Three Complement-like Repeats Compose the Complete $\alpha_2$-Macroglobulin Binding Site in the Second Ligand Binding Cluster of the Low Density Lipoprotein Receptor-related Protein*

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The low density lipoprotein receptor-related protein (LRP) is an essential member (1) of the low density lipoprotein (LDL) family of mosaic receptor proteins that are responsible for binding and internalization of a large number of protein ligands (2). Each of these receptors contains one or more clusters of ligand binding domains, known both as complement-like repeats (CR) and LDL receptor type A modules (3). Whereas LDLR contains only one such cluster of 7 CR domains, LRP has four clusters containing 2, 8, 10, and 11 repeats, counting from the N terminus (4–6). Commonly used nomenclature, thus, describes cluster 2 as being composed of domains CR3 through CR10.

Each CR domain contains three conserved disulfide bridges and a functionally required calcium binding site within 40–42 residues. These domains are linked by variable length, flexible linkers that are likely to afford considerable orientational freedom to each CR domain with respect to others in the cluster (7). Although the cysteines and calcium-coordinating residues are highly conserved, most of the remaining ~30 residues are variable (8). This potentially gives LRP a wide variety of ligand recognition sites within its four clusters of 31 CR domains and may in part explain the very much broader range of ligands that can bind and be internalized by LRP compared with the much simpler LDLR. Thus, LRP binds ligands as diverse as ~760-kDa $\alpha_2$M-protease complexes, various ~100-kDa serpin-protease complexes such as plasminogen activator inhibitor 1-urokinase-like plasminogen activator (uPA), thrombin-antithrombin, and protease nexin-1-uPA, apolipoprotein E, and lipoprotein lipase, amyloid precursor protein, complement C3, and lactoferrin (2).

One of the best studied LRP ligands is $\alpha_2$M. $\alpha_2$M is an abundant, homo-tetrameric pan-protease inhibitor (9) that is also reported to bind various growth factors (10) and that is evolutionarily related to the complement proteins C3, C4, and C5 (11). Binding of $\alpha_2$M to LRP requires the exposure of a previously hidden receptor binding domain (RBD) that constitutes the C-terminal 138 residues of each $\alpha_2$M chain. Exposure of RBD results from the conformational transformation that occurs upon complex formation with protease. LRP can, thus, discriminate strongly between native and activated $\alpha_2$M species. Internalization of complexed $\alpha_2$M can result not only in internalization and degradation of the complexed protease but also in intracellular signaling responses, making this ligand an especially important one to study (12).

It is thought that the RBD of $\alpha_2$M, whose structure is known
and consists of a β-sandwich edged by a single α-helix (13), is solely responsible for binding of α2M-proteinase complexes to LRP (5, 6, 14, 15). Nevertheless, although intact α2M can bind to LRP with an affinity that reaches subnanomolar at high LRP density, isolated RBD binds with a Kₐ of 100–200 nM (15, 16). The difference is thought to be due to the tetrameric nature of α2M allowing interaction with two or more LRP molecules when LRP is present at high density or to an interaction with more than one cluster of CR domains within a single LRP molecule. Thus, it has been reported that α2M can bind to both cluster 2 and cluster 4 when each cluster is expressed as a mini-receptor (17).

Given the importance both of LRP as an essential endocytosis and signaling receptor and of α2M-proteinase complexes as an LRP ligand, an understanding of the specificity of their interaction is a very worthwhile goal. An excellent starting point is the known importance of RBD in binding, the identification of two lysine residues within RBD that are required for tight binding to LRP (18), the localization of high affinity α2M binding to CR cluster 2 of LRP (17), and our earlier observation that RBD can bind to CR3, the first CR domain of cluster 2, with modest affinity (19). In the present study we have built on this and attempted to identify the minimum additional CR domains that together with CR3, might constitute the full binding site for affinity (19). In the present study we have built on this and attempted to identify the minimum additional CR domains that together with CR3, might constitute the full binding site for affinity (19). In the present study we have built on this and attempted to identify the minimum additional CR domains that together with CR3, might constitute the full binding site for affinity (19). In the present study we have built on this and attempted to identify the minimum additional CR domains that together with CR3, might constitute the full binding site for affinity (19). In the present study we have built on this and attempted to identify the minimum additional CR domains that together with CR3, might constitute the full binding site for affinity (19).
Purified CR3-4, CR3-4-5, and CR5-6-7 were refolded using a modification of the previously described protocol (23). Each protein was dissolved in a minimal volume of 6 M GdnHCl, 50 mM Tris-HCl, pH 8.5, and reduced with a 2× molar excess (over Cys) of diithiothreitol for 30 min. Each fragment was then diluted with refolding buffer (50 mM Tris-HCl, 10 mM CaCl₂, pH 8.5) and mixed with a 50% molar excess of glutathione S-transferase-receptor-associated protein (RAP) for a final concentration of complement repeat fragment of 0.1 mg/ml. The refolding mixture was dialyzed at room temperature against 4 liters of degassed refolding buffer containing 1 mM GSH and 0.5 mM GSSG for 24 h with N₂ bubbling to avoid oxidation by O₂ followed by 24 h of refolding at 4 °C without bubbling. To purify correctly folded complement repeat fragments, the mixture was dialyzed against 3 × 4 liters of 20 mM Tris-HCl, 50 mM NaCl, 10 mM CaCl₂, pH 8.0, and loaded on a GSH-Sepharose column equilibrated in the same buffer. After a brief wash to remove unbound material, folded fragment bound to glutathione S-transferase-RAP was eluted with a buffer containing 20 mM Tris-HCl, 50 mM NaCl, 4 mM EDTA, pH 8.0. LRP fragments were further purified by reverse phase HPLC as described above. In each case, the folded protein eluted faster than the reduced protein before refolding.

Folded CR3-4-5 and CR5-6-7 were dialyzed against 20 mM Tris-HCl, 50 mM NaCl, 0.1 mM CaCl₂, pH 8.0, and loaded onto a Q-Sepharose-HP column (10 × 100 mm). The protein was eluted with a gradient of 50–1000 mM NaCl.

As a final purification step to ensure that only correctly disulfide-bonded and, hence, functional protein was present, folded CR3-4-5 was mixed with His₆-RBD in the presence of 1 mM CaCl₂ and passed over a small Ni-NTA column. After washing with 5 column volumes of buffer, CR3-4-5 capable of binding RBD was eluted with a buffer containing 10 mM citrate. Traces of His₆-RBD were removed by ion exchange chromatography on a Q-Sepharose HP column, eluted with a gradient of 0–1 M NaCl in 20 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Protein Concentrations—For each protein the extinction coefficient was calculated using the formula $\epsilon_{280} = 5500 \times \text{(number of tryptophans)} + 1490 \times \text{(number of tyrosines)} + 120 \times \text{(number of disulfide bridges)}$. This empirical formula has been shown to be accurate to within a few percent of experimentally determined extinction coefficients for most proteins (24).

Fluorescence Spectroscopy—Binding experiments were performed on a PTI Quantamaster instrument equipped with double monochromators on both the excitation and emission sides. Because RBD does not contain tryptophan residues, the tryptophan residues in CR3-4-5 (and CR5-6-7) could be selectively excited at 295 nm and RBD binding followed by the increase in tryptophan fluorescence at 325 nm. The experiments were performed in 20 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, pH 7.4, containing 0.1% PEG 20,000 to avoid protein adsorption to the acrylic cuvette. Low pH binding experiments were performed in similar buffers containing 20 mM Bis-Tris (pH 6.5) or 20 mM MES (pH 6.0 and 5.5). Spectra of the apo-forms of CR3, CR3-4, CR3-4-5, and CR5-6-7, were obtained in 20 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 7.4, containing 0.1% polyethylene glycol 20,000.
Involvement of Lysines 1370 and 1374 in RBD Binding to CR3-4-5—In the context of both isolated RBD and intact α2M, it has been shown that only two of the lysines within RBD that are highly conserved among macroglobulins are important for binding to LRP (18, 28). These are lysines 1370 and 1374, located on the outer face of the single α-helix of RBD. We argued that, if CR3-4-5 contains the full LRP binding site for RBD/α2M, it must show the same requirement as LRP for these lysines for tight RBD binding. We, therefore, prepared RBD containing the K1370M/K1374M double mutation and examined binding to CR3-4-5 by gel shift assay and by fluorescence titration.

As expected, RBD containing the two mutations was no longer able to form a tight enough complex with CR3-4-5 to give a band shift on non-denaturing PAGE (Fig. 3, lanes 2, 5, and 7). To quantitate the reduction in affinity, fluorescence was again used. The fluorescence spectrum of 0.5 μM CR3-4-5 with a large excess of K1370M/K1374M RBD (18 μM) showed a similar type of shift seen with the wild type RBD although with smaller magnitude (Fig. 4). The lower magnitude was due to the severe weakening of the interaction such that 18 μM variant RBD was insufficient to saturate the binding site. A best fit to a titration gave a Kd of 18 μM (Table 1), which represents a weakening of the Kd by ~140-fold. Given the weakness of this interaction, no attempt was made to determine the affinity of the variant RBD to CR3-4.

For subsequent use as an important negative control for RBD binding to CR3-4-5, we also expressed and characterized CR5-6-7. As with the isolated constituent domains.

FIGURE 2. Fluorescence spectra of CR3, CR3-4, CR3-4-5, and CR5-6-7 in the absence (lower trace) and presence of 1 mM calcium (upper trace). All spectra are normalized to that of CR3 without calcium.
pH Dependence of RBD-CR3-4-5 Interaction—In vivo, ligands dissociate from LRP and other LDL receptor family members in endosomes, and the free receptors are then recycled to the cell surface. Experiments with isolated LRP have shown a sharp drop in affinity for $\alpha_2$M-proteinase complexes between pH 7.0 and 6.0 (29). It has been suggested that pH-dependent Ca$^{2+}$ binding at extracellular pH and release at endosomal pH might account for this pH dependence (30). However, measurements of calcium affinity to CR domains in this pH range do not support such a mechanism. An alternative mechanism has been proposed based on the structure of the extracellular portion of LDLR, crystallized at pH 5.3, that shows specific interactions between the single YWTD-propeller domain and complement-like repeats 4 and 5 (called LB4 and LB5 in LDLR) (20). The interactions include salt bridges between lysine and histidine residues in the YWTD-propeller and acidic residues in the complement-like repeats and one reversed salt bridge between a histidine in LB5 and a glutamic acid in the YWTD-propeller. This suggested a model in which protonation of histidine residues at low pH greatly enhances the intramolecular interaction between YWTD-propeller domains and ligand binding complement-like repeats and results in successful competition for, and thus, displacement of the previously bound ligands. In such a model, loss of affinity between LRP and a ligand as the pH is lowered would not necessarily be required. We, therefore, sought to examine the pH dependence of binding for the RBD-CR3-4-5 pair. Fluorescence titrations of RBD with CR3-4-5 were carried out at pH 6.5, 6.0, or 5.5, analogous to that reported above at pH 7.4. Consistent with the YWTD-displacement mechanism, no significant alteration in binding affinity was seen (Table 2).

**DISCUSSION**

Qualitatively by gel shift assay and quantitatively by fluorescence titration we have demonstrated a tight interaction between LRP fragment CR3-4-5, representing the first three CR domains from cluster 2 of LRP, and RBD, the domain from $\alpha_2$M that contains the full binding epitope. The experimental indistinguishability of the determined $K_d$ of 130 nM for this interaction from those published for binding of RBD to intact LRP demonstrates that CR3-4-5 represents the com-
plete binding site within cluster 2 for \( \alpha_2 \)M-proteinase complexes. This is also consistent with an earlier study using LRP minireceptors, which showed that a minireceptor that contained only CR3-4-5 from cluster 2 and the two CR domains from cluster 1 was about as effective as intact LRP in internalizing \(^{125}\)I-labeled \( \alpha_2 \)M\(^\ast\) (31). The drastic reduction in affinity of the complex (100-fold increase in \( K_d \)) upon mutating the two RBD lysine residues, shown elsewhere to be important for binding to LRP (28), reinforces this conclusion and provides a measure of the importance of these residues for tight binding. The uniqueness of the CR3-4-5 binding site within cluster 2 is demonstrated by the 30-fold lower \( K_d \) for this site compared with that in CR5-6-7. Finally, the lack of pH dependence of the interaction upon lowering the pH from extracellular to intracellular supports the proposed mechanism of intracellular ligand release that depends on enhanced, pH-dependent affinity of an intramolecular YWTD domain to compete-off internalized bound protein ligands.

Although the affinity found here for the CR3-4-5-RBD pair exactly matches the affinity of isolated RBD for LRP and, therefore, represents the full RBD binding site within LRP, it does not match that of intact, tetrameric \( \alpha_2 \)M for LRP (Table 1). However, it is important to note that “missing” binding energy in both cases represents a small fraction of the total interaction energy. Taking the highest reported affinity of \( \alpha_2 \)M for LRP of 40 pm, the binding energy found here for the CR3-4-5-RBD pair represents 66% of the energy of the interaction (9.3/14 kcal mol\(^{-1}\)). However, this ultrahigh affinity has only been found at very high LRP densities, under which conditions the tetravalent \( \alpha_2 \)M might be capable of engaging CR3-4-5 sites on two or more LRP molecules. At lower LRP density, under which conditions \( \alpha_2 \)M might be expected to bind to only one LRP, a much lower affinity of \( \approx 2 \) nm has been reported (32). Compared with such a bimolecular interaction, the present pair binds with 80% of the affinity (9.3/11.7 kcal mol\(^{-1}\)). Although \( \approx 20\% \) is still unaccounted for, this is only 2.4 kcal mol\(^{-1}\). Here it should be noted that \( \alpha_2 \)M-proteinase complexes have also been reported to bind with similar qualitative affinity to cluster 4 from LRP (17). If one RBD in \( \alpha_2 \)M were to engage the present site of CR3-4-5 in cluster 2, a second RBD from the same \( \alpha_2 \)M would need to find only a poorly complementary additional site within

### Table 1

| Proteins          | RBD-CR3\(^a\) | RBD-CR34 | RBD-CR3-4-5 | K-M-CR3-4-5 | RBD-CR5-6-7 | RBD-LRP\(^b\) | \( \alpha_2 \)M-LRP\(^c\) |
|-------------------|--------------|----------|-------------|-------------|-------------|--------------|-----------------|
| \( K_d \) (nm)    | 108 ± 48     | 103 ± 36 | 118 ± 31    | 18 ± 2 \( \mu \)M | 100–200 M  | 2 M/40–460 M  | NA              |
| \( \Delta G \) (kcal/mol) | 5.2          | -7.9     | -9.3        | -6.4        | -7.3        | -9.4 to -9.0   | -11.8 to -11.4  |
| \( \Delta G_{\text{bound}} \) (kcal/mol) | -7.6         | -10.3    | -11.7       | -8.8        | -9.7        | NA            | -14.1 to -16.4  |

\(^a\) From NMR titration (19).
\(^b\) From Refs. 15 and 16.
\(^c\) From single/multiple receptor model (28, 32).

### Table 2

| pH | RBD-CR3-4-5 binding | RBD-LRP binding |
|----|---------------------|-----------------|
| 5.5 | 108 ± 48 | 100–200 M |
| 6.0 | 103 ± 36 | 2 M/40–460 M |
| 6.5 | 118 ± 31 | NA |

With the caveat that the mode of RBD binding is assumed to remain the same irrespective of the size of the LRP fragment, the determination of \( K_d \) values and, hence \( \Delta G \) and unitary free energies (\( \Delta G_{\text{un}} \)) (33) for RBD binding to CR3, CR3-4, and CR3-4-5, allows an estimate to be made of the contributions from individual CR domains. Using the values from Table 1 it can be estimated that CR3 contributes 65% of the unitary binding energy (7.6 kcal mol\(^{-1}\)), CR4 23% (2.7 kcal mol\(^{-1}\)), and CR5 12% (1.4 kcal mol\(^{-1}\)). None of these is a particularly large contribution, and each might reasonably be composed of a very few specific interactions. In this context the recent structure determination of the complex of LA4-LA5 (CR domains 4 and 5 from LDLR) with the third domain (D3) of the chaperone RAP (34) is of particular relevance. The structure demonstrated that the mode of binding appears to be dominated by a specific interaction between two lysines (256 and 270) on RAP and the highly negative locus surrounding the calcium binding site of each CR domain. This finding correlates perfectly with mutagenesis studies of Strickland and co-workers (35) who identified these same two lysines as the only ones critical for RAP D3-LRP binding and showed several orders of magnitude increase in apparent \( K_d \) for complexes with RAP D3 species containing the individually mutated lysines. Although there are a number of other lysines in RAP D3, 256 and 270 are in particularly basic patches. It was, thus, suggested that an ultra basic lysine-calcium binding site interaction may underlie all LDL receptor family ligand binding.

In keeping with this proposal, we have now shown that two lysine residues, Lys-1370 and Lys-1374 in intact \( \alpha_2 \)M, previously shown to be important for RBD and \( \alpha_2 \)M binding to LRP, are involved in the interaction between CR3-4-5 and RBD, with a contribution to binding of 2.9 kcal/mol. The proximity of these two lysines to one another in RBD and the demonstration that they appear to act synergistically is consistent with the 2.9 kcal mol\(^{-1}\) of binding energy arising from a single lysine-calcium binding site interaction. Similarly, basic residues in tissue-type plasminogen activator inhibitor 1 (PAI-1) have been implicated in tissue-type plasminogen activator-PAI-1 and urokinase-like

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\( \alpha_2 \)M RBD Binding to CR3-4-5
plasminogen activator-PAI-1 complexes binding to LRP and the VLDL receptor (36, 37), and Arg-172 has been shown to be crucial for ApoE binding to LDLR (38).

One concern with the present findings is that they are not in agreement with an earlier study on the affinity of tandem pairs of CR domains from cluster 2 of LRP to RBD, which found that both CR3-4 and CR4-5 bound to RBD with similar low affinity of \( \sim 20 \mu M \) (39). Although we did not examine CR4-5 in the present study, we did examine the CR3-4-RBD interaction and found a \( K_d \) about 12-fold tighter than previously reported. It is not clear why there is a discrepancy between the two studies. It may result from the presence of an N-terminal protein tag in the previous study affecting binding.

Our demonstration that the affinity of RBD for CR3-4-5 does not decrease as the pH is lowered, such as would occur upon acidification in the endosome, supports a model of direct displacement of bound ligands by the intramolecular interaction between the ligand binding complement-like repeats and the flanking YWTD-propeller domains. It has been proposed that protonation of histidine residues serve as the switch that allows tighter binding of the neighboring YWTD-domains than the ligands (20). Although several studies using minireceptors constituting individual ligand binding clusters or parts of ligand binding cluster 2 have failed to observe degradation of radiolabeled \( \alpha_M \) (31, 40, 41), it should be noted that these studies have been performed on receptors lacking the YWTD-propeller domains between clusters 1 and 2. Given our finding that RBD binds CR3-4-5 tightly even at pH 5.5 and the structure of the extracellular domain of LDLR at low pH showing intramolecular interactions between the YWTD domain and two complement-like repeats, it is likely that one of the two YWTD-propeller domains found between clusters 1 and 2 in intact LRP is responsible for the interaction with CR3-4-5 and the intracellular displacement of \( \alpha_M \) from the receptor.

A final point arising from the present studies concerns specificity and the somewhat restricted distribution of ligand binding sites within LRP. Thus, most ligands have been mapped to cluster 2 and, to a lesser extent, cluster 4, even though cluster 3 contains more copies (10) of the CR domain than does cluster 2 (8). Given the demonstrated importance of interactions between lysine residues and the calcium binding sites of CR domains, whether in the LDLR-RAP D3 complex (34), the complex of domain 3 of VLDLR with rhinovirus (42), or the YWTD-propeller domain and two complement-like repeats in the low pH structure of LDLR (20), the negative potential of the calcium binding region may be critical in determining whether or not ligands can bind. Although four acidic residues coordinate the calcium directly through their side chains, two other residues do so through the backbone carbonyl. One of these is the middle residue of a DXXXD pentapeptide. In CR3, CR4, and CR5 the middle residue is Asp in each case. This is likely to enhance the negative potential of each of these calcium binding sites and thereby enhance the attractiveness to ultra-basic lysines on potential ligands. Indeed, it has been shown that this middle aspartate is required for RAP binding to CR tandem repeats from LRP (43). It is, therefore, intriguing that in clusters 2 and 4, where most ligand binding has been mapped, an acidic residue is also found at that position in 7 of 8 and 8 of 11 repeats, respectively, whereas it is found only in 4 of 10 repeats in cluster 3, and 1 of the 2 CR domains in cluster 1. In turn, this raises the possibility that such charge-charge interactions are necessary and sufficient for ligand binding and would consequently result in multiple ligand binding sites within a given cluster based solely on the distribution of DXXXD pentapeptides. However, we have shown in the present study that such patterns, although important, are not the sole determinant, since CR3-4-5 binds with 30-fold lower \( K_d \) than CR5-6-7 even though each of the three CR domains in each fragment have the DXXXD motif. Cluster 2, thus, has a high affinity binding site for \( \alpha_M \) RBD that must be composed of both charge-charge and other interactions that make the CR3-4-5 combination unique for this ligand within this cluster.

More generally, such a scheme for generating a high affinity ligand binding site on LRP and other LDLR family members may account for the ability of these receptors to bind with high affinity and specificity to many structurally unrelated ligands. We had previously suggested that the unique surfaces of CR domains allowed for a “mix-and-match” creation of multiple high affinity binding sites for different ligands (8). In light of the present findings and those of Fisher et al. (34), this model should be modified so that a necessary interaction, which can be met by most CR domains, between acidic residues around a CR domain Ca\(^{2+}\) binding site and a hot-spot of basic residues on the ligand, can be augmented in a ligand-specific way by additional interactions that take advantage of the myriad combinations of surface residues available in a group of 2–4 CR domains. This could then give high affinity binding sites to multiple ligands, each with unique specificity. Indeed use of such short CR domain clusters to engineer artificial high affinity, high selectivity binding sites has recently been proposed (44, 45).

In conclusion, we have shown here that CR3-4-5 constitutes the binding site for RBD on LRP. Although \( \alpha_M \) binding has been mapped to both clusters 2 and 4, the similarity between the affinity of RBD for CR3-4-5 and LRP precludes the site in cluster 4 being tighter. Within cluster 2, the CR3-4-5 site is specific, with a \( K_d \) 30-fold tighter than to CR5-6-7. We have shown that the interaction between RBD and CR3-4-5, like that of RBD or \( \alpha_M \) with intact LRP, relies on lysines at positions 1370 and 1374. The interaction between RBD and CR3-4-5 does not weaken in the pH interval 7.4–5.5, suggesting that one of the YWTD-propeller domains located between clusters 1 and 2 is indeed involved in displacing bound \( \alpha_M \) from CR3-4-5 in the endosome.

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α2M RBD Binding to CR3-4-5

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