studies (see below).
Fig. 4. Binding of KIV\textsubscript{9}ΔCys to the immobilized apoB peptide apoB\textsubscript{3732–3745}. (A) Binding of KIV\textsubscript{9}ΔCys to apoB\textsubscript{3732–3745} immobilized in the wells of microtitration plates was quantified by ELISA using either the apo(a) monoclonal antibody mAb 40 (triangles) or an anti-apo(a) polyclonal antibody raised in rabbits (diamonds). Bound primary antibody was detected by the addition of a horseradish peroxidase-conjugated secondary antibody; following addition of the substrate O-phenylenediamine dihydrochloride, the absorbance at 490 nm of each well was determined using a Titertek plate reader. (B) ELISA analysis was performed as described for (A), except that in addition to control samples lacking amino acids, KIV\textsubscript{9}ΔCys was incubated with immobilized apoB\textsubscript{3732–3745} in the presence of 0.1 M glycine, arginine, ε-ACA, lysine or proline or 0.05 M phenylalanine. Binding was detected using the rabbit polyclonal antibody.

We tested the effect of six amino acids on the binding of KIV\textsubscript{9}ΔCys to the immobilized apoB\textsubscript{3732–3745} peptide. Little effect on this binding interaction was observed upon the addition of glycine, ε-ACA, proline or phenylalanine, although the last three amino acids were found to compete effectively with LDL for binding to KIV\textsubscript{9}ΔCys-Sepharose (Figure 2) and also decreased the binding of KIV\textsubscript{9}ΔCys to immobilized LDL (Figure 3C). However, as was the case for the binding of KIV\textsubscript{9}ΔCys to immobilized LDL, a significant inhibitory effect was observed with lysine and arginine (~36 and 56% apparent inhibition at 5 μM KIV\textsubscript{9}ΔCys, respectively). Again, arginine had the greatest inhibitory effect (Figure 4B).

Molecular modeling of the structure of KIV\textsubscript{9}ΔCys

Given the high degree of sequence conservation between human plasminogen kringle 4 and apo(a) kringle IV type 9 (McLean et al., 1987), the 3-D structure of these two kringles was expected to be very similar. Indeed, the 3-D homology modeling was accomplished smoothly and the resulting energy-minimized model is very close to that of the starting template (Mulichak et al., 1991). All three disulfide bridges are conserved. Very few secondary structural features are observed in the KIV\textsubscript{9} model (Figure 5A), akin to what was observed for the X-ray crystallographic structure of plasminogen kringle 4 (Mulichak et al., 1991).

The Cys67 residue, which has been implicated in the formation of a disulfide bridge with apoB-100 (Brunner et al., 1993; Koschinisky et al., 1993), is located on the protein surface. It is estimated from the model that 95% of Cys67 is solvent exposed and readily accessible for disulfide bridge formation. The initial docking position of the peptide PSCKLDF (encompassing Cys3734 in apoB-100) was largely dictated by Cys67. Similarly to the findings of Guevara et al. (1993a), attempts to dock other peptide segments containing...
potentially free cysteine residues in apoB-100 such as Cys4326 were much less successful.

As shown in Figure 5A, the peptide seems to fit well in a surface channel on KIV9. According to the model, all residues of the peptide are involved in interactions with various residues of KIV9, which include His31, Arg35, Trp60, Tyr62, Asp65, Pro66, Cys67 (involved in disulfide bond formation), Val68, Arg69 and Trp70. As shown in Figure 5B, Phe3738 of the peptide is sandwiched between Trp60 and Trp70 of KIV9, allowing extensive interactions between the respective aromatic rings.

The lysine- or lysine analogue-binding pocket as determined in plasminogen kringle 4 by X-ray crystallography (Wu et al., 1991) partially overlaps with the Phe3738-binding pocket in KIV9 (Figure 5B). Our modeling also demonstrates that arginine (shown in yellow in Figure 5B) can bind to the lysine pocket in a similar manner to lysine, making critical interactions with the KIV9 protein.

Discussion

It is generally recognized that Lp(a) assembly occurs extracellularly (Chiesa et al., 1992; Brunner et al., 1993; Koschinsky et al., 1993; Ernst et al., 1994; Frank et al., 1994; Gabel et al., 1996), perhaps on the hepatocyte cell surface (White and Lanford, 1994). Additionally, it has been well established that the process of Lp(a) formation occurs in two steps, in which an initial non-covalent interaction between apo(a) and apoB-100 precedes specific disulfide bond formation. We have recently demonstrated that a core of sequences within apo(a) kringle IV type 6–8 and to a lesser extent apo(a) kringle IV type 9 mediates initial non-covalent interactions between apo(a) and apoB-100 (Gabel and Koschinsky, 1998). Additionally, a number of investigators have shown that lysine, lysine analogues and proline (Frank et al., 1995; Gabel et al., 1996), and also phenylalanine and arginine (Koschinsky et al., 1997; Gabel and Koschinsky, 1998), can inhibit the first step of Lp(a) assembly.

In the present study, we have demonstrated for the first time that purified apo(a) kringle IV type 9 [lacking the unpaired cysteine residue at position 67 which is involved in disulfide bond formation with apoB-100 (Brunner et al., 1993; Koschinsky et al., 1993)] can interact directly with the apoB-100 component of LDL. This was demonstrated both by ELISA, using immobilized human LDL, and by column chromatography, in which purified human LDL was shown to bind to KIV9ΔCys which had been immobilized on Ni2+-agarose columns. The ability of KIV9ΔCys to interact non-covalently with LDL was suggested by a previous study in which we demonstrated that an apo(a) derivative containing kringle IV types 5–8 bound less tightly (Kd ~ 7900 nM) to immobilized LDL than the corresponding derivative also containing kringle IV type 9 (Kd ~ 2000 nM) (Gabel and Koschinsky, 1998). However, this study did not directly examine the nature of the interaction of apo(a) KIV9 with LDL.

We have recently provided direct evidence that lysine, the lysine analogue ε-ACA, proline, arginine and phenylalanine disrupt the non-covalent step of Lp(a) assembly, probably owing to their ability to interact with kringles which are required for mediating initial interactions with apoB-100 (Gabel and Koschinsky, 1998). In the present study, we have demonstrated using ELISA that a variety of amino acids including arginine, lysine, the lysine analogue ε-ACA, proline, phenylalanine and arginine inhibit the interaction of apo(a) kringle IV type 9 with immobilized LDL. Additionally, we have demonstrated that the interaction of LDL with KIV9ΔCys columns can be disrupted by the addition of phenylalanine, proline and ε-ACA. In either system, glycine appeared to have no significant effect on the apo(a) KIV9ΔCys–LDL interaction. These results are comparable to those previously observed for the interaction of either apo(a) kringle IV types 6–8 or a 17-kringle form of apo(a) with LDL. Using these apo(a) derivatives, arginine, ε-ACA, lysine, phenylalanine and proline had a significant effect on the interaction of apo(a) with immobilized LDL, while no effect was observed upon the addition of glycine (Gabel and Koschinsky, 1998). Based on the results of the present study, it appears that these amino acids can interact directly with apo(a) kringle IV type 9 in addition to sequences within apo(a) kringle IV types 6–8 as has been suggested previously (Gabel and Koschinsky, 1998).

Relative to apo(a), little is known concerning the identity of sequences in apoB-100 which mediate its non-covalent interaction with apo(a). Based on recent studies using site-directed mutagenesis, Cys4326 in apoB has been implicated in disulfide bond formation with apo(a) (Callow and Rubin, 1995; McCormick et al., 1995). However, these studies contradict earlier reports using either immunological methods (Guevara et al., 1996) or biochemical and biophysical methods (Guevara et al., 1993a), which strongly suggested a role for Cys3734 in apoB in this process. In the latter study, segments of apoB-100 containing potentially uncomplexed cysteine residues were subjected to docking and energy minimization analysis using the apo(a) kringle IV type 9 sequence. This resulted in the identification of the apoB-100 peptide sequence PSCKLDFREIQYK (apoB3732–3745) as the best fit, providing the largest number of van der Waals contacts with modeled KIV9. This model, coupled with the observation that Cys3734 could be fluorescently labeled in LDL but not in the context of the Lp(a) particle (Guevara et al., 1993a), led to the hypothesis that Cys3734 in apoB-100 was involved in a disulfide linkage with apo(a). Additional modeling studies (Guevara et al., 1993b) indicated that of all the apo(a) kringles, kringle IV type 9 would be expected to interact most favorably with the apoB3732–3745 peptide.

In the present study, we have demonstrated that apo(a) KIV9ΔCys binds directly to the apoB3732–3745 peptide immobilized in microtiter wells (Figure 4). This is in keeping with previous studies in which we demonstrated that a recombinant apo(a) derivative encoding apo(a) kringle IV types 9 and 10, followed by the kringle V and protease-like domains (i.e. KIV9,p) bound to this peptide, while the same derivative lacking apo(a) kringle IV type 9 (i.e. KIV10,p) bound poorly to the peptide (Koschinsky et al., 1997). This was interpreted to suggest a role for apo(a) kringle IV type 9 in mediating the interaction with the apoB3732–3745 peptide.

Since we found that certain amino acids, including arginine, lysine, ε-ACA, phenylalanine and proline, inhibit the non-covalent association of KIV9ΔCys and LDL (see above), it was of interest to investigate the ability of these compounds to inhibit the binding of KIV9ΔCys to the apoB3732–3745 peptide. We found that whereas arginine and lysine had a pronounced inhibitory effect, glycine, proline, phenylalanine and ε-ACA had very little effect on this interaction. Similar results were obtained previously using KIV9,p in which lysine, arginine and proline were tested for their ability to inhibit the interaction of this derivative with immobilized apoB3732–3745 (Koschinsky et al., 1997). In the latter study, arginine and lysine were also
found to have a significant inhibitory effect, as was observed for the binding of KIV\(_{\Delta}\)Cys to the immobilized peptide in the present study.

In order to characterize further the KIV\(_{\Delta}\)Cys interaction with the apoB peptide, we have modeled this binding interaction. The sequence PSCKLDF (corresponding to apoB\(_{3732-3738}\)) fits well into a surface channel where all of the residues of this peptide can interact with a number of residues in the kringle IV type 9 sequence. Our model indicates the presence of a phenylalanine-binding pocket; in this model, the phenylalanine at position 3738 of the peptide is sandwiched between Trp60 and Trp70, allowing extensive contacts between the respective aromatic rings (see Figure 5). This is consistent with previous computer-generated models which have suggested that the ligand-binding site of KIV\(_{\Delta}\) may be capable of binding large hydrophobic residues such as phenylalanine (Guevara et al., 1993b). Interestingly, the lysine-binding pocket in this kringle partially overlaps with the phenylalanine-binding pocket. This would almost certainly result in mutually exclusive binding of phenylalanine and lysine, which explains the inhibitory function of lysine observed using intact LDL (Figure 3). Interestingly, only a small inhibitory effect on KIV\(_{\Delta}\)Cys binding was observed using the lysine analogue ε-ACA in conjunction with intact LDL, while no effect of this compound was observed using the apoB peptide. Our modeling also demonstrates that arginine can bind to the lysine-binding pocket in a similar manner to lysine. As is the case for lysine, the inhibitory effect of arginine on binding of KIV\(_{\Delta}\)Cys to LDL or the apoB\(_{3732-3734}\) peptide can be explained by the overlapping phenylalanine- and arginine-binding sites present within the KIV\(_9\) structure. This apparent discrepancy may reflect a role for the α-amino group in lysine (which is absent in ε-ACA) in binding to KIV\(_{\Delta}\)Cys, and also differences in the conformation of the apoB sequence in the context of the peptide relative to intact LDL.

Owing to the extensive possible interactions, the phenylalanine-binding pocket appears to be an attractive binding site for aromatic residues. On this basis, it is conceivable that single amino acids such as phenylalanine could also bind to this site with reasonable affinity, thereby impairing to some degree the binding of KIV\(_{\Delta}\)Cys to immobilized LDL. Indeed, our results demonstrate that phenylalanine competes with LDL for binding to KIV\(_{\Delta}\)Cys immobilized on Ni\(^{2+}\)-Sepharose and prevents the binding of KIV\(_{\Delta}\)Cys to immobilized LDL. However, it is less obvious how proline inhibits the KIV\(_{\Delta}\)Cys interaction with LDL. Two possibilities can be put forward to explain these observations: either proline binds to the site that proline in the peptide currently occupies or proline can interact in the phenylalanine-binding pocket. Binding to either of these two sites or both sites may underlie the inhibitory effect of this amino acid in the binding interactions. Again, it is interesting to note that whereas both of these amino acids competed for binding of KIV\(_{\Delta}\)Cys to intact LDL (Figures 2 and 3), little effect of either phenylalanine or proline on the binding of KIV\(_{\Delta}\)Cys to the apoB\(_{3732-3734}\) peptide was observed. Again, this may represent differences in the presentation of residues in the immobilized apoB peptide relative to analogous residues in intact LDL.

In summary, we have expressed apo(a) kringle IV type 9 lacking the free cysteine at position 67 in a bacterial system and have purified the corresponding recombinant protein to homogeneity. We have demonstrated for the first time that this kringle can bind non-covalently to the apoB-100 component of LDL and to a peptide spanning apoB residues 3732–3745. KIV\(_{\Delta}\)Cys binding to both LDL and the apoB peptide are sensitive to the addition of similar amino acids to those which have been shown previously to inhibit the first non-covalent step of Lp(a) assembly. Computer modeling of the KIV\(_9\) sequence with the apoB peptide PSCKLDF (spanning residues 3732–3738) provides a rationale for these observations and calls into question the prevailing view that Cys4326 in apoB is involved in covalent linkage with apo(a) in the Lp(a) particle.

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