The Second and Fourth Cluster of Class A Cysteine-rich Repeats of the Low Density Lipoprotein Receptor-related Protein Share Ligand-binding Properties*

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The low density lipoprotein receptor-related protein (LRP) is a multifunctional endocytic cell-surface receptor that binds and internalizes a diverse array of ligands. The receptor contains four putative ligand-binding domains, generally referred to as clusters I, II, III, and IV. In this study, soluble recombinant receptor fragments, representing each of the four individual clusters, were used to map the binding sites of a set of structurally and functionally distinct ligands. Using surface plasmon resonance, we studied the binding of these fragments to methylamine-activated α2-macroglobulin, pro-urokinase-type plasminogen activator, tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor-1, t-PA-plasminogen activator inhibitor-1 complexes, lipoprotein lipase, apolipoprotein E, tissue factor pathway inhibitor, lactoferrin, the light chain of blood coagulation factor VIII, and the intracellular chaperone receptor-associated protein (RAP). No binding of the cluster I fragment to any of the tested ligands was observed. The cluster III fragment only bound to the anti-LRP monoclonal antibody α2M RO3 and weakly to RAP. Except for t-PA, we found that each of the ligands tested binds both to cluster II and to cluster IV. The affinity rate constants of ligand binding to clusters II and IV and to LRP were measured, showing that clusters II and IV display only minor differences in ligand-binding kinetics. Furthermore, we demonstrate that the subdomains C3–C7 of cluster II are essential for binding of ligands and that this segment partially overlaps with a RAP-binding site on cluster II. Finally, we show that one RAP molecule can bind to different clusters simultaneously, supporting a model in which RAP binding to LRP induces a conformational change in the receptor that is incompatible with ligand binding.

The low density lipoprotein receptor-related protein (LRP)†

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that, although there are small differences concerning the kinetics of the interactions, clusters II and IV are highly similar in their ligand-binding properties, revealing a major functional duplication in the receptor.

Several studies have shown that RAP facilitates the proper folding and subsequent trafficking of LRP within the early secretory pathway (8, 11–14). RAP inhibits the binding of all ligands to the receptor and is thought to prevent premature binding of ligands to the receptor during the trafficking to the cell surface (15–17). Some ligands for LRP occupy distinct binding sites (9, 18) while other ligands compete with each other for binding to LRP (19–22). Although RAP competes with all known ligands, most ligands are not able to compete for RAP binding to LRP. Examples of RAP competitors include lactoferrin and lipoprotein lipase (23, 24). Other ligands, such as t-PA, α2-macroglobulin (α2M), and very low density lipoprotein, do not block the binding of RAP to LRP (23). It is as yet unclear how RAP is capable of antagonizing all ligand binding to LRP. In a previous study we have shown that, even though the affinity of RAP for cluster II and LRP is similar, RAP is a poor inhibitor of ligand binding to the isolated cluster II compared with its inhibition of ligand binding to the intact LRP (9). This suggests that RAP does not inhibit ligand binding solely in a competitive or sterical manner. The current study provides further evidence for a model of inhibition of ligand binding by RAP, involving a RAP-conformational change in the LRP molecule.

**EXPERIMENTAL PROCEDURES**

**Materials—** Oligonucleotides were from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). Restriction enzymes and DNA-modifying enzymes were from Life Technologies, Inc. (Breda, The Netherlands). Other chemicals used were reagent grade from Sigma or Merck (Darmstadt, Germany).

**Proteins—** Purified human LRP and the anti-LRP monoclonal antibody αM3 were kindly provided by Dr. S. K. Moestrup (Institute of Medical Biochemistry, University of Aarhus, Aarhus, Denmark). Two-chain t-PA was from Biopool (Umeå, Sweden). Procedures to purify active PAI-1 were essentially as described (25, 26). t-PA/PAI-1 complex was prepared as described, and the inhibitor was a generous gift from Dr. L. Aarden (Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands). Human apoE, human lactoferrin and recombinant glutathione S-transferase-fused RAP (GST-RAP) were kindly provided by Dr. J. Kuiper (Sylvius Laboratory, University of Leiden, Leiden, The Netherlands). Since the GST tag does not interfere with binding properties of RAP (27), GST-RAP was used throughout the present study and is referred to as RAP. Isolation of the monoclonal antibody Fab fragment Fab ASP as well as the isolation of the monoclonal antibody CLB-Cag 69, directed against a peptide derived from the human coagulation factor VIII (VIII), were essentially as described (28, 29). Mouse pro-u-PA was a gift from Dr. J. Henkin (Abbot Laboratories, Abbott Park, IL). Bovine LpL was purified from milk and biotinylated as described (30, 31). FVIII light chain was kindly provided by Dr. P. Lenting (Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands). Native human αs-macroglobulin was a kind gift from Dr. W. Boers (Academic Medical Center, Amsterdam, The Netherlands). Native human αs-macroglobulin was a kind gift from Dr. W. Boers (Academic Medical Center, Amsterdam, The Netherlands). Native human αs-macroglobulin was a kind gift from Dr. W. Boers (Academic Medical Center, Amsterdam, The Netherlands). Native human αs-macroglobulin was a kind gift from Dr. W. Boers (Academic Medical Center, Amsterdam, The Netherlands). Native human αs-macroglobulin was a kind gift from Dr. W. Boers (Academic Medical Center, Amsterdam, The Netherlands). Native human αs-macroglobulin was a kind gift from Dr. W. Boers (Academic Medical Center, Amsterdam, The Netherlands). Native human αs-macroglobulin was a kind gift from Dr. W. Boers (Academic Medical Center, Amsterdam, The Netherlands).

**Expression and Purification of Recombinant Receptor Fragments—** The expression and purification of recombinant LRP cluster II and fragment preparations were performed as essentially described (9). Purification of clusters I and III from conditioned media was done by affinity chromatography, using Sepharose-coupled monoclonal antibody CLB-Cag 69. After binding, columns were washed with HEPES-buffered saline (HBS, 20 mM HEPES (pH 7.4), 150 mM NaCl) and eluted with HBS, containing 1 mM NaCl. To further purify the cluster I and III fragments, an FPLC Superox 12 gel filtration column was used. The cluster IV fragment was purified by a single affinity-purification step, using a RAP-Sepharose column. This column was washed with HBS and eluted with HBS containing 10 mM EDTA. All purified cluster preparations were concentrated in HEPES-buffered 1 mM NaCl in Centricron 10 or 30 concentrators (Amicon, Beverly, MA) by successive rounds of centrifugation in a Sorvall high speed centrifuge for 1 h at 4 °C at a speed of 7000 rpm. Finally, the preparations were dialyzed against filtered and degassed, modified HBST buffer (containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.005% (v/v) sodium azide, 0.005% (v/v) TWEEN 80). All cluster fragment preparations were analyzed by non-reducing (12.5% (w/v)) SDS-polyacrylamide gel electrophoresis and subsequent silver staining. Typical yields ranged from 100 to 800 μg/liter of conditioned media, depending on the particular fragment and purification method.

**Concentration Determination of Proteins—** All protein concentrations were determined, using a microBCA protein assay reagent kit with BSA (BioRad, Hercules, CA) as standard. The concentrations of cluster II and IV fragments were determined, using surface plasmon resonance (SPR) as described previously (9).

**Mapping of Ligand Binding to Soluble Recombinant Receptor Fragments Using SPR—** To determine the ligand-binding characteristics of the different recombinant receptor fragments, all ligands were immobilized at high density on CM5 sensor chips. Whereas LpL was biotinylated and bound to a streptavidin-coated SA5 sensor chip, the anti-LRP monoclonal Fab fragment A8 and the anti-LRP monoclonal αM3 were bound to biotinylated rat anti-mouse kapa light chain monoclonal antibody (CLB Products, Amsterdam, The Netherlands), which was bound to a SA5 sensor chip. Measurements were performed at 25 °C at a flow rate of 5 μl/min. Concentrations of the different immobilized receptor fragments, which were passsed over the immobilized ligands, varied from nanomolar to micromolar range.

**Kinetic Determinations Using SPR—** Purified human LRP was immobilized on a CM5 sensor chip by amine coupling at a low density of approximately 16 fmol/mm² to determine kinetics of ligand binding to LRP. Interactions between recombinant receptor fragments and ligands were measured as follows: 0.25 μg/cm² PA (18 fmol/mm²), VIII light chain (155 fmol/mm²), t-PA/PAI-1 (37 fmol/mm²), LpL (63 fmol/mm²), and RAP (8 fmol/mm²) were immobilized to the sensor chip and different concentrations of receptor fragments were passed over the sensor chip. The different ligands or receptor fragments were passed over three separate channels with immobilized LRP or ligand, respectively, and one control (non-immobilized) channel at 25 °C at a flow rate of 20 μl/min, using modified HBST as running buffer. Each determination...
was performed at least in duplicate at different concentrations \((n = 5)\) in the appropriate concentration range (around \(K_d\) values). The BIA-evaluation software (Biacore AB, Uppsala, Sweden) was used for analysis of the association and dissociation profiles of the sensorgrams. Interaction constants were determined by performing non-linear fitting of data, corrected for bulk refractive index changes, according to a one- or a two-site model employing previously described equations (9). Data were fitted to a two-site model if a one-site model proved inadequate as judged from residual plots and statistical parameters (data not shown). The data were validated by subjecting them to tests of self-consistency (36).

**Analysis of Cluster II or IV Binding to RAP Which is Bound to LRP, Cluster II, or Cluster IV**—First, 100 ng of LRP, cluster II, or cluster IV was immobilized for 16 h at 4 °C in microwells in 50 mM NaHCO\(_3\) (pH 8.6) in a volume of 50 μl. Second, wells were blocked for 1 h at 37 °C with 3% (w/v) BSA in modified HBST buffer in a volume of 300 μl, washed with modified HBST buffer, and incubated with 100 nM RAP in modified HBST buffer. Next, wells were washed and incubated for 1 h at 37 °C in modified HBST buffer with a range of biotinylated cluster II or IV concentrations. Proteins were biotinylated using an EZ-Link\textsuperscript{TM} sulfo-NHS-LC-biotinylation kit, following the instructions of the supplier (Pierce). Bound proteins were detected using streptavidin-horse-radish peroxidase (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) as described (28). Experiments were performed in duplicate. As controls, cluster II or IV binding to RAP bound to BSA and direct cluster II or IV binding to immobilized BSA, LRP, cluster II, or cluster IV was measured.

**RESULTS**

**Expression and Purification of Soluble Recombinant Receptor Fragments**—To investigate the ligand-binding properties of all four putative ligand binding domains of LRP, we expressed soluble recombinant receptor fragments, comprising clusters I, II, III, and IV (Fig. 1), in transfected baby hamster kidney cells. Based on the findings that clusters II and IV fragments strongly bind to RAP (6–8), these fragments were purified by one-step affinity chromatography with RAP coupled to Sepharose. Affinity chromatography with Sepharose coupled to the monoclonal antibody CLB-Cag 69, directed against a tag was used to purify the cluster I and III fragments containing this particular tag. Most of the latter fragments consists of aggregated, SDS-resistant, high molecular weight material. To remove high molecular weight protein from the monomeric receptor fragment, gel filtration was performed using an FPLC Superose 12 column. The purified, monomeric preparations of clusters I, II, III, and IV were analyzed by non-reducing SDS gel electrophoresis (Fig. 2). Heterogeneity of these monomeric preparations is due to a different degree of N-linked glycosylation, as we have reported before (9). Furthermore, the mobility of the fragments is somewhat slower than would be expected on the basis of the calculated molecular masses (approximately 12.1, 44.2, 48.7, and 52.6 kDa for clusters I, II, III, and IV, respectively), consistent with variable N-linked glycosylation.

**Mapping of Ligand Binding to Soluble Recombinant Receptor Fragments Using SPR**—To examine the ligand-binding characteristics of the four receptor fragments, we tested the binding of the following ligands: t-PA, PAI-1, t-PA-PAI-1 complexes, pro-u-PA, α\(_2\)M, RAP, tissue factor pathway inhibitor, apoE, lactoferrin, LpL, and fVIII light chain. In addition, we assayed the binding of an anti-LRP Fab fragment and an anti-LRP monoclonal antibody, denoted A8 and α\(_2\)MRe3, respectively. Each ligand was immobilized at high density on a sensor chip, and receptor fragments were passed over the sensor chip surface at concentrations varying from nanomolar to micromolar range (Table I). Clearly, we did not observe binding of cluster I to any of the tested ligands. However, when the monoclonal antibody CLB-Cag 69 was immobilized on a sensor chip, which is directed against the tag present in the recombinant cluster I fragment, binding of cluster I was observed when the fragment was passed over the antibody (not shown). This indicates that, although the cluster I fragment is recognized by the tag-binding antibody, it does not contain ligand-binding properties. Cluster III binds to the anti-LRP monoclonal antibody α\(_2\)MRe3 and to RAP, provided the latter was immobilized in very high density, conditions that are indicative for a weak interaction. Surprisingly, cluster IV binds to an identical repertoire of ligands as cluster II, except for the Fab fragment A8,

**Fig. 1.** Recombinant receptor fragments used in the ligand mapping studies. Schematic overview of the domain structure of LRP, containing clusters I, II, III, and IV. Enlargements of the four clusters have been drawn separately. The symbols for the various subdomains are indicated in a separate inset (top right) as are the previously described (9) cluster II subfragments (top left). The presence of the tag sequence that facilitates detection and purification is indicated as well as the epidermal growth factor repeat (E) and the LDLRA repeats (C) within certain fragments.
TABLE I
Ligand binding to cluster fragments of LRP

| Ligand        | Cluster I | Cluster II | Cluster III | Cluster IV |
|---------------|-----------|------------|-------------|------------|
| t-PA · PAI-1  | –         | +          | –           | +          |
| t-PA          | –         | –          | –           | –          |
| PAI-1         | –         | +          | –           | +          |
| RAP           | –         | +          | +           | –          |
| Pro-u-PA      | –         | +          | –           | –          |
| α₂M           | –         | –          | –           | –          |
| TFPI          | –         | +          | +           | –          |
| ApoE          | –         | –          | +           | –          |
| Lactoferrin   | –         | +          | –           | +          |
| LpL           | –         | +          | –           | +          |
| fVIII l.c.    | –         | –          | +           | –          |
| Anti-LRP      | –         | +          | –           | –          |
| Anti-LRP mA b | –         | +          | –           | –          |

which is apparently specifically raised against cluster II. Collectively, these observations strongly indicate that LRP consists of duplicated domains, of which cluster II and IV have fully retained their functional properties.

Kinetics of Ligand Binding to LRP, Cluster II, and Cluster IV—To further characterize the ligand-binding properties of clusters II and IV, we determined the rate constants for the binding of pro-u-PA, t-PA-PAI-1 complexes, LpL, RAP and fVIII light chains to LRP, clusters II and IV (Table I). LRP was immobilized at low density on a sensor chip to determine the kinetics of ligand binding to the native LRP molecule. The resulting binding curves were fitted according to a two-site model if a single-site model did not adequately describe the interaction (Table II). The kinetics of LpL binding to LRP could not be accurately determined due to a high degree of nonspecific binding of LpL to the sensor chip. Pro-u-PA binds to LRP according to a two-site model with similar median affinities of 63.8 and 54.3 nM, respectively. The interaction of t-PA-PAI-1 complexes could be accurately described by a single-site model, yielding a KD value of 9.2 nM. RAP binds to high and median affinity sites on LRP with KD values of 2.2 and 34.9 nM, respectively, and fVIII light chain binds to LRP with median and low affinities of 52.1 and 130.3 nM, respectively. To determine the kinetics of ligand binding to clusters II and IV, ligands were immobilized at low density on sensor chips. As shown in Table II, pro-u-PA displays a slightly higher affinity for cluster II (33.3 nM) than for cluster IV (83.3 nM). This is also concluded for t-PA-PAI-1 complexes that bind to clusters II and IV with KD values of 16.7 and 48.8 nM, respectively. LpL binds with comparable affinities to both clusters (cluster II; 32.6 nM, cluster IV; 21.9 nM). A similar conclusion can be drawn for RAP (cluster II; 12.7 nM, cluster IV; 18.0 nM). The light chain of fVIII binds to clusters II and IV with KD values of 121.4 and 87.8 nM, respectively. From the quantitative data presented in Table II, it can be concluded that clusters II and IV are highly similar with respect to their ligand-binding properties, further substantiating the concept of a major functional duplication in LRP.

Mapping of Ligand Binding to Subfragments of Cluster II—To investigate whether the ligands occupy distinct binding sites or whether they bind to the same segment of cluster II, we determined the binding of α₂M, pro-u-PA, LpL, and factor VIII light chain to recombinant subfragments of cluster II (see Fig. 1) by SPR. The data in Table III shows that only binding of fragment CL-II-1/2 to the tested ligands could be detected, indicating that these ligands bind to a fragment of cluster II spanning the amino-terminal flanking epidermal growth factor repeat E4 and LDLRA domains C3–C7. The same fragment has been reported to bind PAI-1 and t-PA-PAI-1 complexes and contains a RAP-binding site on C5–C7 (9). Only the anti-LRP monoclonal Fab A8 was shown to bind to a part of cluster II (C8–C10) that is distinct from the RAP-binding site (9). In conclusion, each of the tested ligands bind to a region on cluster II that partially overlaps with a RAP-binding site, except for monoclonal A8.

RAP Can Bind to Two Receptor Fragments Simultaneously—The findings presented in the previous paragraph suggest that RAP inhibits ligand binding by direct competition or steric hindrance of each of the LRP ligands. However, previous results (9) show that it is unlikely that RAP regulates ligand binding solely by a mechanism involving mutually exclusive binding to overlapping binding sites. Instead, those results favor a model in which one molecule of RAP would induce a conformational change in the receptor by interacting with multiple receptor domains simultaneously. This conformational change would render the receptor incapable of ligand binding. This model would imply that one RAP molecule should be capable of binding multiple clusters simultaneously. To test this hypothesis, we used an enzyme-linked immunoassay to measure the binding of increasing concentrations of either cluster II or cluster IV to RAP, which, on its turn, was bound to either LRP, cluster II, or cluster IV (Fig. 3). From the data, we can conclude that RAP is able to bind simultaneously to clusters II and IV. Furthermore, RAP is also able to bind to two clusters II or two clusters IV and both clusters II and IV can bind to RAP bound to LRP, suggesting that RAP is capable of forming an intermolecular bridge between LRP molecules. In addition, half-maximal saturation of cluster II binding to RAP, which is bound to LRP, cluster II, or cluster IV, is obtained at virtually identical concentrations, namely at 1.4, 1.7, or 1.4 nM, respectively. Finally, it can be deduced that also nearly identical values are found for cluster IV binding to RAP, bound to LRP, cluster II, or cluster IV, namely 1.8, 2.5, or 4.3 nM, respectively. Apparently, one RAP molecule can bind two clusters simultaneously with comparable affinities and might even be able to bind to multiple LRP molecules.

DISCUSSION

We have generated a set of recombinant LRP fragments, comprising the four putative ligand-binding domains, generally referred to as clusters I, II, III, and IV, to study the structure and function of this universal clearance receptor. Until now, a systematic investigation on the properties of the individual clusters is lacking, since predominantly reports on ligand binding to cluster II have been published (6–10). We demonstrate that each of the ligands tested binds with similar affinity to both cluster II and cluster IV. Furthermore, in both cases binding of these ligands is calcium-dependent (data not shown). These observations suggest that LRP contains two duplicated domains that have fully retained their functional properties. In a study by Willnow et al. (7), using clusters II and IV LRP-mimic receptors, binding of t-PA-PAI-1 and u-PA-PAI-1 complexes to cluster II could be detected, but not to cluster IV. Moreover, in contrast to our findings, no binding of α₂M to either one of the minireceptors could be detected. These discrepancies might be due to a different composition of the constructs employed or to a different experimental design to measure ligand binding. Furthermore, it is relevant to mention that we do not observe binding of t-PA to any of the LRP cluster fragments, despite the fact that t-PA binds with a relatively
Ligands were immobilized at low densities on sensor chips to determine the rate constants of cluster II and IV binding. LRP was immobilized at low density on a sensor chip to determine the rate constants of ligand binding. The $k_{on}$ values are in $M^{-1} s^{-1}$, and $k_{off}$ values are in $s^{-1}$. Data represent the means ± S.E. of six experiments. The term ND means that no accurate rate constants could be determined due to a high degree of nonspecific binding of LpL to the sensorchip.

### Table II

| Ligand | LRP | Cluster II | Cluster IV |
|--------|-----|------------|------------|
| pro-u-PA | $k_{on} = (4.4 \pm 0.2) \times 10^{-2}$ | $k_{on} = (1.9 \pm 0.1) \times 10^{-2}$ | $k_{off} = (2.5 \pm 0.2) \times 10^{-2}$ |
|         | $k_{on} = (6.9 \pm 0.2) \times 10^{-2}$ | $k_{on} = (5.7 \pm 1.8) \times 10^{5}$ | $k_{off} = (3.0 \pm 0.3) \times 10^{3}$ |
|         | $K_{D} = 63.8 \pm 3.4 \text{ nm}$ | $K_{D} = 33.3 \pm 10.7 \text{ nm}$ | $K_{D} = 83.3 \pm 10.7 \text{ nm}$ |
| t-PA ‐ PAI-1 | $k_{on} = (2.4 \pm 0.3) \times 10^{-3}$ | $k_{on} = (2.0 \pm 0.2) \times 10^{-3}$ | $k_{off} = (3.8 \pm 0.3) \times 10^{-3}$ |
| LpL | $k_{on} = (2.6 \pm 0.1) \times 10^{6}$ | $k_{on} = (1.2 \pm 0.1) \times 10^{6}$ | $k_{off} = (7.8 \pm 0.6) \times 10^{6}$ |
| RAP | $k_{on} = (2.4 \pm 0.3) \times 10^{8}$ | $k_{on} = (6.7 \pm 2.2) \times 10^{6}$ | $k_{off} = (48.8 \pm 5.9) \times 10^{6}$ |
| fVIII light chain | $k_{on} = (1.3 \pm 0.1) \times 10^{10}$ | $k_{on} = (7.5 \pm 1.1) \times 10^{-3}$ | $k_{off} = (3.5 \pm 0.5) \times 10^{-3}$ |

### Table III

| Ligand | CI-II-1 | CI-II-2 | CI-II-3 | CI-II-1/2 | CI-II-2/3 |
|--------|---------|---------|---------|-----------|-----------|
| RAPa   | –       | +       | –       | +         | +         |
| t-PA ‐ PAI-1a | –       | –       | –       | +         | +         |
| PAI-1b  | –       | –       | –       | +         | +         |
| Fab A8a | –       | –       | –       | +         | +         |
| α_M    | –       | –       | –       | +         | +         |
| Pro-u-PA | –       | –       | –       | +         | +         |
| LpL    | –       | –       | –       | +         | +         |
| fVIII light chain | –       | –       | –       | +         | +         |

*a* These data were published previously (9).

low affinity to intact LRP. Since the separate cluster fragments were designed to maintain the integrity of the LDLRA repeats, and not that of other subdomains, we tentatively conclude that the LDLRA repeats do not contribute to the t-PA-binding site of LRP.

Although we observe in this study that t-PA‐PAI‐1 complexes can bind both to the separate cluster II and IV, the interactions of these complexes with LRP could be most accurately described by a single-site model. Consequently, a $K_D$ value for LRP is derived that corresponds to a higher affinity than those of the separate clusters II and IV. In the case of RAP, the $K_D$ values for the ligand interactions with the fragments are not in accordance with those for the intact LRP molecule. This may be explained by the fact that the data for RAP binding to LRP were fitted according to a two-site model, whereas we observed that RAP binds, next to cluster II and IV, also weakly to cluster III, suggesting a three-site model. Furthermore, according to the RAP inhibition model in Fig. 4, the kinetics of RAP binding to LRP is even more complex than a three-site model. Nevertheless, the interactions can be faithfully fitted by a two-site model, apparently since the algorithm employed contains sufficient parameters to describe these interactions.

To further delineate the ligand-binding sites on cluster II, we mapped the binding of ligands to subdomains of cluster II. Clearly, each of the ligands tested binds to the same region of cluster II, spanning epidermal growth factor repeat E4 and LDLRA domains C3–C7. RAP binds to a fragment comprising the LDLRA domains C5–C7 and the anti-LRP monoclonal Fab fragment A8 to the domains C8–C10 (9). Recently, Vash et al. (10) reported on the binding of RAP, lactoferrin, and PAI-1 to soluble LRP subfragments of cluster II. These investigators showed binding of these ligands to LDLRA repeats C5–C7 and concluded that the lactoferrin-binding site extends beyond C5–C7. With respect to RAP, our results agree well with those of Vash et al., since in both studies C5–C7 has been identified as the binding site. However, we did not observe PAI-1 binding to C5–C7 alone, but concluded that the PAI-1-binding site only partially overlaps the RAP-binding site on cluster II and extends toward the N terminus. Possible reasons for this discrepancy might be due to differences in immobilization, tag position, and the degree of glycosylation. It is of note to mention that Orlando et al. (37) showed that the multi-ligand endocytic receptor megalin (gp330), which is closely related to LRP, also contains a binding site for apoE-β-migrating very low density lipoprotein, LpL, aprotinin, lactoferrin, and RAP spanning the fourth and fifth LDLRA repeats of cluster II of megalin. These repeats are homologous to the C6 and C7 subdomains within LRP. In addition, and similar to LRP, megalin appears to have more than one RAP-binding site. From the current and above mentioned studies, it can be deduced that a limited number of LDLRA repeats is apparently sufficient to provide specific binding of a diverse array of ligands to LRP and to other members of the LDL receptor family.

Two models have been proposed for the inhibition of ligand binding to LRP by RAP. The first model assumes a close spatial association of a RAP-binding site to each of the independent LRP ligand-binding sites (38). Consequently, RAP would inhibit ligand binding by direct competition or steric hindrance of each of the LRP ligands. This concept is supported by findings reported here, notably that the RAP-binding site on cluster II is adjacent to and partially overlaps with an important ligand
binding domain. On the other hand, we have shown that RAP and the anti-LRP Fab A8 each have their own distinct binding site on cluster II, consistent with the observation that cross-competition on the isolated cluster II domain could not be shown for these molecules (9). However, RAP efficiently competes for binding of Fab A8 to intact LRP, whereas the reverse could not be demonstrated (9), despite the fact that the affinity of RAP and A8 for LRP is similar. Collectively, these observations render the option unlikely that RAP regulates ligand binding solely by a mechanism that involves mutually exclusive binding to overlapping binding sites. Instead, these findings would favor a RAP inhibition model that requires LRP moieties that are located outside the cluster II of the receptor. Indeed, an alternative model for the regulation of ligand binding by RAP proposes a RAP-induced conformational change in the LRP molecule (9, 18, 39). It was demonstrated that both LRP and RAP harbor multiple interaction sites for their mutual interaction (7, 8, 13). Based on analysis of the primary structure, it was revealed that RAP contains an internal triplication of structural autonomous domains comprising residues 1–100 (D1), 101–200 (D2), and 201–323 (D3) (13, 40). When expressed individually, each of the single domains of human RAP maintains its functional integrity and binds to LRP (13, 40). D1 binds to cluster II, D2 to cluster IV, and D3 to all three RAP binding cluster domains (II, III, and IV) (13). Based on these reported multiple binding sites on RAP and LRP, one may envision a model in which one molecule of RAP can induce a conformational change in the receptor by interacting simultaneously with multiple receptor domains (Fig. 4B). This conformational change would render the receptor incapable of ligand binding. We show in this study that a single RAP protein can simultaneously bind to clusters II and IV with comparable affinities, an observation that is in accordance with the latter model. It should be noted, however, that these experiments were performed with separate cluster fragments and do not strictly prove that these events occur within an intact LRP molecule. Superficially, the adaptation of a different shape of LRP has been suggested by electron microscopic experiments, indicating that LRP may display considerable structural variability (41). In aggregate, our data support aspects of both models and therefore it cannot be excluded that RAP inhibition of ligand binding involves both competition or sterical hindrance and a conformational change.

In conclusion, we have shown that LRP contains ligand-binding sites on cluster II and IV that are very similar with regard to ligand-binding properties, suggesting a functional duplication within the receptor. Finally, we have provided data on the mechanism of the complex interactions between ligands and the intracellular chaperone RAP and their receptor LRP.

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