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Cluster III of Low-Density Lipoprotein Receptor-Related Protein 1 Binds Activated Blood Coagulation Factor VIII

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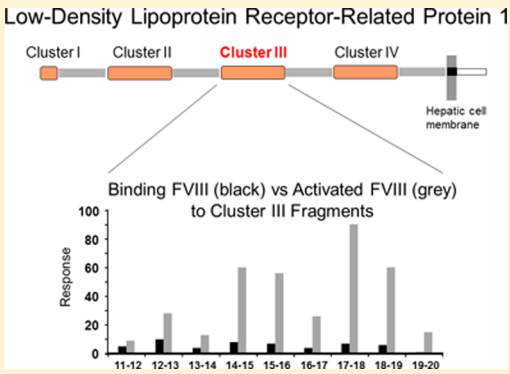
Supporting Information

ABSTRACT: Low-density lipoprotein receptor-related protein 1 (LRP) mediates clearance of blood coagulation factor VIII (FVIII). In LRP, FVIII binds the complement-type repeats (CRs) of clusters II and IV, which also bind a majority of other LRP ligands. No ligand is known for LRP cluster I, and only three ligands, including the LRP chaperone alpha-2 macroglobulin receptor-associated protein (RAP), bind cluster III. Using surface plasmon resonance, we found that in addition to clusters II and IV, activated FVIII (FVIIIa) binds cluster III. The specificity of this interaction was confirmed using an anti-FVIII antibody fragment, which inhibited the binding. Recombinant fragments of cluster III and its site-directed mutagenesis were used to localize the cluster's site for binding FVIIIa to CR.14−19. The interactive site of FVIIIa was localized within its A1/A3′-C1-C2 heterodimer (HDa), which is a major physiological remnant of FVIIIa. In mice, the clearance of HDa was faster than that of FVIII and prolonged in the presence of RAP, which is known to inhibit interactions of LRP with its ligands. In accordance with this, the cluster III site for RAP (CR.15−19) was found to overlap that for FVIIIa. Altogether, our findings support the involvement of LRP in FVIIIa catabolism and suggest a greater significance of the biological role of cluster III compared to that previously known.



Low-density lipoprotein receptor-related protein 1 (LRP) belongs to a large group of structurally related endocytic receptors known as the low-density lipoprotein receptor (LDLR) family. Expressed in the circulation, hepatic LRP is involved in endocytosis of lipoproteins, proteinases, and factors of blood coagulation and fibrinolysis.[1] The ligand-binding moiety of the LDLR receptors is typically presented by highly homologous complement-type repeats (CRs). Each CR consists of approximately 40 amino acids forming an autonomous domain, which coordinates Ca^{2+}.[2−4] The CR domains are connected by flexible linkers and grouped in clusters. According to the general model for ligand recognition by the LDLR family, a CR domain "docks" a ligand's lysine via several conserved residues (Figure S1 of the Supporting Information).[2,4−11] Among them, four acidic residues interact with the ε-amino group of the lysine, and an aromatic residue interacts with the lysine's aliphatic chain. A number of other CR residues also contribute to the interaction. A pair of adjacent CRs is minimally necessary for ligand binding,[2,4−15] whereas additional adjacent CRs can contribute to the binding and provide an avidity effect.[11,16,17]

Among the four CR clusters of LRP, the binding sites for a majority of its ligands are located in clusters II and IV (Figure 1). These ligands include α2-macroglobulin (α2M), protease−inhibitor complexes (serpins), and coagulation factor VIII (FVIII).[11,16,18,19] Only two ligands in the plasma bind cluster III: ApoE-containing lipoproteins (β-VLDL) and the thrombin−protease nexin 1 complex.[20] A folding chaperone of the LDLR family, known as α2M receptor-associated protein (RAP), binds all three clusters, II, III, and IV, and inhibits interactions of LRP with all its ligands.[20−22] No ligand is known for cluster I.

One of the functions of LRP is to internalize FVIII from the circulation.[23−26] FVIII is an important component of blood coagulation, with its deficiency resulting in the bleeding disorder known as hemophilia A.[27] The disease is treated by frequent infusions of FVIII, preformed up to four times per week in prophylaxis. The need for reducing the frequency of injections calls for generation of longer-lasting therapeutic FVIII.[28] This requires a better understanding of the mechanisms of FVIII clearance.

A molecule of FVIII is composed of a heavy chain (HCh) and a light chain (LCh) with A1-A2-B and A3-C1-C2 domain structures, respectively (Figure 2). The LCh contains an extended LRP-binding site, which was suggested to involve the A3, C1, and C2 domains.[29−32] This site is masked when FVIII is in complex with its carrier protein, von Willebrand factor (vWF).[23] Therefore, the clearance of FVIII via LRP is believed to occur via a small FVIII fraction (<5%) not bound to vWF that exists in equilibrium with the vWF-bound FVIII. Similar

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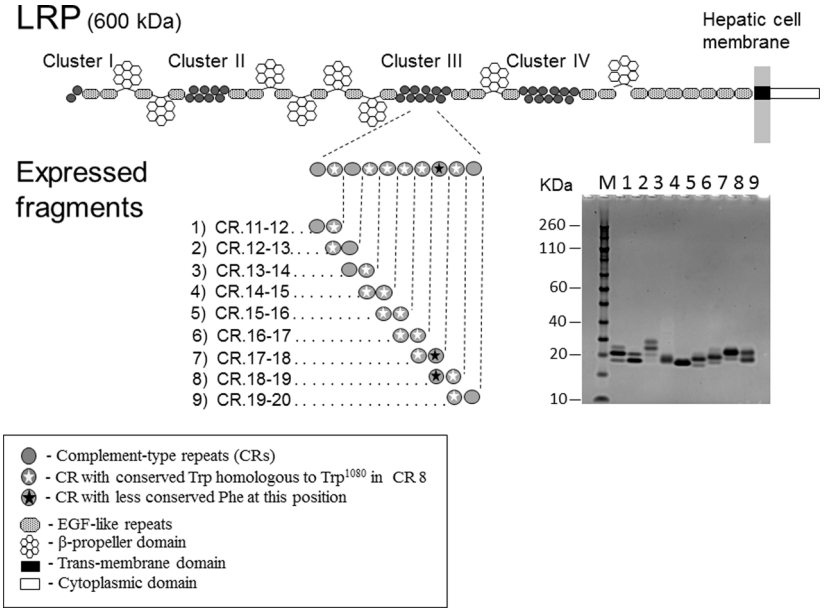

Figure 1. Domain structure of LRP and its expressed fragments. CRs with conservative aromatic residues homologous to W^{1080} in CR.8 (position 1 in Figure S1 of the Supporting Information) are marked by asterisks: white for tryptophans and black for phenylalanine. SDS−PAGE analysis of the expressed fragments is shown (nonreducing conditions and Coomassie staining).

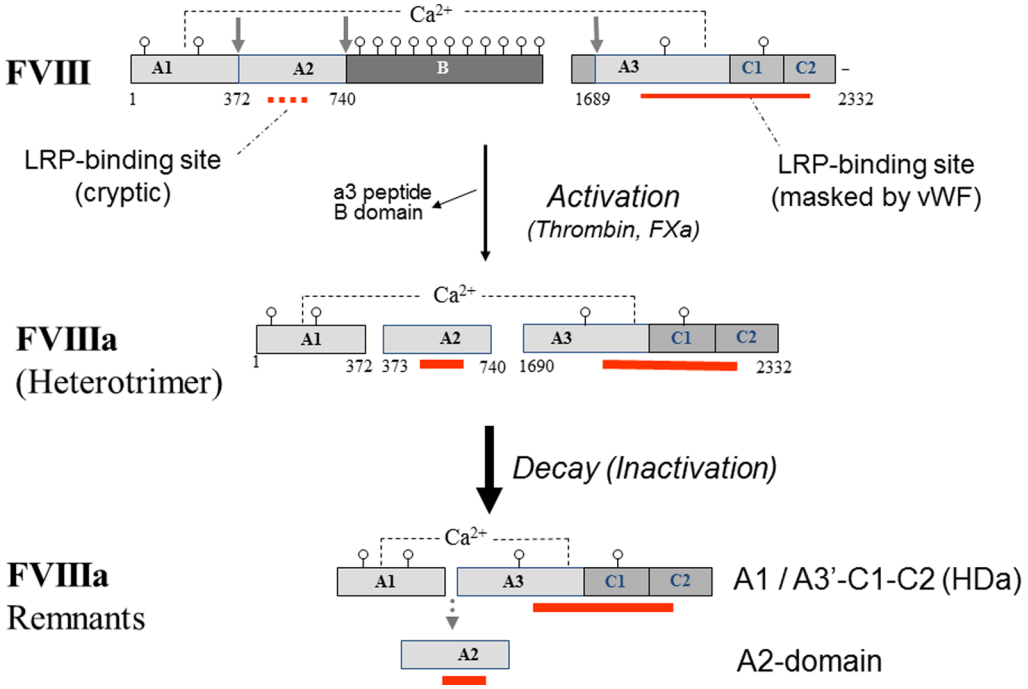

Figure 2. Domain structure and LRP-binding sites of FVIII and FVIIIa. The FVIII molecule (top) is composed of a heavy chain (HCh, domains A1, A2, and B) and a light chain (LCh, domains A3, C1, and C2). Upon site-specific cleavage (activation) by thrombin or factor Xa (gray arrows), FVIII is converted into an A1/A2/A3′-C1-C2 heterotrimer (FVIIIa) (middle). The unstable FVIIIa eventually dissociates into the A2 and A1/A3′-C1-C2 heterodimer (HDa) (bottom). The LRP-binding sites are colored red, and glycosylation sites are shown as circles.

considerations are likely applicable to the clearance of FVIII via LDLR, which is involved in this process in concert with LRP.[25,33]

At the site of blood vessel injury, FVIII is activated by site-specific cleavage by thrombin. This event leads to the release of an N-terminal LCh peptide (α3 peptide) and the B domain, followed by dissociation of vWF. The resulting heterotrimer (FVIIIa), A1/A2/A3′-C1-C2, now exposes the LRP-binding sites in the LCh and in the A2 domain (Figure 2).[30,34] Upon fulfilling its biological function of being a cofactor for activated factor IX, the unstable FVIIIa dissociates into the A2 domain and A1/A3′-C1-C2 heterodimer (HDa).[35] Thus, in contrast to FVIII, FVIIIa has two LRP-binding sites; each of them appears on a respective remnant of FVIIIa. This suggests that both of the remnants of FVIIIa are cleared via LRP, which was confirmed for the A2 domain.[34]

The difference in exposure of the LRP-binding sites on FVIII and FVIIIa suggests a difference in their interactions with LRP.

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As previously shown, FVIII binds to LRP clusters II and IV,[16,19,23] but not cluster III. In the present study, we found that in addition to clusters II and IV, FVIIIa also binds to cluster III. Focused on the latter finding, we determined the binding site of cluster III for FVIIIa. The interactive site of FVIIIa was found to involve the HDa. Consistent with this, in mice, the clearance of HDa was prolonged upon inhibition of the LDLR family receptors. In addition, we mapped the RAP-binding region in cluster III, which finalized the mapping of RAP-binding sites in whole LRP. Thus, our study provides new data for interactions of LRP with FVIII (FVIIIa) and RAP, and demonstrates a new function of LRP cluster III and the involvement of the LDLR family in the clearance of FVIIIa.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. Recombinant FVIII (Advate, Baxter, CA) was purchased from the National Institutes of Health (Bethesda, MD) pharmacy. The heterodimer of FVIIIa, A1/A3′-C1-C2 (HDa), was isolated as described previously.[34] LRP was provided by Dr. I. Mikhailenko, and LRP clusters II–IV were produced as described previously.[36] The anti-FVIII scFv iKM33 antibody fragment was produced as described previously.[37] RAP was purchased from R&D Systems (Minneapolis, MN). Human α-thrombin and FPR-chloromethylketone (PPACK) were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Ni Sepharose 6 Fast Flow resin was purchased from GE Healthcare (Waukesha, WI). The BCA protein assay kit was purchased from Pierce (Rockford, IL).

Generation of Plasmid Constructs Encoding LRP Cluster III and Its Fragments. A modified pFastBac1 plasmid containing a melittin secretion signal, a six-His tag, a protease cleavage site, a multiple cloning site, a c-*myc* tag, and a stop codon (GenBank accession number AY598466) was used as a vector.[33] The coding regions of the LRP cluster III mutant and its CR doublets were chemically synthesized, subcloned into the vector, and inserted into a bacmid according to the Bac-to-Bac Expression system (Life Technologies, Carlsbad, CA).

Expression and Purification of the LRP Cluster III Fragments. The expression of the LRP fragments and their purification were performed as described previously.[33,38] The proteins were verified by PAGE with Coomassie staining and Western blotting using anti-*myc* mAb9E10. The protein concentrations were measured spectrophotometrically using extinction coefficients calculated using Vector NTI (Life Technologies).

Preparation of FVIIIa. Advate was reconstituted and dialyzed overnight in HBS-Ca. The next day, its concentration was measured using the BCA kit. Thrombin (0.16 μg) was added to the sample (~50 μg of FVIII). Upon incubation for 10 min at 20 °C, thrombin was inactivated with PPACK (262 ng), and the samples were used in SPR. The activation of FVIII was confirmed by PAGE.

Surface Plasmon Resonance. Binding assays were performed in HBS-P buffer (GE Healthcare) supplemented with 5 mM $CaCl_2$ (HBS-Ca) at room temperature using the Biacore 3000 instrument (GE Healthcare). LRP cluster III, its mutant, and fragments were immobilized on a CM5 chip using an amine coupling kit (GE Healthcare) at ~1000 RU for CR doublets and ~2000 RU for the CR cluster and its mutant. Association with RAP, FVIII, FVIIIa, and the A1/A3′-C1-C2 heterodimer at different concentrations (series of 2-fold dilutions, specified in Results for each particular experiment) in HBS-Ca was recorded at a flow rate of 10 μL/min for 3 min. Dissociation was recorded for 5 min in the running buffer. Regeneration of the sensors was performed with 0.1 M H_3PO_4. The assessed K_D values were derived from the simultaneous fitting of respective sensorgram series using a 1:1 (Langmuir) model using BIAevaluation version 4.1.1. Each binding assay was repeated on a different day.

Clearance of FVIII and HDa in Mice. Experiments were performed as described previously.[34] Briefly, prior to an experiment, FVIII and HDa were labeled with ^{125}I; 100 nM solutions each of [^{125}I]FVIII and [^{125}I]HDa (alone or in the presence of 200 μM RAP) in HBS-Ca (100 μL) were injected into the tail vein of BALB/c mice (n = 4). Blood aliquots (~50 μL) were withdrawn via retro-orbital puncture at selected time intervals starting from 5 min after the injection and measured for radioactivity using a γ-counter. The values were normalized to the sample volumes, averaged, and expressed as a percentage of the initial count for each animal group. The data were fit using a double-exponential decay model.

RESULTS

Interaction of FVIII and FVIIIa with LRP. The interaction between FVIII and FVIIIa with LRP was studied using SPR. LRP was immobilized on a sensor, and its binding properties were confirmed with the assessed RAP affinity (Table S1 of the Supporting Information) comparable to that known from the literature (K_D = 1–18 nM).[39,40] Next, we compared the binding of FVIII and FVIIIa to LRP (Figure 3). Previously, it

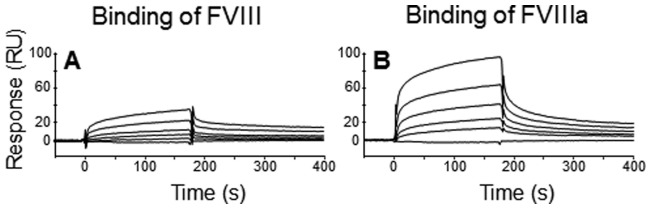

Figure 3. Binding of FVIII and FVIIIa to LRP. In SPR, LRP was immobilized on a sensor at ~2000 RU and tested for binding with (A) FVIII and (B) FVIIIa used at 18.75–300 nM (2-fold serial dilutions) for each ligand; the control injections of buffer only were also performed.

was reported that the signals for binding of FVIII to LRP and LDLR did not fit well using any of the standard fitting models.[16,33,41] In our study, the curve fittings for both FVIII and FVIIIa with LRP (and its fragments in further experiments) were also unsatisfactory in each of the two experimental runs performed. Therefore, using a 1:1 (Langmuir) model, we made an assessment of these affinities from only one representative experiment that still allowed us to compare the assessed affinities between FVIII and FVIIIa for LRP and its fragments. The assessed affinities of FVIII and FVIIIa for LRP indicated their similarity [K_D values of ~50 nM (Table S1 of the Supporting Information)]. However, the markedly stronger binding signals of FVIIIa indicated a difference in its interaction with LRP compared to that of FVIII. To elucidate the cause of this difference, we next tested the binding of fragments of LRP and FVIIIa.

Interaction of FVIII and FVIIIa with LRP Clusters. LRP fragments were expressed using a baculovirus system, capable of producing functionally active fragments of the LDLR

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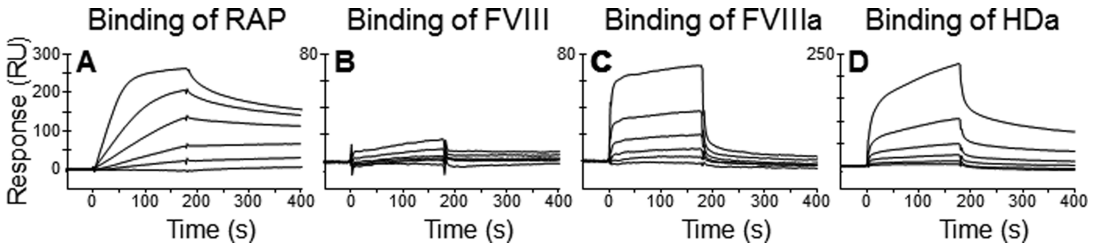

Figure 4. Binding of RAP, FVIII, FVIIIa, and HDa to LRP cluster III. In SPR, LRP cluster III was immobilized at ~2000 RU and tested for the binding with (A) RAP (used at 0.3125−5 nM), (B) FVIII and (C) FVIIIa (both used at 6.25−100 nM), and (D) HDa (used at 5−80 nM). For each ligand in solution, the concentration difference was 2-fold, and a control injection of buffer only was also performed.

family.[33,36,42,43] As the active binding moieties of LRP for all known ligands, including FVIII,[16,19] are within clusters II−IV, all these clusters were generated. The functional integrity of the expressed proteins was tested by binding to RAP, which is known to interact with each of clusters II−IV.[21] RAP demonstrated high affinity for each of the clusters, comparable with that for LRP in the former experiment. Both FVIII and FVIIIa bound to clusters II and IV (data not shown), and the assessed affinities were within the range of those of FVIII for LRP and its clusters II and IV determined previously (K_D values of ~60−116 nM).[16,19,23,24] With similar affinity [K_D ~ 130 nM (Table S1 of the Supporting Information)], FVIIIa also bound to cluster III that was in contrast to FVIII (Figure 4B,C). The interaction of FVIIIa and cluster III was characterized in further experiments.

Identification of the FVIIIa-Binding Site for Cluster III. At neutral pH, unstable FVIIIa undergoes fast dissociation into A2 and HDa[35] (Figure 2). The isolated A2 domain was previously found to bind only clusters II and IV, but not cluster III.[36] Therefore, we tested if HDa binds cluster III and found that indeed it does (Figure 4D); the affinity was not assessed as the association and dissociation curves were not fitted well. The specificities of interactions of both FVIIIa and HDa with cluster III were tested in a competitive binding assay. Previously, a single-chain variable antibody fragment (scFv) iKM33,[37] which recognizes the C1 domain of FVIII, was shown to inhibit binding of FVIII to LRP.[44−46] Therefore, we tested if iKM33 had a similar effect on the binding of both FVIIIa and HDa to LRP cluster III. We found that the binding of both ligands to the cluster was dose-dependently inhibited by iKM33 (Figure 5). Thus, the FVIIIa-binding site for cluster III is located on the HDa and involves its C1 domain.

Generation of Cluster III Fragments. To identify the specific CRs of cluster III responsible for the binding of FVIIIa, overlapping CR doublets of the cluster were generated (Figure 1). This strategy was based on the fact that a CR doublet represents a minimal binding unit of a receptor and its previous use to map the binding sites of LRP cluster II for RAP[11] and of LDLR for FVIII.[33] Thus, nine overlapping CR doublets of cluster III were expressed. The isolated proteins were essentially pure (Figure 1) with some samples containing multiple bands. This was due to differences in glycosylation as shown previously for other LRP fragments.[16]

Interaction of Cluster III Fragments with RAP. The functional integrity of the expressed fragments was assessed by their ability to bind RAP. All the CR doublets overlapping the CR.15−19 region were capable of binding (Figure 6A). For a representative fragment, CR.17−18, the data are shown in Table S1 and Figure S2A of the Supporting Information; the assessed affinity of this interaction (K_D ~ 0.7 nM) was similar to the affinities of RAP for cluster III [K_D ~ 0.3 nM (Table S1 of the Supporting Information)] and to those previously found for CR doublets of LRP cluster II (K_D values of 1−5 nM).[11] Our data are also in agreement with a previous finding that showed the C-terminal half of cluster III binds RAP.[21] Thus, we

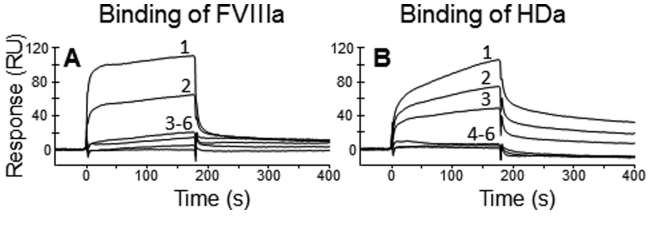

Figure 5. Binding of FVIIIa and HDa to LRP cluster III in the presence of scFv iKM33. In SPR, immobilized LRP cluster III was tested against (A) FVIIIa (150 nM) and (B) HDa (40 nM) in the absence of iKM33 (1) or in its presence at increasing molar ratios of 1:1 (2), 1:2 (3), and 1:5 (4). In each experiment, the control injections of iKM33 only, used at the amount corresponding to the ratio of 1:5 (5) and buffer only (6), were performed.

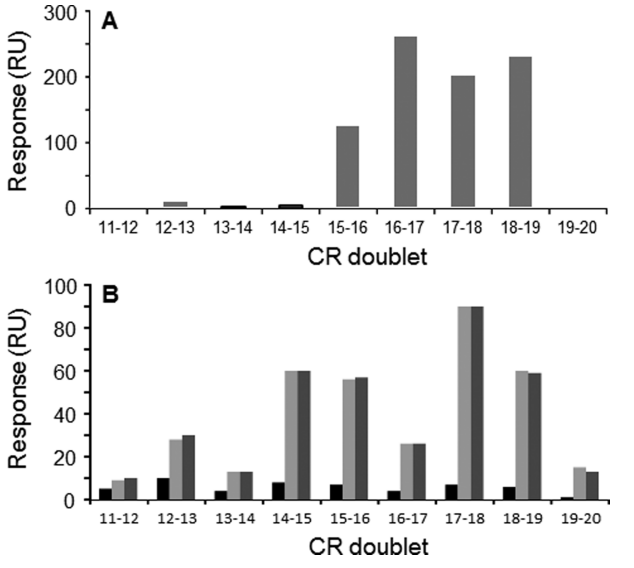

Figure 6. Binding of RAP, FVIII, FVIIIa, and HDa to fragments of LRP cluster III. In SPR, CR doublets of LRP cluster III were immobilized at ~1000 RU and tested for binding with (A) RAP (10 nM) and (B) FVIII (125 nM, black), FVIIIa (80 nM, light gray), and HDa (80 nM, dark gray). Each bar height corresponds to the maximal response at the end of the association phase (3 min). The experiment was performed with 2-fold serial dilutions of each ligand, while the bars are shown for a selected concentration of each ligand.

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mapped the RAP-binding site in cluster III and justified the suitability of all expressed fragments of cluster III for further experiments.

Interaction of Cluster III Fragments with FVIIIa. The panel of cluster III fragments was tested for the binding with FVIIIa and HDa (Figure 6B); because of unsatisfactory fitting of the binding curves discussed above, we measured the relative intensities of the signals at the end of the association. We found that both ligands had a similar mode of binding: they bound relatively strongly to doublets within CR.14−19 and weakly to CR.12−13. Notably, among those, CR.13 has the weakest homology with the consensus sequence (Figure S1 of the Supporting Information) and deviates from the model of Fisher et al.[5] For the representative doublet CR.17−18, the binding signals are shown in Figure S2B−D of the Supporting Information and the assessed affinities for FVIIIa and HDa were similar [K_D values of ∼65 nM (Table S1 of the Supporting Information)]. These results indicate that CR.14−19 of cluster III forms the major binding site for FVIIIa and confirm that the interactive site of the latter is located on the HDa.

Interaction of the Cluster III Mutant with RAP and FVIIIa. To confirm the cluster III mapping results, we used its site-directed mutagenesis. In the general model for ligand recognition by the LDLR family, a conserved aromatic residue of a CR at position 1 (Figure S1 of the Supporting Information) interacts with the aliphatic chain of a lysine on the ligand.[5] In fragments of LRP and LDLR, replacement of such residues (tryptophans) with serines resulted in the abolishment of RAP binding and a reduction in the level of FVIII binding.[18,33] We previously showed that such a mutation does not affect the structure of a CR domain, as it still retains Ca^{2+}.[33] Thus, we generated a cluster III mutant in which all aromatic residues at position 1 within the major binding site for FVIII, CR.14−19, were replaced with serines. The minor binding site for FVIII, CR.13−14, was not affected, as CR.13 naturally contains a serine at position 1. Similar to the LDLR cluster mutant,[33] the cluster III mutant [6xW(F) → S] was unable to bind RAP, and the level of binding of both FVIIIa and HDa was reduced (Figure 7). This indicates that the interaction of FVIIIa (HDa) involves additional residues besides those involved in RAP binding. Also, the residual interaction of FVIIIa (HDa) was supplemented by the nonmutated CR.12−13 having weak affinity for FVIIIa (HDa). Altogether, the results support the mapping of cluster III-binding sites for RAP and FVIIIa.

Clearance of HDa in Mice. The higher binding capacity of LRP for FVIIIa than for FVIII suggests that the clearance of FVIIIa also involves LRP and may occur faster than that of FVIII. To verify this hypothesis, both remnants of FVIIIa, A2 and HDa, were tested separately for their clearance in mice. In a previous study, we found that such clearance of A2 is indeed LDLR family-dependent, as it was inhibited by RAP.[34] In the study presented here, we report data for HDa, which was tested in a parallel experiment. [^{125}I]HDa, alone or in the presence of RAP, and [^{125}I]FVIII (control) were injected into mice, and the radioactivity in plasma was monitored over time (Figure 8). We found that the clearance of HDa ($T_{1/2}$ = 39 ± 10 min) was ∼2.3 times faster than that of FVIII (90 ± 21 min). Co-injection of RAP prolonged the half-life of HDa by ∼1.5 times ($T_{1/2}$ = 60 ± 8 min). These results demonstrate that a RAP-sensitive factor(s), most likely the LDLR family receptors, contributes to the clearance of HDa.

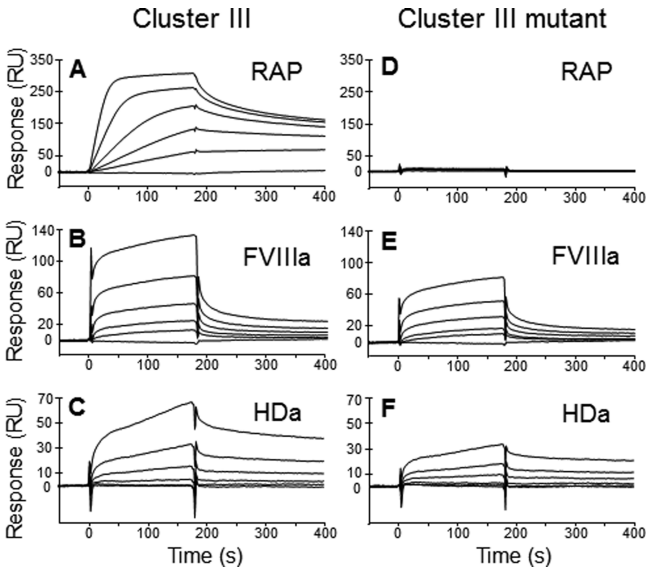

Figure 7. Binding of RAP, FVIIIa, and HDa to the LRP cluster III mutant. In SPR, LRP cluster III (A−C) and its CR.14−19 [6xW(F) → S] mutant (D−F) were immobilized at ∼2000 RU and tested for binding with RAP [0.625−10 nM (A and D)], FVIIIa [18.75−300 nM (B and E)], and HDa [2.03−32.5 nM (C and F)]. For each ligand in solution, the concentration difference was 2-fold, and a control injection of buffer only was also performed.

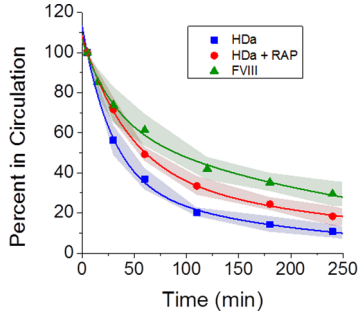

Figure 8. Clearance of HDa from mice. [^{125}I]FVIII [100 nM (green)] and [^{125}I]HDa [100 nM, alone (blue) or in the presence of 200 μM RAP (red)] were injected (100 μL) into the tail vein of mice (n = 4). Blood aliquots (∼50 μL) were withdrawn at selected time intervals starting from 5 min after the injection, and their radioactivity was measured. The values were normalized to the sample volumes, averaged, and expressed as a percentage of the initial count for each experiment. The data were fit using a double-exponential decay. The shadowed areas correspond to the standard deviation from two experiments.

DISCUSSION

In this study, we demonstrated that FVIIIa interacts with LRP cluster III. In cluster III, the main binding region was located within CR.14−19, and in FVIIIa, the interactive site was found to involve the A1/A3′-C1-C2 heterodimer (HDa), the C1 domain, in particular. We confirmed that the nonactivated FVIII does not bind cluster III. We then showed that the clearance of activated FVIII *in vivo* involves the LDLR family, further implicating this group of receptors in the regulation of the FVIII life cycle. In addition, we located the RAP-binding site of cluster III within CR.15−19, which finalized the previous mapping of RAP-binding sites in LRP.[11,16,21]

The difference between FVIII and FVIIIa in interactions with LRP is based on the structural changes in FVIII that occur upon

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its activation. The activation results in the exposure of two LRP-binding sites in FVIIIa, i.e., in A2[34] and HDa, and their eventual dissociation.[35] At the same time, in HDa (A1/A3′-C1-C2), the LRP-binding site appears to be exposed differently compared to that in the LCh (A3-C1-C2) of FVIII. Indeed, even though both FVIII and FVIIIa bind LRP via the LCh moiety, only that of FVIIIa binds cluster III. This finding may also reflect a uniqueness of cluster III, which is notably less homologous to clusters II and IV (Figure S1 of the Supporting Information).

In cluster III, the major FVIIIa-binding site is relatively long as it consists of six consecutive CRs. This site is almost equal in size to the RAP-binding site and comprises the ApoE site.[47] The cluster III site for FVIIIa (HDa) is significantly longer than the sites of clusters II and IV for binding FVIII (LCh).[16] In turn, the RAP-binding sites of clusters II and IV overlap the sites for α_2M, the uPA−PAI1 complex, factor IXa, FVIII, and its A2 domain.[16,18,36,48] Notably, the A2-binding sites of clusters II and IV are equal to those for RAP and also exceed those for FVIII;[36] at the same time, the affinity of A2 for LRP ($K_D \sim 20$ nM)[34] is markedly higher than the affinity of FVIII for LRP ($K_D \sim 100$ nM).[23,24] Thus, the higher capacity of LRP for binding FVIIIa is based on the higher capacity of this receptor for binding each FVIIIa remnant (A2 and HDa).

In turn, the length of the FVIIIa-binding region on cluster III raises a reasonable question if this region "wraps" FVIIIa (i.e., HDa) or provides several alternative sites for binding. The first model would be in accordance with the data that show that the LRP-binding site of FVIII is formed by an extended region on the LCh, which involves all its domains[29−32] and can interact with either cluster II or IV.[25,29,49] However, the similarity of FVIIIa affinities for cluster III and its fragments (i.e., CR.17−18) and the complete inhibition of binding of FVIIIa (HDa) to the cluster by scFv iKM33 favors the second model. Indeed, the relatively small iKM33 (~30 kDa) would effectively block only the C1 domain[46] and the areas in the proximity of it.

With regard to other known ligands of cluster III, the first model can be applicable for ApoE. Indeed, the cluster's binding site for ApoE is presented by a CR triplet (CR.16−18), and the CR doublet (CR.17−18) binding most strongly to a relevant ApoE peptide was 10-fold weaker than with the triplet.[47] At the same time, the RAP binding can be relevant to both models. In support for the first model, such binding was proposed to involve simultaneously up to six adjacent CRs of a receptor.[40] The relevance of the second model is favored by studies (including our study) that showed that the RAP affinity for a CR doublet does not increase significantly upon addition of a third CR and is similar to that for the whole receptor.[11,33,40,50]

In turn, it is interesting to elucidate what determines the binding specificity of RAP toward particular CRs. During LRP biosynthesis, RAP facilitates the folding of its CR moiety;[51,52] thus, one would expect RAP to bind any CR of LRP. However, RAP was found to bind only the CRs that are closely homologous with the conserved residues: with our present results, these are CRs 3−9, 15−19, and 23−29, located within clusters II−IV, respectively (Figure S1 of the Supporting Information).[11,16,21,36] Notably, our mapping of the cluster III-binding site for RAP is consistent with a previous finding that RAP binds only the C-terminal half of the cluster.[21]

In our mutagenesis experiments, we demonstrated the critical role of a conserved aromatic residue at position 1 of a CR (Figure S1 of the Supporting Information). Replacement of this residue with serine in selected CRs of cluster III, while preserving the structure,[33] resulted in the complete abolishment of RAP binding (Figure 7A), which is similar to what was observed upon mutating LDLR.[33] In contrast, the interactions of FVIIIa (HDa) with the mutated cluster III (Figure 7B,C) and of FVIII with the mutated LDLR in a previous study[33] were less affected. The higher sensitivity of RAP to such mutations indicates that its binding to LDLR receptors is mostly based on the classical docking of the lysines,[5] which would explain why RAP recognizes such a broad spectrum of receptors. In turn, the lower sensitivity of FVIII and FVIIIa to the mutations indicates the involvement of more interface residues compared to their number in the interaction with RAP.

Our data support the involvement of LRP in the clearance of FVIIIa, in addition to that shown previously for FVIII. Indeed, the clearance in mice of both FVIIIa remnants, HDa (present work) and A2,[34] was faster by ~2.3- and ~4.5-fold, respectively, than that of FVIII. These results are consistent with better exposure of LRP-binding sites in FVIIIa than in FVIII and the greater capacity of LRP for binding FVIIIa. These experiments are likely representative of human conditions, because the affinity of human FVIII for murine vWF is similar to that of human vWF ($K_D \sim 0.2$ nM).[53,54] Therefore, the injected FVIII most likely rapidly complexed with the intrinsic murine vWF, which blocked its LRP-binding site (in the LCh) and resulted in the slower clearance of FVIII.

In the presence of RAP, the clearance of both HDa and A2 was prolonged by ~1.5- and ~3.5-fold, respectively. This is consistent with the ability of RAP to inhibit LDLR family ligands *in vitro* and *in vivo*. These data further support the role of LRP in the clearance of FVIIIa, though the involvement of other LDLR family receptors cannot be excluded. In particular, hepatic LDLR binds the A2 domain *in vitro*[55] and was shown to cooperate with LRP in the regulation of FVIII *in vivo*.[25] Two other members of the LDLR family, VLDLR and megalin, also present in the circulation, are expressed in the endothelium and kidney, respectively. Although both receptors bind FVIII *in vitro*,[55,56] the role of VLDLR in FVIII clearance has been excluded, and the role of megalin is questionable because of its accessibility to only small ligands[57,58] (which could be the A2 domain).

One should take into account the fact that LRP-mediated clearance of FVIII via its direct interaction with LRP may not be the primary mechanism of FVIII catabolism. Previously, we demonstrated the existence of LRP-independent FVIII clearance and showed that both pathways involve cell surface heparan-sulfate proteoglycans.[24,26] At the same time, these pathways are relevant to a small FVIII fraction (<5%) not bound to vWF, whereas the bulk of FVIII is bound to vWF. Therefore, the major mechanism of FVIII clearance may involve the FVIII−vWF complex as a number of observations indicate that the half-life of FVIII (~14 h) is limited by the half-life of vWF (<20 h).[59,60] Indeed, the half-lives of several FVIII variants with modifications of the molecule to prolong its lifetime in recent clinical trials did not exceed 19 h.[61] Notably, one of the pathways of vWF clearance also involves LRP,[60] and the FVIII−vWF complex may follow this pathway. All these mechanisms require further investigation.

In conclusion, we demonstrate that LRP has a greater binding capacity for FVIIIa than for FVIII, and the clearance of FVIIIa involves the LDLR family. Our results show that the role of LRP cluster III in binding ligands is more significant than previously thought. Future studies will include characterizing the interactions of FVIIIa with LRP clusters II and IV, LDLR,

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and VLDLR, as the latter has potential to regulate FVIIIa (in endothelium). These directions will facilitate elucidation of FVIII clearance mechanisms and, in particular, generation of new longer-lasting FVIII products for better treatment of hemophilia A.

■ ASSOCIATED CONTENT

Supporting Information

LRP CR alignment and additional data from binding experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

uPA−PAI1, urokinase-type plasminogen activator−plasminogen activator inhibitor-1 complex; α_2M, α_2-macroglobulin; RAP, α2M receptor-associated protein; LRP, low-density lipoprotein receptor-related protein 1; LDLR, low-density lipoprotein receptor; VLDLR, very low-density lipoprotein receptor; CR, complement-type repeat; FVIII, blood coagulation factor VIII; HCh, heavy chain of FVIII; LCh, light chain of FVIII; FVIIIa, activated FVIII; A2, A2 domain of FVIIIa; HDa, A1/A3′-C1-C2, a heterodimer of FVIIIa; FIXa, activated blood coagulation factor IX; scFv, single-chain variable antibody fragment; HBS-HEPES, 10 mM NaCl and 0.1 M Tween 20 (0.005%, pH 7.2); HBS-Ca, HBS-P buffer with 5 mM $CaCl_2$ and 0.005% Tween 20; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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