INTRODUCTION

The low-density-lipoprotein-receptor (LDLR)-related protein (LRP) is composed of several classes of domains, including complement-type repeats (CR), which occur in clusters that contain binding sites for a multitude of different ligands. Each ∼40-residue CR domain contains three conserved disulphide linkages and an octahedral Ca$^{2+}$ cage. LRP is a scavenging receptor for ligands from extracellular fluids, e.g. $\alpha_{2}$-macroglobulin ($\alpha_{2}$M)–proteinase complexes, lipoprotein-containing particles and serine proteinase-inhibitor complexes, like the complex between urokinase-type plasminogen activator (uPA) and the plasminogen activator inhibitor-1 (PAI-1). In the present study we analysed the interaction of the uPA–PAI-1 complex as well as for the binding of the receptor-associated protein (RAP) – the folding chaperone/escort protein required for transport of LRP to the cell surface. Accordingly, the present work provides (1) an identification of a preferred binding site within LRP CR cluster II; (2) evidence that the uPA–PAI-1 binding site involves residues from two adjacent protein domains; and (3) direct evidence identifying specific residues as important for the binding of uPA–PAI-1 as well as for the binding of RAP.

Key words: complement-type repeat, protein–protein interactions, receptor-associated protein.

The multidomain organization of LRP features four clusters of complement-type (CR) repeats, several epidermal-growth-factor (EGF) repeats, six modules each of ∼50 amino acid residues with an YWTD motif that have been proposed to fold into a compact β-propeller [23], a transmembrane segment, and a cytoplasmic tail containing motifs important for the binding to adaptor proteins involved in clathrin-coated-pit-mediated endocytosis [24] and intracellular signalling proteins [25] (see Figure 1). The CR clusters have attracted attention, since ligand binding to LRP is mediated by these clusters. Some structural information for individual ∼40-residue CR domains is available, as the tertiary structures of CR3 and CR8 from LRP [26,27] and of the related domains of LB1, LB2, LB5 and LB6 from LDLR [28–31] have been elucidated. Each CR domain contains three conserved disulphide linkages and an octahedral Ca$^{2+}$ cage in which the Ca$^{2+}$ ion is co-ordinated mostly by conserved negatively charged residues.

uPA is a serine proteinase which can catalyse the activation of plasminogen to plasmin. Inhibition of the proteolytic activity of uPA by PAI-1 results in the formation of a covalently linked domains, but show a preferential interaction between the uPA–PAI-1 complex and a two-domain fragment comprising CR domains 5 and 6 of LRP. We demonstrate that surface-exposed aspartic acid and tryptophan residues at identical positions in the two homologous domains, CR5 and CR6 (Asp$^{583,584}$, Asp$^{585,586}$, Trp$^{585,586}$ and Trp$^{598,599}$), are critical for the binding of the complex as well as for the binding of the receptor-associated protein (RAP) – the folding chaperone/escort protein required for transport of LRP to the cell surface. Accordingly, the present work provides (1) an identification of a preferred binding site within LRP CR cluster II; (2) evidence that the uPA–PAI-1 binding site involves residues from two adjacent protein domains; and (3) direct evidence identifying specific residues as important for the binding of uPA–PAI-1 as well as for the binding of RAP.

Key words: complement-type repeat, protein–protein interactions, receptor-associated protein.

Abbreviations used: LDLR, low-density-lipoprotein receptor; LRP, $\alpha_{2}$-macroglobulin ($\alpha_{2}$M) receptor/LDLR-related protein; RAP, receptor-associated protein; RAPd3, RAP residues 216–323; uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; uPAR, uPA receptor; CR, complement-type repeat from LRP; VLDLR, very-low-density-lipoprotein receptor; apoER2, apolipoprotein E receptor 2; SFP, surface plasmon resonance; $\alpha_{2}$M, transformed $\alpha_{2}$M; EGF, epidermal growth factor; LB, complement-type repeat from LDLR; wt, wild-type; W004S, a mutant in which Trp$^{598}$ has been mutated to Ser (and similarly for other mutants); serpin, serine-proteinase-inhibitor.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed (e-mail hct@biobase.dk).
complex of 1:1 stoichiometry. PAI-1 is a member of the serine-proteinase-inhibitor (serpin) superfamily (reviewed in [32,33]). Besides LRP [20–22], the uPA–PAI-1 complex also binds with high affinity to VLDLR [34,35] and megalin [36]. In vivo, PAI-1 will most likely react with uPA bound to the glycosyl-phosphatidylinositol membrane-anchored uPA receptor (uPAR), followed by binding to LRP of the ternary complex between uPA, PAI-1 and uPAR [21,22]. uPAR does not contain any known binding sites for LRP, but is co-internalized with the LRP-bound uPA–PAI-1 complex. Following internalization, the LRP-bound uPA–PAI-1 complex undergoes lysosomal degradation, whereas the uPAR and LRP receptors are recycled back to the cell surface [37,38].

The affinity of uPA–PAI-1 for LRP (K₀ ≈ 0.4 nM) is ≈ 100-fold higher than the affinities of either of the free components, uPA (K₀ ≈ 50 nM) and PAI-1 (K₀ ≈ 50 nM) [39]. A possible explanation for this higher affinity may be the presence of multiple LRP contact sites in the uPA–PAI-1 complex [39,40]. In accordance with this, the free components are only partly able to inhibit the binding of the complex to LRP [39]. The binding of uPA–PAI-1 complex to members of the LDLR family of receptors can be completely inhibited by RAP, whereas the uPA–PAI-1 complex is invariably unable to abolish all binding of RAP, suggesting that RAP may bind with high affinity to the site recognizing uPA–PAI-1, but may also bind to additional sites which are not recognized by the uPA–PAI-1 ligand. The approximate location of the uPA–PAI-1-binding site in LRP was reported to reside in CR cluster II, a region containing a cluster of eight CR domains [41], where the related tissue-type plasminogen activator (tPA)–PAI-1 complex was reported to bind within the N-terminal region of this CR cluster [42]. The mutual cross-competition between the two PA–PAI-1 ligand complexes does, in fact, suggest that their binding sites on LRP are, at least partially, overlapping [22].

The present study was undertaken to identify the location of the binding site for the uPA–PAI-1 complex in LRP and to probe the functional importance of selected residues in the smallest receptor fragment that contains a complete ligand-binding site. Previously we described the production of a RAP-affinity-purified set of ubiquitin-fused overlapping CR-domain pairs (U-CRxy, where x and y are the CR domain numbers) [43]. These proteins were shown to adopt a correct folding, as demonstrated by their ligand-binding properties (e.g. Ca²⁺ binding) and, with the exception of U-CR910, these proteins were all demonstrated to bind RAP. Here, we extend the ligand mapping analysis to include LRP ligands from the plasminogen activation system. Furthermore, site-directed-mutagenesis studies were carried out to identify residues in LRP crucial for the binding of uPA–PAI-1.

**EXPERIMENTAL**

**Construction of mutant expression vectors and protein production**

RAP- and RAP-derivative-affinity chromatography were as described previously [43].

U-CR56 fragments with specific amino acid substitutions were expressed in *Escherichia coli* AG1 cells using the expression vector pT7H6/ubCR56 [43]. Site-directed mutagenesis was carried out using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.) following the protocol provided by the manufacturer. Mutagenesis primers with mutation sites located in their central regions were from DNA Technology A/S (Aarhus, Denmark). Sequences of inserts in expression vectors were authenticated by DNA sequencing using the Thermo Sequenase™ II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Mutant protein products were subjected to RAP-domain-3 (RAPd3; RAP residues 216–323; [43])-affinity chromatography under conditions similar to those used in the purification of U-CR56wt (wild-type U-CR56) and, although variation in the affinity for the RAPd3-Sepharose column was observed, all derivatives, with U-CR56W994S (a U-CR56 mutant in which Trp994 has been mutated to Ser) as the only exception, could be purified in sufficient quantities. U-CR56W994S was purified using ion-exchange chromatography on Q-Sepharose where protein was loaded to the column in a buffer A containing 50 mM sodium acetate, pH 5.0, 1 mM CaCl₂, and 20 mM NaCl. Upon application of a linear gradient from 20 mM to 1 M NaCl in buffer A, pure U-CR56W994S monomer (as judged by SDS/PAGE and Coomassie Brilliant Blue staining) was eluted at a position in the gradient corresponding to approx. 600 mM NaCl.

**Surface-plasmon-resonance (SPR) analysis**

Preparation of uPA–PAI-1 complex was carried out as described in [39]. Measurements of competition of uPA–PAI-1 binding to human LRP by U-CR_XY proteins were performed by SPR analysis on a BIAcore 2000 instrument (BIACORE AB, Uppsala, Sweden). The BIAcore sensor chip (type CM5; BIACORE AB) was activated with a 1:1 mixture of 0.2 M N-ethyl-N’-(3-dimethylaminopropyl)carbodi-imide and 0.05 M N-hydroxysuccimide in water as described by the manufacturer. Human LRP was immobilized at a concentration of 40 μg/ml in 10 mM sodium acetate, pH 3.0, passing through the BIAcore flow cell at a rate of 5 μl/min. Remaining binding sites were blocked by reaction in 1 M ethanolamine, pH 8.5, and SPR signals generated corresponded to a protein load of 28 fmol/μm². A flow cell containing reduced receptor protein served as a negative control.

Analyte proteins were desalted into Binding Buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 1.5 mM CaCl₂, 0.5 mM EGTA, and 0.05 mM Tween-20), which also was used as running buffer for the competition analysis for 10 nM uPA–PAI-1 binding either in the presence or in the absence of U-CR_XY competitors (applied at either 0.5 μM or 1.0 μM). Regeneration of the sensor chip was carried out by the application of 1 M glycine/HCl, pH 2.0. SPR signals generated corresponded to a 10-fold molar excess of PAI-1. Complex-formation was verified by SDS/PAGE analysis. The ¹²⁵I-labelled complex was isolated from the reaction mixture by sequential passage over columns containing Sepharose 4B-immobilized monoclonal antibodies against each of the individual components [39].

Solid-state competition analysis [45] and purification of human LRP from placenta [46] were carried out as described previously [43].

For ¹²⁵I-ligand blotting analysis, two-domain fragments were applied to SDS/18%-(w/v)-PAGE gels and electrophoresed on to nitrocellulose filters using a semi-dry electrophoretic method (Kem-En-Tec A/S, Copenhagen, Denmark). Nitrocellulose filters were: (a) blocked by incubation for 2 h in a mixture containing 100 mM Tris/HCl, pH 8.0, 0.9% (w/v) NaCl, 2.0% (v/v) Tween-20, and 2.0% (w/v) BSA; (b) washed with a Washing Buffer containing 10 mM Hepes, pH 7.4, 2 mM CaCl₂, 1 mM MgCl₂, 140 mM NaCl and 0.5% (w/v) BSA; (c) incubated for 16 h at 4 °C with 10 ml of Washing Buffer containing ¹²⁵I-labelled RAP (1 × 10⁶ c.p.m./ml) or ¹²⁵I-labelled uPA–PAI-1 (3 × 10⁶ c.p.m./ml); and finally (d) washed four times for 15 min in Washing Buffer. Autoradiography was performed using a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

**RESULTS**

**Ligand-blot analysis of uPA–PAI-1 binding to overlapping CR-domain pairs of LRP cluster II**

We have previously described the production of a RAP-affinity-purified set of ubiquitin-fused overlapping CR-domain pairs from the second CR cluster of LRP (Figure 1).

With the exception of U-CR910, these proteins were all demonstrated to bind RAP. To study the binding of uPA–PAI-1 to U-CR_XY fragments, equimolar aliquots of the fusion proteins

![Figure 2 Ligand-blot analysis of the binding of ¹²⁵I-uPA–PAI-1 to two-repeat fragments of LRP cluster II](image-url)

(A) Non-reducing SDS/PAGE of equimolar amounts of the U-CR34, U-CR45, U-CR56, U-CR67, U-CR78, U-CR89 and U-CR910 fragments as indicated. Molecular markers are shown on the left side (98, 64, 50, 36, 30, 16 and 6 kDa, from top to bottom). The gel was stained with Coomassie Blue. (B) Samples identical with those shown in (A) were tested for ¹²⁵I-uPA–PAI-1 binding by ligand-blotting analysis. The blot was incubated with 100 nM ¹²⁵I-labelled uPA–PAI-1.
Figure 3 SPR analysis of CR-domain pair competition of uPA–PAI-1 binding to LRP

Sensorgrams shown as broken thick curves represent 10 nM uPA–PAI-1 binding to immobilized LRP without any inhibitor. Full-line duplicate sensorgrams are shown for identical samples of 10 nM uPA–PAI-1 in the presence of 0.5 µM or 1.0 µM of U-CR56 as indicated. Dotted curves represent duplicates of 10 nM uPA–PAI-1 in the presence of 0.5 µM and 1.0 µM of U-CR34, U-CR45, U-CR67, U-CR78 or U-CR89. U-CR910 was not tested.

were subjected to SDS/PAGE separation followed by transfer to a nitrocellulose filter, which was then incubated with 125I-labelled uPA–PAI-1. The application of equimolar amounts of fusion proteins on the SDS/PAGE gels was verified by Coomassie Blue staining of SDS/PAGE gels run in parallel (shown in Figure 2A). The results of radioligand blotting (Figure 2B) show that U-CR56 binds 125I-uPA–PAI-1, whereas U-CR34, U-CR45, U-CR67, U-CR78, U-CR89 and U-CR910 do not. The immobilized U-CRXxy proteins have been shown to retain authentic affinity for Ca2+ and RAP [43].

Figure 4 CR-domain-pair competition of 125I-uPA–PAI-1 binding to LRP

Representative binding assays with radiolabelled uPA–PAI-1 and a series of unlabelled competitor proteins (U-CR34, U-CR45, U-CR56, U-CR67, U-CR78, U-CR89 and U-CR910). Each data point is mean ± S.E.M. of at least four measurements. The ordinate shows the ratio between bound and free 125I-uPA–PAI-1 in wells in percentage of the same ratio in wells without receptor fragment. Competitor proteins used are indicated in the key.

Figure 5 Ligand-blot analysis of the binding of 125I-RAP and 125I-uPA–PAI-1 to U-CR56 mutants

(A) Non-reducing SDS/PAGE of aliquots of U-CR56 mutant proteins. Molecular markers are shown on the left-hand side (98, 64, 50, 36, 30, 16 and 6 kDa, from top to bottom). The gel was stained with Coomassie Blue. (B and C) Samples identical with those shown in (A) were transferred to nitrocellulose filters and incubated with either 1 nM 125I-RAP (B) or 100 nM 125I-uPA–PAI-1 (C). The lanes contain U-CR56wt, U-CR56R947Q, U-CR56W953S, U-CR56D958N, U-CR56D959N, U-CR56R964Q, U-CR56R988Q, U-CR56W994S, U-CR56R995Q, and U-CR56D999N as indicated (the underlining indicates the CR domain containing the mutation).

CR-domain pair competition of the binding between uPA–PAI-1 and LRP

To investigate whether any of the U-CRXxy two-domain fragments may perturb the binding of uPA–PAI-1 to full-length native LRP, the binding of 10 nM uPA–PAI-1 to a BIAcore sensor chip with immobilized LRP was studied in the presence and absence of U-CRXxy two-domain fragments. The sensorgrams obtained show (Figure 3) that binding of uPA–PAI-1 to LRP is decreased, in the presence of 0.5 or 1.0 µM U-CR56, and the decrease observed is apparently dose-dependent. At these concentrations, none of the other U-CRXxy two-domain fragments were able to inhibit the binding of uPA–PAI-1 to LRP, suggesting that U-CR56 binds uPA–PAI-1 most strongly. The response curves show a ≈ 200 response unit bulk contribution due to small changes in buffer composition at the start (150 s) and at the end (600 s) of injection. These results were subjected to further investigation by solid-state competition analysis in which the binding of 125I-uPA–PAI-1 to LRP immobilized in microtitre wells was measured. No single CR domain was able to inhibit the interaction (results not shown), but, among the U-CRXxy two-domain fragments tested, inhibition was observed with U-CR34, U-CR45, U-CR56, U-CR67 and U-CR78, but not U-CR89 and U-CR910. However,
U-CR56 was the most efficient inhibitor of radioligand binding (Figure 4) with an approx. 5-fold lower IC$_{50}$ value of 1 µM compared with U-CR54, U-CR45, U-CR67 and U-CR78. Notably, these competition curves are in accord with the SPR results, since at concentrations of 500 nM and 1 µM competitor, U-CR56 is the only CR-domain pair which is able to partly block the binding. However, the fact that other receptor fragments also are able to displace the binding of $^{125}$I-uPA--PAI-1 to LRP at higher concentrations might be indicative of either (i) the native binding interface between LRP and uPA--PAI-1 is so small that a single CR-domain pair is sufficient to mask the binding site in the ligand for full-length LRP, whereby the data in Figure 4 reflect the different affinities between this unique site and the various CR-domain pairs, or (ii) the binding site is extended, and as many as four to six CR domains of LRP cluster II are involved in the recognition of uPA--PAI-1. In the latter case, each CR domain is able to bind to different sites on the complex, each decreasing the overall affinity significantly, but with CR56 being of major importance.

Identification of residues in CR5 and CR6 of importance for the recognition of RAP and uPA--PAI-1

As the binding of proteinase--PAI-1 complexes to the LRP and VLDLR receptors has been proposed to depend on electrostatic interactions involving basic residues in the serpin [47–49], we decided to test the importance for binding of specific charged residues located in CR5 and CR6. Accordingly, we constructed the U-CR56 mutant derivatives U-CR56R947Q, U-CR56D958N, U-CR56G964Q, U-CR56D959N and U-CR56W994S. Previously we identified important contributions to ligand binding by side chains of residues assumed to co-ordinate Ca$^{2+}$ through backbone carbonyl oxygen atoms [43], and, additionally, prepared the mutant derivatives U-CR56W953S and U-CR56W994S for inclusion in the present study. Mutant derivatives U-CR56D958N and U-CR56D999N, which exhibit decreased RAP binding [43], were also included. We chose not to mutate any of the eight residues assumed to co-ordinate Ca$^{2+}$ via side-chain carboxy groups (Asp$^{956}$, Asp$^{966}$, Asp$^{1001}$, Glu$^{967}$, Glu$^{968}$, Glu$^{994}$, Glu$^{995}$) (see Figure 1B). Residues Asp$^{963}$, Asp$^{1001}$, Asp$^{1004}$, Asp$^{1005}$, Glu$^{968}$, Glu$^{969}$, Glu$^{976}$, Glu$^{994}$, Glu$^{995}$, which are conserved in all CR domains, were also excluded from the analysis as homologous residues have been shown to be critical for folding of the CR-domain [30].

The expression levels of substituted U-CR56 fragments were virtually identical with that obtained for wt U-CR56. Although some variants had to be pooled from several passages of crude product through the RAP3-affinity column to obtain the yield obtainable from the wt U-CR56 sample in a single passage, all mutant derivatives, except one, could be obtained in pure active form by RAP3-affinity chromatography. The exception was U-CR56W994S, which failed to bind to the RAP3–Sepharose. This derivative was purified essentially to homogeneity by ion-exchange chromatography. As judged from the appearance of bands corresponding to purified products on non-reducing SDS/PAGE gels (Figure 5A), all mutated derivatives were in fact obtained in a correctly folded form.

The ability of all but one of the U-CR56 derivatives to bind to immobilized RAP3 indicated correct folding, but the variation of affinity chromatographic properties among the various derivatives indicated that affinities for RAP3 differed significantly among the U-CR56 mutant proteins studied. The affinities of the various U-CR56 mutant derivatives for RAP and for uPA--PAI-1 were assessed by ligand-blotting analysis (Figure 5). Neither the Asp$\rightarrow$Asn mutant derivatives (Asp$^{956}$, Asp$^{966}$ and Asp$^{994}$, Asp$^{995}$ respectively) nor the Trp$\rightarrow$Ser mutant derivatives (Trp$^{953}$, Trp$^{956}$, Trp$^{994}$, Trp$^{995}$ respectively) exhibited any detectable binding to $^{125}$I-labelled RAP or uPA--PAI-1. The U-CR56D959N mutant derivative exhibited decreased binding to both $^{125}$I-RAP and $^{125}$I-uPA--PAI-1, whereas U-CR56R988Q was found to bind both radioligands more efficiently than U-CR56wt.

The affinities of the U-CR56 mutants to RAP and uPA--PAI-1 were also estimated by the ability of the mutants to compete the binding of $^{125}$I-RAP and $^{125}$I-uPA--PAI-1 to LRP immobilized in microtitre wells (Table 1). Each of the four Arg$\rightarrow$Gln mutant derivatives (R947Q, R964Q, R988Q and R995Q) was almost as effective in displacing $^{125}$I-RAP from immobilized LRP as U-CR56wt. Although some variants had to be pooled from several passages of crude product through the RAP3-affinity column to obtain the yield obtainable from the wt U-CR56 sample in a single passage, all mutant derivatives, except one, could be obtained in pure active form by RAP3-affinity chromatography. The exception was U-CR56W994S, which failed to bind to the RAP3–Sepharose. This derivative was purified essentially to homogeneity by ion-exchange chromatography. As judged from the appearance of bands corresponding to purified products on non-reducing SDS/PAGE gels (Figure 5A), all mutated derivatives were in fact obtained in a correctly folded form.

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Table 1 Competition of binding of RAP and uPA--PAI-1 to immobilized LRP by U-CR56 mutants

<table>
<thead>
<tr>
<th>U-CR56 derivative</th>
<th>RAPd3 affinity chromatography*</th>
<th>Inhibition (%) of:</th>
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<tr>
<td></td>
<td></td>
<td>$^{125}$I-RAP</td>
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<td></td>
<td></td>
<td>500 nM</td>
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<td>U-CR56wt</td>
<td>+ + +</td>
<td>70</td>
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<td>U-CR56G999N</td>
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* Affinity score of RAPd3 binding evaluated from RAPd3 affinity chromatographic analysis; equal amounts of protein were loaded on the column, and the amount of bound protein was classified ranging from effective binding (+ + +) to no detectable interaction (−).
and D999N), showing most pronounced reduction in affinity for RAP, were also the group of U-CR56 mutant proteins least capable of competing the binding between 125I-uPA–PAI-1 and LRP (Table 1). The four Arg → Gln U-CR56 mutant derivatives were found to compete the binding of uPA–PAI-1 to LRP as efficiently as U-CR56wt. Notably, U-CR56R988Q was found to be a better competitor than wild-type U-CR56.

**DISCUSSION**

The present study identifies a preferential binding site for uPA–PAI-1 within CR cluster II, located in CR5 and CR6, of LRP. Among the complete overlapping set of two-domain fragments, representing LRP CR cluster II in the form of ubiquitin-fused CR-domain pairs, the uPA–PAI-1 complex was found to bind with highest affinity to U-CR56, but also not insignificantly to CR-domain pairs harbouring neighbour CR domains.

The fact that no single CR-domain protein could block the binding of uPA–PAI-1 to LRP indicates that at least two CR domains must be present to generate the high-affinity binding site, either because a single binding site is made up from residues from both domains, or because individual weaker binding sites residing in each domain co-operate to generate the equivalent of a high-affinity site. Indications are, in fact, that the latter organizational model is correct, as every CR-domain pair tested, except U-CR89 and U-CR910, was found to exhibit an inhibition effect if present in higher concentration, suggesting that each CR domain does in fact contain a ligand-binding site. Structural studies of proteins comprising two CR-domain fragments indicate that the two CR domains are structurally autonomous and joined together only by flexible linkers [50,51], which obviously does not rule out the alternative possibility that the two CR domains may adopt some fixed spatial organization on ligand binding.

The present results, showing that the preferential binding site for uPA–PAI-1 in LRP is located in CR5 and CR6, is in accord with previous reports concerning the locations of binding sites for other ligands and the patterns of cross-competition among them. For example, the fourth CR domain of LRP harbours a specific binding site for transformed uPA–PAI-1 [52], and uPA–PAI-1 and zM* exhibit no cross-ligand competition in their binding to LRP [22]. The inhibition of uPA–PAI-1 binding to LRP by RAP is in agreement with our previous demonstration of high-affinity binding of RAP to U-CR56. The failure of uPA–PAI-1 to inhibit the binding of RAP to LRP is most likely due to the presence of several additional binding sites for RAP in LRP [43].

High-affinity (0.4 nM dissociation constant) binding of uPA–PAI-1 to LRP requires the presence of the serpin as well as the protease [39]. The present results raise the question as to whether each CR domain in a CR-domain pair binds to the same site in the uPA–PAI-1 complex – either in uPA or in PAI-1 – in which case avidity accounts for a major part of the observed binding strength. The alternative possibility is that two binding sites on a two-domain protein, one residing in CR5 and one residing in CR6, bind to different sites in the complex, e.g. one site in uPA and one site in PAI-1, or two different sites residing in one of the components of the complex. In terms of affinity of the uPA–PAI-1 for the complete LRP receptor, additional bindings to neighbouring CR domains most likely contribute to overall affinity. In conclusion, we have identified CR56 as the most interesting subject for future structural investigations of complexes comprising minimal LRP fragments and this particular protein ligand.

In the search for residues contributing to the overall affinity of U-CR56 for uPA–PAI-1 and for residues determining the specificity of this particular CR-domain pair, we investigated the binding properties of a series of single substitution U-CR56 mutants. Previously, we showed the importance of a conserved Asp residue, located in the centre position between the fourth and the fifth Cys residue in each CR domain, for efficient binding of the ligands RAP [43] and zM* [52]. Alteration of the β-carboxy groups in these residues in domains CR5 and CR6 separately by site-directed mutagenesis (Asp → Asn) was shown here to reduce the ability of the U-CR56 to compete the binding between LRP and uPA–PAI-1 and in direct binding measurements to abolish interaction with uPA–PAI-1.

The proposal of electrostatic complementarity between ligand and CR domains as the primary determinant for ligand binding to LDLR-like receptors has been challenged on the basis of X-ray crystal-structure analysis of LB5, which showed that many of the conserved acidic residues are in fact involved in Ca⁺⁺ coordination [30]. However, as pointed out by North and Blacklow [31], there is still a region of negative electrostatic surface potential that surrounds the co-ordinated Ca⁺⁺ ion. The acidic side chain of the Asp residue at the centre position between Cys⁴⁴⁴ and Cys⁴⁴⁵ studied here is located at the molecular surface (Figure 6) and contributes further to this negative potential, thereby indicating that electrostatic interactions can be involved in ligand-CR domain recognition. (The roman numeral superscripts IV and V designate relative sequence positions of conserved cysteine residues within the CR domain.)

The substitutions of Trp⁵⁴⁵,⁵⁴⁶ and Trp⁹⁸⁴,⁹⁸⁵ with Ser residues dramatically decreased the affinities of U-CR56 for RAP and for uPA–PAI-1. The conserved Trp residues are assumed to coordinate Ca⁺⁺ via their backbone carbonyl oxygen atoms, as this is a structural feature of the corresponding Trp residues in CR domains of known tertiary structure, e.g. CR3, CR8 and LB5 [26,27,30]. A sequence alignment of the eight CR domains of LDLR cluster II (Figure 1B) shows that this Trp residue is conserved in seven of the eight sequences. Given the high sequence similarity of the domains, the overall folding is expected to be similar for at least these seven domains (CR3–CR9). The structure of CR3 (Figure 6) shows that the conserved Trp residues are located on the molecular surface, and it is therefore highly likely that the homologous residues in CR5 and CR6 are also surface-located.

The fact that the two residues (Asp and Trp) of critical importance for ligand binding are intimately linked to the Ca⁺⁺-binding sites via their backbone carbonyl groups that serve as ligand donors engaged in co-ordination of Ca⁺⁺, provides a direct explanation for the observed Ca⁺⁺ dependence of ligand binding [46,53], as ligation of Ca⁺⁺ would be expected to impose (and depend on) a high level of structural order of residues contributing to configuration of the metal-binding site. The intimate structural linkage between Ca⁺⁺-binding sites and macromolecular-ligand-binding sites may further contribute to explain the dissociation of ligands at low pH in the acidic environment of the endosomes, to the extent that such loss of affinity is due to loss of affinity for Ca⁺⁺.

Interestingly, LB6 from LDLR contains an Arg residue at the sequence position of the conserved Trp residue of the LRP CR-cluster II domains. In LB6 this Arg residue is also likely to coordinate Ca⁺⁺ via its backbone carbonyl group [31]. However, a naturally occurring mutation in LDLR of this residue to a Trp residue (Arg⁹⁸⁴LB6 → Trp) results in familial hypercholesterolaemia, by an as yet unexplained mechanism, further underscoring the critical role in ligand recognition of the residue at this position within ligand-binding CR domains.
In the present work we have demonstrated that the two ligands, RAP and uPA–PAI-1, bind to the CR56 fragment essentially on a common structural basis, given the almost identical effects obtained by substituting conserved Ca\(^{2+}\)-ligating residues. It has previously been questioned whether such structural similarity between the mode of binding an extracellular ligand, like uPA–PAI-1, and the mode of RAP binding would in fact exist, as RAP is an intracellular escort protein. Extrapolating from our present results, we propose that the general mechanism by which RAP may function as an antagonist for all other ligand binding to LRP is by competing directly for the same binding sites on LRP. Our results suggest that the affinity of a specific subfragment of a CR-domain cluster for a specific ligand is composed of at least two terms, a specific term governed by specific interactions, residing in specific configurations of key residues on specific CR domains, in addition to a less specific term, governed by less specific interactions with Trp-Asp epitopes, which contribute significantly to overall affinity for the specific ligand, and which are the primary determinants for the interaction of CR domains with RAP. Because the critical residues are present in most CR domains from LRP cluster II, the specific preference of CR56 for uPA–PAI-1, must rely on residues as yet remaining to be identified and/or a specific spatial orientation of the relative position of these two CR domains.

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