Localization of the low-density lipoprotein receptor-related protein regions involved in binding to the A2 domain of coagulation factor VIII

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Summary

Catabolism of coagulation factor VIII (FVIII) is mediated by low-density lipoprotein receptor-related protein (LRP). The ligand-binding sites of LRP are formed by complement-type repeats (CR), and CR clusters II and IV bind most of LRP ligands. FVIII contains two major LRP-binding sites located in the A2 and A3 domains. This study was aimed to identify specific complement-type repeats of LRP involved in interaction with the A2 site and to probe their functional importance in A2 catabolism. We generated individual LRP clusters II, III and IV, along with nine overlapping CR triplets encompassing clusters II and IV in a baculovirus expression system and studied their interaction with isolated A2. In surface plasmon resonance (SPR) assay, A2 bound to clusters II and IV with K_Ds 22 and 39 nM, respectively, and to the majority of CR triplets with affinities in the range of K_Ds 25–90 nM. Similar affinities were determined for A2 interaction with a panel of CR doublets overlapping cluster II (CR 3–4, 4–5, 5–6 6–7 and 7–8). These LRP fragments inhibited the binding of 125I-A2 to LRP in solid-phase assay, LRP-mediated internalization of 125I-A2 in cell culture and 125I-A2 clearance from the mouse circulation. Point mutations of critical A2 residues of the LRP-binding site resulted in differential reduction or abolishment of its binding to LRP fragments. We conclude that A2 interacts with LRP via multiple binding sites spanning CR 3–8 in cluster II and CR 23–29 in cluster IV, and the minimal A2-binding unit of LRP is formed by two adjacent CR.

Keywords
Coagulation factor VIII, low-density lipoprotein receptor-related protein

Introduction

Factor VIII (FVIII) plays an essential role in reactions of blood coagulation, and its deficiency leads to a coagulation disorder, haemophilia A (1). FVIII consists of A- and C-type domains and the B domain arranged in a heterodimer with A1-A2-B portion (heavy chain (HCh), 90–220 kDa with variable in length B-domain) and A3-C1-C2 portion (light chain (LCh), 80 kDa). In blood, FVIII circulates in a tight complex with von Willebrand factor (VWF), and at sites of coagulation FVIII is activated by limited proteolysis into a heterotrimer A1/A2/A3-C1-C2. This event leads to dissociation of VWF and participation of activated FVIII (FVIIIa) in the intrinsic Xase complex, where it serves as a cofactor for FIXa (2).

Regulation of FVIII level in the circulation is mediated by low-density lipoprotein receptor-related protein (LRP), a hepatic scavenger receptor (3–6). LRP is a large (600 kDa) multifunctional receptor which belongs to the low-density lipoprotein receptor (LDLR) family of endocytic receptors. LRP is involved in clearance from the circulation of over 35 different ligands, including several coagulation factors (7). A 39 kDa receptor-associated protein (RAP), which is a folding chaperone for all members of the LDLR family, antagonizes the binding of most ligands (8, 9) and serves as a useful tool in studying ligand/recept-

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The role of LRP in clearance of FVIII from the circulation has been confirmed by several lines of experimental evidence. Administration of RAP in mice, either by co-injection with FVIII or by adenovirus-mediated over-expression of RAP, led to a 3.5-fold prolongation of FVIII half-life in the circulation (3, 5, 10) and to a comparable increase of intrinsic FVIII level (6) suggesting involvement of the LDLR family receptors. Direct inactivation of the LRP gene in cre/loxP-mediated conditional LRP-deficient mice resulted in ~two-fold increase in plasma FVIII level and ~two-fold prolongation of half-life of injected FVIII in the circulation (6, 11). In humans, polymorphism in LRP affects FVIII levels and activity in blood (12). It has been found that in clearance of FVIII, LRP cooperates with LDLR, as evidenced by ~four-fold increase of FVIII plasma level and ~five-fold prolongation of FVIII half-life in LRP/LDLR double-knockout mice (11).

The ligand-binding sites within the extracellular domain of LRP are formed by clusters of complement-type repeats (CRs) (13). Each CR is formed by approximately 40 amino acids and represents a compact structure enforced by three conserved internal disulfide bonds and stabilized by Ca\(^{2+}\), coordinated in an octahedral cage formed by conserved four acidic residues and two backbone carbonyl groups (14). The presence of Ca\(^{2+}\) in CR is required for maintaining their correct ligand-binding structure (15). LRP bears 31 CRs arranged in clusters I-IV which consist of 2, 8, 10 and 11 CRs, respectively (Fig. 1). Clusters II and IV were shown to be responsible for the binding of majority of LRP ligands, and for a number of ligands, their binding sites were mapped within cluster II (16). Noteworthy, RAP has multiple high-affinity binding sites within cluster II formed by at least a pair of adjacent CR, which contain conservative residues homologous to Trp\(^{1080}\) and Asp\(^{1085}\) coordinating Ca\(^{2+}\) via backbone chain carbonyl groups in CR 8.

![LRP molecule with clusters and expressed fragments](image)

Figure 1: Schematic representation of LRP, its clusters II, III and IV and 3xCR fragments overlapping clusters II and IV. LRP clusters and 3xCR fragments were generated using a baculovirus expression system. Inset: Analysis of purified LRP clusters II, III and IV and 3xCR fragments by SDS-PAGE (gradient 4–12%) under non-reducing conditions followed by Coomassie staining. The lane numbers on the gel correspond to numbers 1–12 of LRP fragments, assigned in their schematic representation.
(22), a low-affinity site is located within the C2 domain (4). In circulating FVIII/VWF complex, the A3 and C2 sites are blocked by VWF bound to LCh (4, 23) that is consistent with a shorter half-life of FVIII in patients with von Willebrand disease (24) and in VWF-deficient mice (5). Exposure of the sites in LCh is driven by dissociation of VWF upon proteolytic activation of FVIII (4). This event also leads to exposure of the high-affinity LRP-binding site within A2 which is not interactive with LRP within intact FVIII (25, 26). In turn, exposure of the high-affinity sites for LRP in FVIIIa suggests that clearance of LRP may be a specific mechanism for removal of the fragments of unstable FVIIIa, i.e. A2 and A1/A3-C1-C2, from the circulation.

Recently, we have identified residues within the A2 site, which are critical for A2 interaction with LRP (21). We also found that isolated A2, the product of dissociation of activated FVIII (27–29), can be cleared from the circulation via LRP. The present study was aimed to gain a deeper insight into the mechanisms of interaction of the A2 site with LRP through identification of specific complement-type repeats of LRP involved in this interaction and probing their functional importance in A2 catalysis. Using baculovirus expression system, we generated individual LRP clusters II, III and IV along with a set of overlapping the A2-interacting region(s) within LRP clusters II and IV. We tested interaction of these fragments with isolated A2, and revealed multiplicity of the A2-interacting region(s) within LRP clusters II and IV.

Materials and methods

Proteins and reagents

Plasma FVIII was purified from therapeutic concentrate Antihemophilic Factor (Human) (Baxter/Hyland Healthcare Inc. Glendale, CA, USA) as described (30). Plasma-derived A2 was prepared from thrombin-activated FVIII and purified by affinity chromatography using anti-A2 mAb8860 (31, 32). Recombinant A2 and its mutants were expressed in insect cells as previously reported (21). LRP was isolated from human placenta as published elsewhere (33). Recombinant RAP was expressed and purified as described (34); the glutathione S-transferase portion was cleaved off by thrombin. Cells producing anti-myc IgG 9E10 were obtained from ATCC (Rockville, MD, USA), and anti-myc mAb9E10 was purified by chromatography on protein G-Sepharose. Anti-RAP mAb7F1A8 was developed in Dr. Strickland’s laboratory (University of Maryland, Baltimore, MD, USA). Penta His HRP-conjugate was purchased from Qiagen Inc. (Valencia, CA, USA). Oligonucleotides were purchased from Sigma-Genosys (Woodlands, TX, USA).

Generation of expression vector

The plasmid pFastBac1/mhx-A2 (21) was cleaved by Neol and Sphl endonucleases, and the excised ~1.2kb fragment was replaced with a fragment obtained by annealing of oligonucleotides 5’CATGGGTCCAGATCGAAGGAGTCTCGAGCC-ACCCGGAGCAGAAAAAACCTGCAAACTGAGGATCTGAAFAGGTGAGCAGT and 5’CTCATCAGCTATCAATCTCCTTCTGAGTAGTTTTTCCTCGGGTGGTCTCGAGA-CTAGGTTCTAGTGGTGACC. Within this plasmid, pFastBac1-HM (Genbank Accession NumberAY598466), the melittin/6xHis tag coding region was followed by Neol-Sall-Xbal-Spel-Xhol-Sphl-polycloning site and by 12-amino acid epitope of myc-tag.

Generation of LRP fragments

The coding regions of LRP fragments were generated by PCR using cdNA of human LRP (35) (Genbank Accession NumberX13916) and pairs of corresponding primers which introduced Ncol and Xhol sites into the PCR-fragments to insert them into the pFastBac1-HM vector. The generated expression cassettes, encoding the fragments (Table 1) with fused tags, poly-His-tag at the N-terminus and myc-tags at the C-terminus, were sequenced, transposed into bacmids and expressed according to the Bac-to-Bac expression system protocol (Invitrogen, Carlsbad, CA, USA).

Expression and purification of LRP fragments

In the obtained recombinat baculovirus stocks, the presence of secreted LRP fragments was confirmed by PAGE-Western blot using either Penta His HRP-conjugate or anti-myc-tag mAb7F1A8. Optimization of expression of LRP fragments was performed according to our previous protocol (36). The proteins were purified from the culture media by affinity chromatography on Talon Superflow resin (BD Biosciences, Palo Alto, CA, USA) using 20 mM HEPES pH 7.4/0.15 M NaCl/0.005% Tween-20 buffer (HBS) containing 2 mM CaCl2 (HBS-Ca buffer) with 0.3 M NaCl/150 mM imidazol for elution as described (21). LRP clusters II-IV and their 3xCR fragments were further purified by size-exclusion chromatography on Superdex-200 and Superdex-75 columns, respectively (Amersham Biosciences Corp, Piscataway, NJ, USA). The 2xCR doublets spanning LRP cluster II were expressed in Escherichia coli, purified on Talon Superflow resin column, refolded and affinity purified on RAP-Sepharose CL-6B column as described (17). The homogeneity of LRP fragments was verified by SDS-PAGE under non-reducing conditions.

Solid-phase binding assay for interaction of RAP with LRP fragments

Each LRP fragment (5 µg/ml) in HBS-Ca buffer was immobilized via poly-His tag in the wells of Ni-NTA HisSorb 96-well plates (Qiagen, Valencia, CA, USA) at 4°C for 12 hours (h) followed by blocking with 1% bovine serum albumin (BSA). In control experiment, the plates were coated with BSA only. The amount of each immobilized LRP fragment was determined using anti-myc mAb9E10 (1.5 µg/ml). Alternatively, LRP fragments were immobilized on regular 96-well plates via mAb9E10, and the quantification of each bound LRP fragment was performed using Penta- His antibody. RAP (at concentrations of 1–50 nM) was incubated with immobilized LRP fragments in HBS-Ca buffer for 1 h at 37°C followed by incubation with anti-RAP mAb7F1A8 and anti-mouse goat polyclonal antibody conjugated with alkaline phosphatase. Each binding step was performed in and preceded by washing the wells with HBS-Ca buffer. The amounts of bound RAP were determined using a substrate pNPP (Sigma, St. Louis, MO, USA) and recording the absorbance at 405 nM. The amounts of bound RAP were corrected for the background level and normalized according to relative amount of each immobilized LRP fragment. The fitting of the data and calculation of the affinities of RAP for LRP frag-
ments were performed using a one-site Langmuir binding model of SigmaPlot 8.0 program (SYSTAT Software, Point Richmond, CA, USA).

### Solid-phase competition assay for interaction of A2 with LRP

LRP (5 μg/ml) in HBS-Ca buffer was immobilized in the wells of 96-well plates at 4°C for 12 h that was followed by blocking with 1% BSA. In control experiment, the plates were coated with BSA only. Prior to the experiment, A2 was labeled with <sup>125</sup>I (Amersham Biosciences, Piscataway, NJ, USA) as previously described by us (37), typically yielding specific radioactivity of 5–15×10<sup>6</sup> cpm/μg of protein. The binding of <sup>125</sup>I-A2 (1 nM) to immobilized LRP was performed in HBS-Ca buffer in the presence of increasing concentrations of LRP fragments for 2 h at 37°C followed by washing and counting bound radioactivity as previously described (3, 21). These values were corrected for nonspecific binding in control BSA-coated wells. The data were fitted to a heterologous displacement model using SigmaPlot 8.0 program at a flow rate of 10 μl/minute (min). Dissociation of bound proteins was recorded after replacing ligand solution for the buffer. Regeneration of the chips was performed by washing with 0.1 M H<sub>3</sub>PO<sub>4</sub>. The kinetic parameters were derived by fitting the binding and dissociation curves with a 1:1 (Langmuir) model using a program BIAevaluation 3.1. In equilibrium binding studies, the binding signal at equilibrium (B<sub>e</sub>) achieved at increasing concentrations of A2 was plotted versus F, the concentration of unbound ligand. The K<sub>D</sub> values were derived from the best fit of the experimental data with Equation 1: B<sub>e</sub> = R<sub>max</sub>F/(K<sub>D</sub>+F), where R<sub>max</sub> is the maximal binding capacity of immobilized receptor surfaces expressed in resonance units. As under experimental conditions the concentration of bound A2 was significantly lower than the concentration of added A2, the concentration of unbound A2 (F) was assumed to be close to the concentration of added A2. The fitting was performed using SigmaPlot 8.0 program.

### Cell-mediated internalization of A2 in the presence of LRP fragments

LRP-expressing mouse embryonic fibroblasts (MEF cells) and the same cell line genetically deficient in LRP (PEA 13 cells) were obtained from Dr. J. Herz (University of Texas Southwestern Medical Center, Dallas, TX, USA) and maintained in DMEM/10% FBS as described (39). The cells were seeded in 12-well plates at a density of 1.5×10<sup>5</sup> cells/well, grown overnight to ~80% confluence and incubated with <sup>125</sup>I-A2 (10–80 nM) in medium containing 1% BSA, in the absence or presence of RAP (1 μM) or a selected LRP fragment (2 μM) at 37°C for 4 h. Inter-

### Table 1: Parameters of interaction of LRP clusters II, III and IV and overlapping CR triplets of clusters II and IV with A2 domain of FVIII.

The K<sub>D</sub> values for LRP fragments and RAP were assessed in ELISA-based experiment shown in Figure 2. The K<sub>D</sub> values for inhibition of A2 binding to immobilized LRP by its fragments were obtained from competition assay shown in Figure 3. The data were fitted to a heterologous displacement model. The K<sub>D</sub> values for the A2 binding to immobilized LRP fragments were derived from the data of SPR-based experiment shown in Figure 4 by fitting the families of kinetic curves to a one-site binding model. Each parameter represents the mean and SD of triplicate measurements.

<table>
<thead>
<tr>
<th>LRP fragment</th>
<th>Number of LRP residues</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; for RAP (nM)</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; for A2 (nM)</th>
<th>K&lt;sub&gt;off&lt;/sub&gt; for A2 (M&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;off&lt;/sub&gt; for A2 (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; for A2 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster II: 1. CR 3–10</td>
<td>831–1165</td>
<td>1.2 ± 0.1</td>
<td>31 ± 3</td>
<td>(3.3 ± 0.3)×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(7.3 ± 0.6)×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>2. CR 3–5</td>
<td>831–956</td>
<td>3.1 ± 0.6</td>
<td>33 ± 4</td>
<td>(3.5 ± 0.4)×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(8.8 ± 0.7)×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>3. CR 5–7</td>
<td>913–1042</td>
<td>0.6 ± 0.2</td>
<td>42 ± 5</td>
<td>(2.9 ± 0.3)×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(9.0 ± 0.8)×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>4. CR 7–9</td>
<td>994–1125</td>
<td>2.1 ± 0.3</td>
<td>65 ± 7</td>
<td>(1.8 ± 0.2)×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(8.1 ± 0.7)×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>5. CR 8–10</td>
<td>1033–1165</td>
<td>3.3 ± 0.5</td>
<td>ni&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nb</td>
<td>nb</td>
<td>nb</td>
</tr>
<tr>
<td>Cluster III: 6. CR 11–20</td>
<td>2501–2924</td>
<td>3.2 ± 0.7</td>
<td>ni</td>
<td>nb</td>
<td>nb</td>
<td>nb</td>
</tr>
<tr>
<td>Cluster IV: 7. CR 21–31</td>
<td>3312–3765</td>
<td>1.6 ± 0.2</td>
<td>68 ± 8</td>
<td>(1.1 ± 0.1)×10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>(4.3 ± 0.5)×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>39 ± 6</td>
</tr>
<tr>
<td>8. CR 21–23</td>
<td>3312–3433</td>
<td>2.1 ± 3.2</td>
<td>ni</td>
<td>nb</td>
<td>nb</td>
<td>nb</td>
</tr>
<tr>
<td>9. CR 23–25</td>
<td>3390–3516</td>
<td>3.4 ± 0.4</td>
<td>123 ± 15</td>
<td>(2.9 ± 0.2)×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(2.7 ± 0.3)×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>93 ± 10</td>
</tr>
<tr>
<td>10. CR 25–27</td>
<td>3471–3593</td>
<td>0.7 ± 0.1</td>
<td>71 ± 9</td>
<td>(1.1 ± 0.1)×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(4.5 ± 0.4)×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>11. CR 27–29</td>
<td>3552–3675</td>
<td>2.2 ± 0.3</td>
<td>81 ± 8</td>
<td>(8.0 ± 0.7)×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(3.9 ± 0.5)×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>49 ± 8</td>
</tr>
<tr>
<td>12. CR 29–31</td>
<td>3630–3765</td>
<td>nb</td>
<td>ni</td>
<td>nb</td>
<td>nb</td>
<td>nb</td>
</tr>
</tbody>
</table>

<sup>a</sup> no inhibition of binding (ni) or no binding (nb) was observed.
nalization of $^{125}$I-A2 was measured as described (40, 41). Briefly, after removal of surface-bound radioactivity by trypsin, the radioactivity which remained cell-associated was considered as internalized.

**Clearance of A2 from the circulation of mice in the presence of LRP fragments**

Twenty nM of $^{125}$I-A2 (alone or with 40 µM of selected LRP fragment after a 1 h pre-incubation at room temperature), or 40 µM of selected LRP fragment labeled with $^{125}$I similarly to A2 (37) were injected into the tail vein of BALB/c mice (12–14 weeks old, weight ~25 g), four mice in each group. Clearance of the radiolabeled proteins from the circulation was monitored as previously described (3, 37). Briefly, blood aliquots (~50 µl) were withdrawn via retro-orbital puncture at time intervals within a 1–180 min range, and the radioactivity of the samples was measured using a gamma-counter. The values were normalized to the sample volumes and expressed as a percentage of the initial count (sample taken at 1 min). The data were fitted using a double-exponential decay model for A2 clearance and single-exponential model for LRP fragments clearance using SigmaPlot 8.0 program as described (3, 6).

**Results**

**Expression of LRP fragments and testing them for binding with RAP**

LRP fragments (Fig. 1) were expressed in a baculovirus system, previously used to produce fragments of megalin (a receptor from the LDLR family) (42). The ligand-binding properties of generated LRP fragments were tested by their ability to interact with RAP in ELISA and SPR-based assays. In ELISA, each LRP fragment was immobilized via a tag (poly-His-tag or myc-tag) and tested for binding with increasing concentrations of RAP that revealed the same pattern of A2-binding fragments. As follows from Figure 2, RAP efficiently bound to LRP clusters II, III and IV, in agreement with the previous report (9). Furthermore, RAP efficiently bound to all CR triplets overlapping cluster II, consistent with the previous finding that the minimal binding site for RAP is formed by any pair of adjacent CR of cluster II, except for CR 9–10 (17). Within cluster IV, RAP efficiently bound to all CR triplets except for CR 21–23 (low binding) and CR 29–31 (no binding). In these triplets, two out of three CRs do not contain conserved amino acid residues, homologous to Trp1080 and Asp1085 of CR8, involved in coordinating Ca$^{2+}$ (Fig. 1); thus they are not able to form the minimal binding site for RAP. For the majority of LRP fragments, the $K_i$ values derived by fitting the ELISA data to a one-site binding model were within the range of 0.6–3.4 nM (Table 1). This is consistent with the reported affinities of RAP for LRP, its clusters II-IV, and for 2xCR fragments of LRP cluster II (1–5 nM) (8, 9, 17). In the SPR-based assay, we studied the binding of RAP to LRP fragments covalently immobilized onto the chip; the binding pattern and obtained $K_i$ values were close to those of ELISA (data not shown). Similarity of the RAP-binding patterns of LRP fragments for different immobilization strategies (via any of the tags or covalently) demonstrated that the tags presence did not affect the binding. Altogether, ELISA and SPR data confirmed normal ligand-binding properties of recombinant LRP fragments validating their use in further experiments.

**Binding of A2 to LRP in the presence of LRP fragments**

To determine which fragments of LRP contain binding sites for A2, we studied effects of LRP fragments on $^{125}$I-A2 binding to immobilized LRP in a solid-phase competition assay. As shown in Figure 3A, LRP clusters II and IV, but not cluster III, dose-dependently inhibited the A2 binding to LRP, indicating that clusters II and IV contain the A2-binding sites. The CR triplets 3–5, 5–7 and 7–9 of cluster II, but not 3xCR 8–10, were found to be similarly efficient competitors for the A2 binding (Fig. 3B). Within cluster IV, CR triplets 23–25, 25–27 and 27–29, but not 21–23 and 29–31, also inhibited the A2 binding to LRP (Fig. 3C) with similar $K_i$ values (Table 1). Altogether, these results suggest...
that the A2-binding sites are located within CR 3–8 and 23–29 of LRP clusters II and IV, respectively.

**Binding of A2 to LRP clusters and 3xCR fragments in SPR assay**

To verify the data obtained in the competition assay and to further characterize the kinetics of A2 interaction with LRP fragments, we studied the direct binding of A2 to covalently immobilized LRP fragments in an SPR-based assay. As follows from Figure 4, A2 bound to clusters II and IV and to their sub-fragments comprised of CR 3–5, 5–7, 7–9 and 23–25, 25–27, 27–29, respectively. In contrast, cluster III and CR 8–10, 21–23 and 29–31 of clusters II and IV did not bind A2. Kinetics of the A2 binding to the fragments were adequately fitted to a one-site model and the corresponding $K_D$ values (Table 1) were similar (in the range of 20–40 nM and 40–90 nM for cluster II and cluster IV fragments, respectively).

To verify the relevance of these $K_D$ values, we studied the equilibrium binding of A2 to representative CR triplets 3–5 and 25–27 of LRP clusters II and IV, respectively. These triplets were immobilized onto the chips at ~three-fold lower density than in the above experiment to ensure the binding equilibrium for A2 association at increasing concentrations (Fig. 5A and C). The equilibrium signals were plotted versus the A2 concentrations (Fig. 5B and D) and fitted with a one-site equilibrium binding model. The determined $K_D$s ($33 \pm 5$ nM for A2/CR 3–5 and $54 \pm 6$ nM for A2/CR 25–27 interaction) were in a good agreement with corresponding values obtained from A2 association/dissociation kinetics ($25 \pm 4$ and $41 \pm 5$ nM, respectively). This indicates that the binding parameters presented in Table 1 reflect real affinities of A2 for LRP fragments. Thus, SPR-based experiments (using immobilized LRP fragments) supported the data of the solid-phase competition assay (using LRP fragments in solution); both approaches demonstrated that the A2-binding sites...
on LRP are located within CR 3–8 of cluster II and CR 23–29 of cluster IV.

Identification of the minimal A2-binding unit using CR doublets spanning LRP cluster II
To narrow down the A2-binding region(s) within LRP cluster II, we tested interactions of A2 with a series of overlapping CR doublets comprising this cluster (Fig. 6). In SPR-based experiment with immobilized 2xCR modules, we found that A2 interacted with CRs 3–4, 4–5, 5–6, 6–7, and 7–8 with K_D values (Table 2) similar to those determined for CR triplets (Table 1).

The kinetic curves for three representative CR doublets (CR 3–4, 4–5 and 5–6) are shown in Figure 6A-C. In control experiment, pre-injection of RAP completely abolished the A2 binding to each of CR doublets (Fig. 6D-F) confirming specificity of these interactions. The finding that A2 interacts with the doublet CR 7–8, but does not interact with the triplet CR 8–10 or doublets CR 8–9 and CR 9–10, indicate that CR 9 and CR 10 are not involved in A2 binding. In a solid-phase competition experiment, CR doublets 3–4, 4–5, 5–6, 6–7 and 7–8 inhibited the A2 binding to immobilized LRP with K_i values (Table 2) similar to those determined for CR triplets containing these doublets (Table 1).

Table 2: Parameters of interaction of A2, recombinant A2 and selected A2 mutants with CR doublets overlapping LRP cluster II. LRP fragments were immobilized on the sensor chip and tested for binding with A2 forms as described under Materials and methods. Interaction of A2 with selected 2xCR fragments is shown in Figure 6, and interaction of recombinant A2 (r-A2) and its representative mutant K466A with cluster II and its 2xCR fragments is shown in Figure 7. The K_D values were derived by fitting the data to a one-site binding model. The K_i values for inhibition of A2 binding to immobilized LRP by CR doublets were derived from heterologous competition experiment similar to that shown in Figure 3. Each parameter represents the mean and SD of triplicate measurements.

<table>
<thead>
<tr>
<th>LRP fragment</th>
<th>K_D (nM)</th>
<th>A2 K_D (nM)</th>
<th>A2 wild-type (nM)</th>
<th>A2 K466A (nM)</th>
<th>A2 R471A (nM)</th>
<th>A2 R484A (nM)</th>
<th>A2 R489A (nM)</th>
<th>A2 H497A (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR 3–10</td>
<td>31 ± 3</td>
<td>22 ± 3</td>
<td>24 ± 3</td>
<td>58 ± 6</td>
<td>78 ± 11</td>
<td>45 ± 5</td>
<td>51 ± 7</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>CR 3–4</td>
<td>30 ± 3</td>
<td>23 ± 4</td>
<td>28 ± 3</td>
<td>72 ± 13</td>
<td>82 ± 17</td>
<td>57 ± 7</td>
<td>54 ± 6</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>CR 4–5</td>
<td>38 ± 4</td>
<td>31 ± 5</td>
<td>34 ± 4</td>
<td>65 ± 7</td>
<td>132 ± 19</td>
<td>39 ± 5</td>
<td>52 ± 7</td>
<td>63 ± 7</td>
</tr>
<tr>
<td>CR 5–6</td>
<td>36 ± 4</td>
<td>28 ± 4</td>
<td>32 ± 4</td>
<td>nb</td>
<td>nb</td>
<td>47 ± 5</td>
<td>57 ± 6</td>
<td>72 ± 9</td>
</tr>
<tr>
<td>CR 6–7</td>
<td>46 ± 5</td>
<td>37 ± 8</td>
<td>35 ± 5</td>
<td>nb</td>
<td>nb</td>
<td>49 ± 6</td>
<td>61 ± 7</td>
<td>75 ± 9</td>
</tr>
<tr>
<td>CR 7–8</td>
<td>67 ± 5</td>
<td>46 ± 8</td>
<td>49 ± 5</td>
<td>126 ± 18</td>
<td>nb</td>
<td>94 ± 13</td>
<td>85 ± 10</td>
<td>79 ± 8</td>
</tr>
<tr>
<td>CR 8–9</td>
<td>ni</td>
<td>nb</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CR 9–10</td>
<td>ni</td>
<td>nb</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* no inhibition of binding (ni) or no binding (nb) was observed; data not applicable (NA).
Collectively, these data indicate that the minimal A2-binding unit is formed by a pair of adjacent complement-type repeats within CR 3–8, which bear conserved residues involved in coordinating Ca\(^{2+}\).

**Interaction of A2 mutants with LRP fragments**

Using Ala-scanning mutagenesis, we have recently determined the A2 amino acid residues which play a critical role in the formation of the binding epitope for LRP (21). In the present study, we tested the effects of point mutations within this site on the interaction of A2 with each LRP fragment in attempt to identify CRs involved in interaction with key A2 residues. In SPR-based experiments, LRP fragments, positive for A2 binding, were immobilized on a sensor chip and tested for binding with recombinant A2 (wt-A2, control) and five A2 mutants K466A, R471A, R484A, R489A and H497A, which had a significantly (3– to 4-fold) reduced affinity for LRP. The representative kinetics for interaction of wt-A2 and A2 mutant K466A with cluster II and its 2xCR fragments are shown in Figure 7, and the \(K_D\) values calculated for all tested A2 mutants are presented in Table 2. We found that the mutations K466A and R471A had the most dramatic effects on the A2 binding to CR doublets of cluster II. Mutation K466A led to a complete abolishment of the A2 binding to CR doublets 5–6 and 6–7 and also significantly reduced the A2 binding to CR 7–8. On the other hand, the binding of K466A mutant to the cluster II CR 3–4 and CR 4–5 persisted, although with a moderately decreased affinity. These data suggest that CR 6–7 doublet of the LRP cluster II is the major and specific contributor to the binding of the A2 residues K466.

Analogously, mutation R471A abolished the A2 binding to CR 5–6, 6–7 and 7–8, and significantly reduced the A2 binding to CR 4–5, suggesting that these complement-type repeats of cluster II play a predominant role in interaction with this A2 residue. In contrast, mutations R484A and R489A did not abolish but decreased the A2 binding to cluster II fragments, with the most significant reduction in the affinity registered for C-terminal CR 7–8. This suggests the major role of CR 7–8 in interaction with R484 and R489 residues of A2.

Analysis of the binding of the A2 mutants to 3xCR fragments of cluster IV also revealed a decrease in their affinity for specific

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**Inset:**

Cluster II (CR 3-10)

CR 3-4
CR 4-5
CR 5-6
CR 6-7
CR 7-8
CR 8-9
CR 9-10

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**Figure 6:** Binding of A2 to immobilized 2xCR fragments of LRP cluster II in SPR-based assay. Inset shows schematic representation of LRP cluster II and its overlapping CR doublets expressed in E. coli and tested for binding of A2. These 2xCR fragments were covalently immobilized on sensor chips at ~25 fM/mm\(^2\). A2 was passed over these chips at increasing concentrations (20–300 nM) in HBS-Ca buffer for 5 minutes as described under Materials and methods. A-C) A2 binding to three representative doublets CR 3–4, CR 4–5 and CR 5–6, respectively. In control experiment, verifying RAP-sensitivity of the A2 binding to 2xCR fragments, RAP (500 nM) was first bound to immobilized fragments to a saturating concentration that was followed by co-injection of A2 (300 nM) and RAP (500 nM) at time-points indicated by arrows (D-F, curve 1). The signals obtained for control injections of RAP (500 nM) or A2 (300 nM) are shown in D-F as curves 2 and 3, respectively.
triplets (data not shown). The most pronounced effects were observed for K466A and R471A, which lost the ability to bind to CR 23–25 and CR 27–29, correspondingly. On the other side, these mutants bound with a 2.5-fold lower affinity to CR 25–27. This suggests that CR 24–25 and CR 28–29, which bear conservative Trp and Asp residues (Fig. 1), play a key role in interactions of cluster IV with A2 residues K466 and R471. Collectively, experiments with A2 single-point mutants identified CRs within LRP clusters II and IV which play a predominant role in binding the key residues of the LRP-binding site within A2.

Effects of LRP fragments on internalization of A2 by LRP-expressing cells
To test the functional significance of our results in purified system, we next examined whether the identified LRP regions are able to interfere with the uptake of A2 by LRP exposed on the cell surface. Based on our previous observation that the A2 domain is effectively internalized via LRP in cell culture (37) and on the reported inhibitory effect of soluble exodomain of LRP on LRP-mediated ligand uptake (43), we compared internalization of A2 by LRP-expressing MEF cells in the presence of LRP fragments. We found that clusters II and IV and their triplets CR 3–5, 5–7, 7–9 and 23–25, 25–27, 27–29, respectively, inhibited A2 internalization by MEF cells by ~2-fold (Fig. 8). Notably, cluster II CR doublets 3–4, 4–5, 5–6, 6–7 and 7–8 were also effective inhibitors of A2 internalization. Cluster III, 2xCR 8–9 and 9–10, and 3xCR 8–10, 21–23 and 29–31 (which did not bind A2 in purified system) had no effect on A2 internalization. The presence of RAP inhibited A2 internalization by ~75%, which is consistent with our previous observations (3) and confirms the key role of LRP in this process. In control experiment with PEA 13 cells (of the same type as MEF but genetically deficient in LRP), A2 internalization was not affected by the presence of LRP fragments. Thus, this experiment confirmed that selected portions of clusters II and IV are able to compete with LRP natively expressed on the cell surface for binding of A2 suggesting their possible involvement in LRP-mediated uptake of A2.

Figure 7: Binding of recombinant wt-A2 and A2 mutant K466A to LRP cluster II and its fragments in SPR-based assay. Cluster II (A and G) and its overlapping fragments comprising CR 3–4 (B and H), CR 4–5 (C and I), CR 5–6 (D and J), CR 5–7 (E and K) and CR 7–9 (F and L) were immobilized on sensor chips at ~7 fM/mm² and tested for binding wt-A2 (A–F) or A2 mutant K466A (G–L) at increasing concentrations (20–300 nM) as described under Materials and methods. The response is expressed in resonance units (RU) corrected for nonspecific binding of A2 to a blank surface and normalized to the same levels of immobilized fragments.

Effect of LRP fragments on A2 clearance in vivo
We previously demonstrated that A2 is cleared from mouse circulation via RAP-sensitive mechanism that provided an in-vivo model to study interaction of A2 with LRP and other receptors of the LDLR family (21). To test whether LRP regions, which bind A2 in vitro, can participate in A2 clearance in vivo, we compared the half-lives of radiolabeled A2 in the absence and presence of selected LRP fragments in mice. The data for A2 survival in the circulation were well fitted with a double-exponential decay model used previously (21). As shown in Figure 9A and B, co-injection of 125I-A2 with LRP clusters II or IV or their A2-binding fragments CR 3–4 or CR 25–27 led to a statistically significant 1.9– to 2.6-fold prolongation of the A2 half-life. The half-lives were 25 ± 6 min for A2 alone, and 62 ± 12, 64 ± 13, 48 ± 9 and 46 ± 8 min for A2 in the presence of clusters II and IV, and CRs 3–4 and 25–27, respectively. In control experiment, co-injection of cluster III or CR 29–31 (that does not bind A2) had no effect on the A2 half-life, with the corresponding values of 28 ± 6 min and 27 ± 7 min. In additional control experiment, we tested clearance of four representative 125I-labeled LRP fragments (LRP cluster II and CR 3–4 which bind A2, and cluster III and CR 29–31 which do not bind A2, Fig. 9C). The half-lives of all the fragments, positive or negative for A2 binding in vitro, were significantly longer than the A2 half-life (23 ± 7 min) but similar between large and between short fragments: for clusters II and III, and for CRs 3–4 and 29–31 the values were 284 ± 47 min, 343 ± 54 min, 139 ± 23 min and 166 ± 22 min, respectively. As LRP fragments persisted in the circulation longer than A2, the observed prolongation of A2 half-life is due to interference of A2-binding LRP fragments with LRP-mediated removal of A2. In turn, this indicates that the corresponding regions in the native LRP (expressed in the liver) may be involved in A2 clearance in vivo.
In the present study, we identified LRP regions involved in binding to the A2 domain of FVIII and revealed existence of the multiple binding sites, with each elementary site being composed of two adjacent complement-type repeats. Most of the known LRP ligands bind to LRP clusters II and IV (16), and the A2 domain proved to follow this mode. This was previously indicated by the two-site binding kinetics of A2/LRP interaction reported by us (21) and was confirmed here by direct binding of A2 to isolated clusters II and IV in SPR-based assay as well as by inhibitory effects of clusters II and IV on LRP-mediated internalization of A2 in cell culture and on A2 clearance from the circulation in mice. As with other LRP ligands, we did not detect the A2 binding to LRP cluster III, which, up-to-date, is known to bind only RAP (7).

In several studies, various fragments of LRP cluster II were expressed for mapping the binding sites for a number of ligands (16, 17, 19, 38) and our mapping of the A2-binding region within LRP cluster II adds to this knowledge. We also expressed five overlapping CR triplets of LRP cluster IVA and tested these fragments for binding of RAP and A2. Comparison of the binding patterns in a number of complementing assays provided the evidence that the binding sites for A2 and RAP on LRP are shared within CR 3–8 and CR 23–29 of clusters II and IV, respectively. Unlike RAP, the A2 domain did not bind to CR 8–10 of cluster II, CR 21–23 and CR 29–31 of cluster IV and had a lower affinity for CR 23–25. These data narrow down the regions in LRP interacting with A2 in comparison with RAP that is consistent with the ability of RAP to inhibit A2 binding to LRP and with its rec-
ognized role as a universal antagonist of the LDL receptor family.

An interesting feature of A2 interaction with LRP is that A2 binds with a similar high affinity to intact LRP, its clusters II and IV and to most of their 3xCR and even 2xCR fragments (Tables 1 and 2). Indeed, the affinity of A2 for intact LRP is ~14 nM (21) that is close to the affinities for LRP cluster II (K_d = 22 ± 3 nM) and for its fragments, CRs 3–5 and 5–7 (K_d = 25 ± 4 and 31 ± 4 nM, respectively) and CRs 3–4, 4–5 and 5–6 (K_d = 23–37 nM). This observation implies that the elementary binding site for A2 on LRP is formed by adjacent CR and these sites are multiple. In turn, this explains why single mutations of A2 residues K466, R471, R484, S488, R489, R490, H497, and K499 which form the LRP-binding site resulted in a modest reduction of A2 affinity for LRP in our previous study (21). It is likely that each A2 point mutation affects its binding only to certain, but not to all, binding sites in LRP, and the A2 binding to the whole receptor persists via its other sites. The relevance of this possibility is illustrated by the differential binding of A2 mutants to 2xCR fragments of cluster II (Fig. 7 and Table 2) which reflects the predominant contribution of certain CR doublets in the binding of key A2 residues K466, R471, R484, R489 and H497.

Our conclusion that an elementary binding site for A2 on LRP is formed by a CR doublet is supported by resolution of the structures of complexes of RAP domains 3 and 1 with 2xCR modules of LDLR (44) and LRP (45), respectively. These studies revealed that the binding interface is dominated by electrostatic interactions between complementary charged residues of a ligand and a receptor that is likely to be a general mode for ligand recognition by the LDL receptor family. Notably, the contact interface between individual CR and RAP suggests that a single module would be insufficient to stabilize the correct ligand-binding conformation of the receptor and that two docking sites are required to maintain the high-affinity binding of a ligand. Our results are also consistent with Ca^{2+} requirement for the binding established for most LRP ligands. Indeed, A2 did not bind to LRP fragments comprised of CR 8–10, 21–23 and 29–31, since CR 10, 22, 23, 30 and 31 do not contain conserved residues, homologous to Trp1080 and Asp1085 in CR8 (Fig. 1), which are critical for coordinating Ca^{2+} (18) via backbone carbonyl group.

Identification of the LRP regions interacting with A2 raises a question which regions of the receptor are involved in high-affinity binding with another LRP-binding site (the A3 region 1811–1818 (22)). Although the mapping of LRP sites for isolated A3 domain has not yet been performed, it was previously reported that LCh of FVIIIa interacts with LRP clusters II and IV (16). Employing the same panel of LRP fragments as in the present study, we later reported that FVIIia heterodimer A1/A3-C1-C2 (activated form of LCh) has multiple binding sites within these clusters with affinities similar to those for A2 (46, 47). Recently, interaction of intact (non-activated) LCh with overlapping CR triplets of clusters II and IV has been analyzed and the LCh binding regions were attributed to CR 5–7, CR 6–8 and CR 24–26 (48). While the assessed affinities for LCh are lower than for A2 and A1/A3-C1-C2 (likely due to differences in experimental approaches), these studies collectively suggest that regions CR 5–8 of cluster II and CR 23–26 of cluster IV are involved in binding both LCh (A3 domain) and A2.

In our previous study, we demonstrated existence of a mechanism for clearance of A2 from the circulation which involves LRP and, possibly, other member(s) of the LDLR family (21). The physiological need for the A2 removal may be related to the fact that A2 subunit itself possesses FVIIIa-like cofactor activity (49), which must be stringently controlled. In that study, we confirmed the functional role of LRP-binding site on A2 by demonstrating that the half-life of a representative A2 mutant (bearing a double-point mutation R471A/R484A within this site) in mouse circulation was substantially longer than that of wt-A2 (21). In the present study, we demonstrated that the A2-binding fragments of LRP, in turn, interfere with A2 clearance in mice. The prolongation of the A2 half-life (1.9–2.6-fold for intact LRP, and 6–12-fold longer half-life for its fragments, CR 3–5 and 5–7 (K_d = 22 ± 3 nM)) was less pronounced as it could be expected considering high molar excess of injected LRP fragments, their relative high affinity for A2 (~20 nM) and 6–12-fold longer half-life in the circulation in comparison with A2. This effect can be explained by the presence of interfering components in plasma and possible existence of an alternative catabolic pathway(s) for A2 which is not mediated by LRP/LDLR. Considering that LRP has over 35 ligands (7) and that some of them are present in the circulation at significantly higher concentrations than injected A2 (such as α_2-macroglobulin and α_M/proteinase complexes [50]), these ligands can effectively compete with A2 for interaction with co-injected LRP fragments. The existence of additional LRP-independent catabolic pathway(s) for A2 was suggested in our previous study when internalization of A2 in cell culture could not be completely inhibited by RAP (37). Altogether, identification of LRP regions which bind A2 and finding that these LRP portions can interfere with LRP-mediated A2 endocytosis in cell culture and with RAP-sensitive clearance of A2 in mice suggest a functional role of the identified LRP regions in the A2 catabolism. As a future prospective, identification of key amino acid residues within the binding epitope(s) of LRP for A2 may further contribute to understanding molecular mechanisms of interaction of LRP and its ligands.

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