THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 30, pp. 22033–22041, July 26, 2013
Published in the U.S.A.

Mapping the Binding Region on the Low Density Lipoprotein Receptor for Blood Coagulation Factor VIII*[S]

Received for publication, March 11, 2013, and in revised form, May 24, 2013 Published, JBC Papers in Press, June 10, 2013, DOI 10.1074/jbc.M113.468108

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Background: Low density lipoprotein receptor (LDLR) mediates clearance of blood coagulation factor VIII (FVIII).
Results: The region of complement-type repeats 2–5 in LDLR was identified as the binding site for FVIII and for α-2-macroglobulin receptor-associated protein (RAP).
Conclusion: Binding sites of LDLR for FVIII, and also for RAP, were characterized.
Significance: This provides new data on LDLR structure and function and on FVIII catabolism.

Low density lipoprotein receptor (LDLR) was shown to mediate clearance of blood coagulation factor VIII (FVIII) from the circulation. To elucidate the mechanism of interaction of LDLR and FVIII, our objective was to identify the region of the receptor necessary for binding FVIII. Using surface plasmon resonance, we found that LDLR exodomain and its cluster of complement-type repeats (CRs) bind FVIII in the same mode. This indicated that the LDLR site for FVIII is located within the LDLR cluster. Similar results were obtained for another ligand of LDLR, α-2-macroglobulin receptor-associated protein (RAP), a common ligand of receptors from the LDLR family. We further generated a set of recombinant fragments of the LDLR cluster and assessed their structural integrity by binding to RAP and by circular dichroism. A number of fragments overlapping CR.2-5 of the cluster were positive for binding RAP and FVIII. The specificity of these interactions was tested by site-directed mutagenesis of conserved tryptophans within the LDLR fragments. For FVIII, the specificity was also tested using a single-chain variable antibody fragment directed against the FVIII light chain as a competitor. Both cases resulted in decreased binding, thus confirming its specificity. The mutagenic study also showed an importance of the conserved tryptophans in LDLR for both ligands, and the competitive binding results showed an involvement of the light chain of FVIII in its interaction with LDLR. In conclusion, the region of CR.2-5 of LDLR was defined as the binding site for FVIII and RAP.

Factor VIII (FVIII)[2] is a plasma protein (~300 kDa) composed of a heavy chain and a light chain with the domain structures of A1-A2-B and A3-C1-C2, respectively (1). FVIII serves as a cofactor for activated factor IX in the intrinsic pathway of blood coagulation, and deficiency in FVIII results in a bleeding disorder known as hemophilia A. This disease is treated by FVIII products, which require frequent infusions due to the short half-life of FVIII in plasma (~14 h). Despite the clinical significance of understanding mechanisms of FVIII clearance, the details of it are unknown.

One of receptors involved in clearance of FVIII is the low density lipoprotein receptor (LDLR). In this process, LDLR acts in concert with the low density lipoprotein receptor-related protein (LRP). Indeed, deficiency in either LDLR or LRP in mice prolonged the half-life of FVIII about 1.5-fold, whereas the combined deficiency resulted in ~4.8-fold prolongation (2). Furthermore, inhibition of both receptors by their highly specific ligand α-2-macroglobulin receptor-associated protein (RAP) (3, 4) increased its half-life in mice (5, 6). In humans, polymorphism in either LDLR or LRP is associated with elevated levels of FVIII (7–10).

LDLR and LRP belong to a large group of endocytic receptors known as the LDLR family. Members of this family are structurally similar, yet have a diverse pattern of tissue expression and a broad spectrum of ligands. LDLR and LRP, expressed in the liver, serve to catabolize various plasma ligands (11, 12), in addition to FVIII. Two other receptors from the family, very low density lipoprotein receptor (VLDLR) and megalin, are also found in the circulation. Although both receptors bind to FVIII in a purified system (13, 14), they are likely not involved in its clearance *in vivo* (15, 16).

The ligand binding moiety of the LDLR family receptors is represented by highly homologous complement-type repeats (CRs). Each CR is composed of ~40 amino acid residues and forms an autonomous domain. All CRs of LDLR were characterized for their tertiary structures (17–22), as well as some CRs of LRP (23). These data showed that each CR domain contains three internal disulfide bonds, formed by six conserved cys-

* This work was supported by funds from the Center for Biologics Evaluation and Research (CBER) of the United States Food and Drug Administration (FDA) and also by the Research Participation Program at the CBER administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the United States Department of Energy and the United States Food and Drug Administration. This work was initially presented at the 54th American Society of Hematology Annual Meeting, December 8–11, 2012, in Atlanta, GA.

[S] This article contains supplemental Tables S1 and S2 and Figs. S1 and S2.

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[2] The abbreviations used are: FVIII, blood coagulation factor VIII; BDD-FVIII, B-domain-deleted recombinant FVIII; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; LDLR cluster, cluster of complement-type repeats of LDLR; VLDLR, very low density lipoprotein receptor; CR, complement-type repeat; CR doublet, a pair of adjacent CRs; RAP, α-2-macroglobulin receptor-associated protein; ScFv, single-chain variable antibody fragment; HBS, HEPES-buffered saline.

teines, and coordinates Ca^{2+} via several conserved acidic residues. During the interaction with a ligand, each CR domain "docks" a specific lysine via conserved tryptophan and acidic residues (18, 21, 24–26).

The CR domains are connected to each other by short flexible linkers (23) and are composed in clusters. LDLR contains seven CRs grouped in one cluster (11), whereas LRP contains 31 CRs grouped in four clusters (12). Typically, the binding sites of the ligands are formed by several adjacent CRs, among which a minimal binding unit is presented by a pair of CRs (CR doublet). Such organization of the sites was found in LDLR for binding RAP (22), apoE (27), and apoB (28) and found in LRP for a number of its ligands including FVIII (29–31).

For FVIII, LRP has two binding sites; each site is formed by 3–4 adjacent CRs and located in a separate CR cluster of the receptor (31, 32). At the same time, the FVIII-binding site in LDLR is unknown. *In vitro*, the affinity of FVIII to LDLR (K_D of ~200 nM) (14) was found to be less than to LRP (K_D of ~80 nM) (2, 5, 33). Such affinities are unlikely to provide effective direct interactions of FVIII with both receptors *in vivo*, considering the concentration of FVIII in plasma (~0.3 nM). For LRP, its *in vivo* interaction with FVIII is facilitated by cell surface heparan sulfate proteoglycans (34). Whether this type of receptors serves a similar role for LDLR is unknown.

In the present work, we aimed to determine the specific CRs of LDLR responsible for FVIII binding. We generated a set of LDLR fragments and tested their ability to bind FVIII in a purified system. The specificity of these interactions was verified using an anti-FVIII antibody fragment and site-directed mutagenesis of the LDLR fragments. As a result, we identified specific CRs of the receptor that form a binding region for FVIII.

EXPERIMENTAL PROCEDURES

Reagents—FVIII products, Advate (Baxter, CA) and Xyntha (Wyeth, PA), corresponding to recombinant full size FVIII and BDD-FVIII, respectively, were purchased from the National Institutes of Health Pharmacy (Bethesda, MD). Plasma FVIII was isolated as described (35). Recombinant LDLR exodomain (expressed in mouse cells) and RAP (expressed in bacteria) were purchased from R&D Systems (Minneapolis, MN). Anti-FVIII ScFv iKM33 was produced as described (36). LDLR cDNA was obtained from Dr. G. Rudenko. Anti-*myc* tag mAb 9E10 was purchased from Sigma-Aldrich.

Generation of Constructs Coding LDLR Fragments—A modified pFastBac1 plasmid containing a melittin secretion signal, His_6 tag, a multiple cloning site, c-*myc* tag, and a stop codon was used as a vector as described (37). The coding regions of the LDLR fragments were generated by PCR using LDLR cDNA as a template and corresponding primers. Point mutations of selected LDLR fragments were performed by overlapping PCR. All resulting PCR fragments were cloned into the modified pFastBac1 vector.

Expression and Purification of the LDLR Fragments—Recombinant baculoviruses for the wild-type and mutant fragments of LDLR were generated using the plasmid constructs and the Bac-to-Bac expression system (Invitrogen). Optimization of the expression of the proteins in Sf9 cells was performed as described (38). The proteins were expressed in 120 ml of the suspension culture. The culture supernatants were harvested 72 h after infection and exchanged into PBS, pH 7.4, buffer using a tangential flow filter Pellicon XL 5k (Millipore, Billerica, MA). The fragments were then purified on nickel-Sepharose 6 Fast Flow resin (GE Healthcare). Upon adjusting the solutions to contain 300 mM NaCl, 20 mM imidazole, they were passed through the column followed by washing the resin with PBS, pH 7.4, 300 mM NaCl, 30 mM imidazole and eluting the proteins with PBS, pH 7.4, 150 mM NaCl, 230 mM imidazole. The eluted product was concentrated and further purified using size-exclusion chromatography on either Superdex-75 or Superdex-200 (GE Healthcare) to isolate the monomeric forms in 20 mM HEPES, pH 7.4, 0.15 M NaCl, 0.005% Tween 20, and 5 mM $CaCl_2$ (HBS/Ca). LDLR CR cluster (CR.1-7) and its mutant were refolded as described (29). All proteins were verified by PAGE with GelCode Blue Safe (Thermo Scientific) staining and Western blotting with anti-*myc* mAb9E10. The protein concentrations were measured by absorbance spectroscopy at 280 nm using calculated respective extinction coefficients.

Circular Dichroism Measurements—Far-UV CD measurements were performed on a Jasco J-815 spectropolarimeter (JASCO Co., Japan) at 25 ± 0.2 °C, as maintained by a PTC 423S/15 Peltier temperature controller (JASCO). The protein concentration in the samples was adjusted to ~ 30 μM in HBS/Ca. Titration of the samples by EDTA was conducted stepwise by adding a calculated amount of the 0.5 M stock solution. The spectra were recorded between 180 and 260 nm in a 0.5-mm path length quartz cuvette using a scan speed of 20 nm/min, bandwidth of 1.0 nm, and resolution of 0.2 nm and accumulated in triplicate. An ellipticity of CD spectra was expressed in millidegrees.

Surface Plasmon Resonance Measurements—Prior to the measurements, FVIII products were reconstituted and dialyzed in a HBS/Ca buffer prepared by adjusting HBS-P buffer (GE Healthcare) to contain 5 mM of $CaCl_2$. The concentration of the dialyzed FVIII was measured using a BCA kit (Thermo Scientific). Binding assays were performed in HBS-P/Ca using Biacore 3000 (GE Healthcare). LDLR fragments were immobilized on a CM5 chip using an amine coupling kit at ~1000 resonance units for CR doublets and at ~2000 resonance units for the CR cluster. Association of the fragments with RAP or with FVIII in the presence or absence of iKM33 was recorded at a flow rate of 10 μl/min for 3 min. The dissociations were recorded for 5 min in the running buffer. Regeneration of the sensors was performed by 0.1 M H_3PO_4. The estimated K_D values were derived by fitting the association and dissociation curves with a 1:1 (Langmuir) model using the BIAevaluation 4.1.1 program.

RESULTS

Expression of LDLR Fragments—The generated LDLR fragments were its CR cluster and six overlapping CR doublets (Figs. 1 and 2). This strategy was assumed suitable, based on its previous use for mapping the RAP-binding site on LRP (29). The LDLR fragments were expressed in insect cells, which were previously shown capable of producing functionally active fragments of LRP and megalin (37, 39, 40). The LDLR fragments obtained were essentially pure (Fig. 1); the observed double

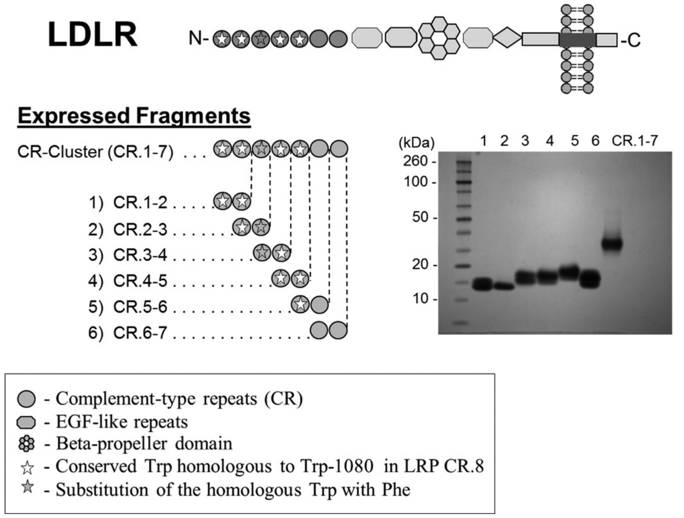

FIGURE 1. **The domain structure of LDLR, strategy of expressing its fragments, and their analysis by PAGE.** The *inset* shows an image of the GelCode Blue Safe-stained gel with the purified LDLR fragments.

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                                        1     2 3 4     5   67
          ●        ●          ●         ▼    ●◊ ▼ ◊   ● ◊   ◊◊        ●
CR-1  GDR CERNE FQ CQDGK      CISYKWV CDGSAE CQDGSDESQET CL
CR-2  SVT CKSGD FS CGGRVNR    CIPQFWR CDGQVD CDNGSDEQG   CP
CR-3  PKT CSQDE FR CHDGK      CISRQFV CDSDRD CLDGSDEAS   CP
CR-4  VLT CGPAS FQ CNSST      CIPQLWA CDNDPD CEDGSDEWPQR CRGL
CR-5  SSP CSAFE FH CLSGE      CIHSSWR CDGGPD CKDKSDEEN   CA
CR-6  VAT CRPDE FQ CSDGN      CIHGSRQ CDREYD CKDMSDEVG   CVNV
CR-7  VTL CEGPNKFK CHSGE      CITLDKV CNMARD CRDWSDEPIKE CGTN

CR-8  PGG CHTDE FQ CRLDGL     CIPLRWR CDGDTD CMDSSDEKS   CEGV
(LRP)
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FIGURE 2. **LDLR complement-type repeats.** The sequences are aligned by the conservative cysteines (●). Other conservative residues (in *gray*) are aspartic (*D*) or glutamic (*E*) acid residues 2, 4–7 (◊) coordinating Ca^{2+} via side-chain carboxyl oxygens, and those, 1 and 3 (▼), coordinating Ca^{2+} via backbone carbonyl oxygens. Conservative residues 4 and 5 coordinate Ca^{2+} alternatively. Conservative tryptophan (*W*) at position 1 (in *bold*) also interacts with the aliphatic moiety of the lysine of the ligand. Shown at the *bottom* is LRP CR.8, which matches the consensus sequence (in *italics*).

banding for some of the CR doublets was attributed to a possible difference in glycosylation, similar to that found previously for expressed fragments of LRP (31).

Interaction of LDLR Fragments with RAP—Because RAP is known to inhibit interactions of the LDLR family with their ligands *in vitro*, the integrity of the LDLR fragments was assessed by testing them for binding to RAP. As a control, we used the LDLR exodomain (full size), expressed in mammalian cells. In preliminary experiments, we found that the preparation of the LDLR cluster (of CRs) was less active for binding than some CR doublets, likely due to a higher content of the misfolded form. Chemical refolding of this preparation resulted in an increase of its binding activity; therefore, this approach was employed in further studies.

In SPR, both the LDLR exodomain and its cluster bound RAP in a similar mode (Fig. 3, *A* and *B*). The assessed kinetic constants including both K_D values (~1 nM, supplemental Table S1) were similar. This indicated that the LDLR cluster contains all necessary elements of the receptor for binding RAP, similar to that previously found for LRP (29). Among the CR doublets overlapping the cluster, CR.2-3, CR.3-4, and CR.4-5 were able to bind RAP, whereas other doublets were inactive (Fig. 3, *C–H*). The assessment of the kinetic constants indicated higher affinity for CR.3-4 and CR.4-5 (K_D values of 3–6 nM) than CR.2-3 (K_D ~29 nM). Although the binding signals did not fit well, several independent experiments produced essentially the same results. These data suggested that the LDLR region CR.2-5 forms a binding site for RAP.

Interaction of LDLR Fragments with FVIII—Under the same experimental conditions, the LDLR fragments were tested for binding to FVIII (plasma-derived). We found that FVIII bound to the LDLR exodomain and LDLR cluster in a similar fashion (Fig. 4, *A* and *B*). Although fitting of the signals was not optimal, similar to that found for FVIII and LRP (41), the respective kinetic constants including K_D values (30–60 nM) were assessed as similar (supplemental Table S2). These data indicated that the LDLR cluster contains all required elements to bind FVIII, similar to that found for LRP clusters (31). Among the CR doublets, CR.2-3, CR.3-4, and CR.4-5 were found active for FVIII (Fig. 4, *C–F*) with similar K_D values (40–90 nM). These data suggested that the LDLR region CR.2-5 forms a binding site for FVIII.

To confirm the results, all the CR doublets were retested with two recombinant FVIII variants, full size FVIII and BDD-FVIII. We asked whether the same CR doublets bind to all FVIII variants. At a selected concentration, each FVIII preparation was tested with each CR doublet for the signal intensity in the association phase. We found that active binding LDLR fragments were indeed the same for all three FVIII variants (Fig. 5). At the same time, the intensity of the signals differed among the FVIII variants, possibly reflecting their variability in characteristics. In particular, BDD-FVIII has a lower molecular mass producing lower signals; therefore, a higher concentration of BDD-FVIII was used. More extensive pre-processing of plasma-derived FVIII possibly caused its lower signals as compared with those of the full size recombinant FVIII. Other characteristics include heterogeneity of full size FVIII due to the natural variability in the B-domain length and other properties, previously compared between various FVIII products (42–45). A residual binding of both recombinant FVIII variants to the CR doublets non-active for plasma-derived FVIII was attributed to a non-specific component. Also, our data suggest that the B-domain of FVIII does not contribute to its binding to LDLR, similarly to that indicated for LRP (41). In conclusion, our data demonstrate that the LDLR-binding site is essentially the same for each FVIII variant, which supports the results of the mapping study.

Interaction of LDLR Fragments with FVIII in the Presence of an Anti-FVIII Antibody Fragment—The specificity of FVIII binding to the LDLR fragments was verified using a recombinant anti-FVIII ScFv iKM33 (36), which recognizes the C1-domain of FVIII and inhibits its binding to LDLR (46–48). We asked whether iKM33 had a similar effect on the binding of FVIII to the LDLR fragments. FVIII (full size recombinant) was incubated with increasing amounts of iKM33, and the samples were tested with the LDLR cluster and CR.2-3, CR.3-4, and CR.4-5. In all cases, iKM33 strongly inhibited the binding in a dose-dependent manner (Fig. 6), thus supporting the specificity of the interactions. Also, these data indicate involvement of the FVIII light chain, in particular its C1-domain, in the binding to LDLR.

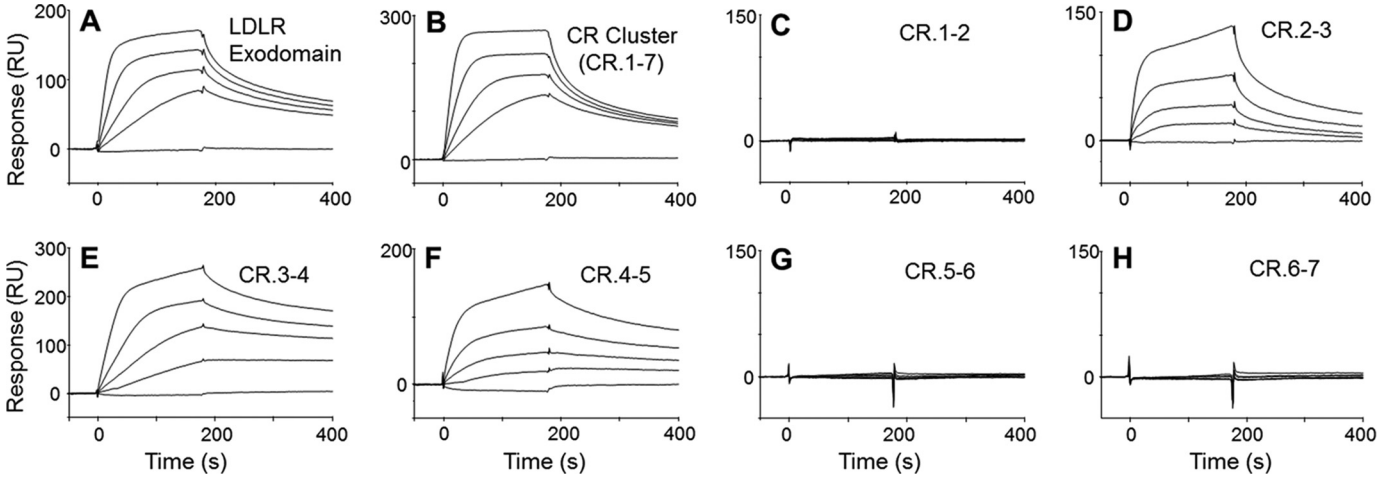

FIGURE 3. **Binding of RAP to LDLR fragments.** In SPR, the LDLR fragments were immobilized on a sensor at ~2000 resonance units (*RU*) for the LDLR exodomain (*A*) and LDLR cluster (*B*) and at ~1000 resonance units for CR doublets (*C–H*) and tested for binding with RAP (0, 2.5, 5, 10, and 20 nM). The RAP association was recorded for 3 min followed by measuring its dissociation in the running buffer. *RU*, resonance units.

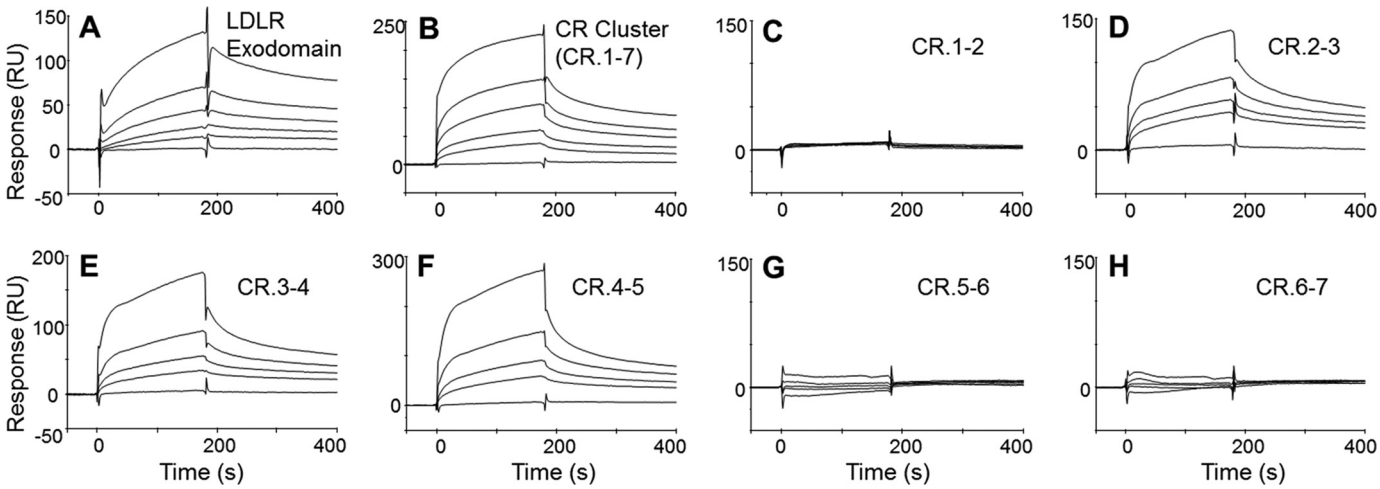

FIGURE 4. **Binding of FVIII to LDLR fragments.** In SPR, the LDLR fragments, LDLR exodomain (*A*), LDLR cluster (*B*), and CR doublets (*C–H*), were immobilized and tested for binding with various concentrations of FVIII (0, 56.3, 112.5, 225, and 450 nM). *RU*, resonance units.

Interaction of Mutated CR Doublets with FVIII and RAP—To further verify specificity of the interactions of the LDLR fragments with FVIII, we asked whether specific mutations of the fragments have an effect on the binding. As shown previously, a conserved tryptophan in position 1 (Fig. 2) of a CR from the LDLR family interacts with the aliphatic chain of a lysine residue of the ligand (18, 21, 24–26). A replacement of such tryptophan by serine in either CR of CR.5-6 of LRP significantly reduced its binding to RAP, thus confirming specificity of this interaction (30).

Within the CR.2-5 region, the conserved tryptophan is presented in CR.2 (Trp-66), CR.4 (Trp-144), and CR.5 (Trp-193), whereas CR.3 contains a phenylalanine (Phe-105) at this position (Fig. 2). Using the above mutagenic approach (30), we targeted these tryptophans in all active binding CR doublets in a way to affect one CR per doublet. The respective replacements (Trp → Ser) were made in CR.2 of CR.2-3, in CR.4 of CR.3-4, and in CR.5 of CR.4-5, resulting in generation of CR.2-3 (W66S), CR.3-4 (W144S), and CR.4-5 (W193S), respectively. Hereafter, the CR domains affected by the mutagenesis are marked with an asterisk (*).

In SPR, we found that binding of RAP and FVIII (full size recombinant) to CR.2*-3 and CR.3-4* was significantly reduced (supplemental Fig. S1). At the same time, the binding of both ligands to CR.4-5* was not affected (data not shown). The additional mutation (Trp → Ser) introduced into CR.4 of this doublet completely abolished binding of the resulting double mutant (CR.4*-5*) to both RAP (Fig. 7, *A* and *B*) and FVIII (Fig. 7, *C* and *D*). Altogether, these data indicated that the interactions of the doublets CR.2-3, CR.3-4, and CR.4-5 with RAP and FVIII are specific.

Assessment of the Conformation of CR Doublets and Their Mutants—Using far-UV CD, the conformation of all CR doublets and their mutants was assessed for the presence of bound Ca^{2+}, which is essential for proper folding. All the initial CD spectra (190–260 nm) were highly similar, suggesting similarity in the secondary structure content. Upon the titration of a CR doublet with a Ca^{2+}-chelating agent (EDTA), we observed both a dose-dependent shift of the negative maximum (~200 nm) to a longer wavelength (~206 nm) and a decrease of the signal intensities by 34–43%. These changes were highly similar among all preparations of the CR doublets. The spectra for a

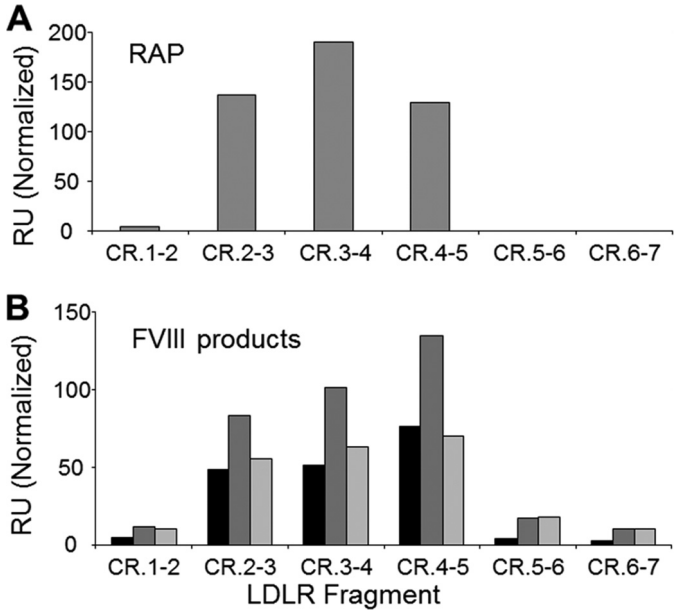

FIGURE 5. **Binding of various FVIII products to CR doublets of LDLR.** In SPR, the LDLR fragments were immobilized and tested for binding with RAP (32 nM) (*A*) and with FVIII variants plasma-derived FVIII (225 nM, *dark gray*), full size recombinant FVIII (275 nM, *medium gray*) and BDD-FVIII (400 nM, *light gray*) (*B*). The bars correspond to signals recorded after 3 min of the association. *RU*, resonance units.

representative doublet CR.4-5 and its double mutant (CR.4*-5*) are shown in Fig. 8. For other active binding doublets, CR.2-3, CR.3-4, and their mutants, the spectra are shown in supplemental Fig. S2, and not shown for the non-binders.

Based on previous studies (19, 20, 49–51), the observed spectra changes reflect a gradual loss of the secondary structures of the proteins due to progressive removal of the bound Ca^{2+}. Therefore, the similarity of the spectra is indicative of the correctness and similarity of the folding of all the CR doublets including their mutants. Due to the structural independence of CRs (23), it is likely that each individual CR domain is correctly folded within both the respective CR doublet and the LDLR cluster. Thus, the CD data further supported the results of our binding studies.

Interaction of the Mutated LDLR Cluster with FVIII and RAP—Finally, we reproduced all the above mutations within the LDLR cluster. The resulting mutant CR.1-7 W66S/W144S/W193S (CR.1-2*-3-4*-5*-6-7) had all active CR pairs affected. Based on our CD data, we assumed similarity of the folding of both the wild type and the mutant variants. In SPR, the binding of the mutated cluster to RAP (Fig. 7, *E* and *F*) and to FVIII (full size recombinant) was diminished (Fig. 7, *G* and *H*). The residual interaction of the mutated cluster with FVIII, also observed for the doublets CR.2*-3 and CR.3-4* (supplemental Fig. S1), was likely supported by the non-mutated CR.3, which was presented in each of these fragments.

Thus, specific point mutations in all active binding LDLR fragments did affect their interactions with RAP and FVIII. This confirms the specificity of the interactions and validates the mapping results. These data also demonstrate an important role of the LDLR tryptophans 66 (CR.2), 144 (CR.4), and 193 (CR.5) for the interactions of LDLR with RAP and FVIII.

DISCUSSION

In the present study, we demonstrate that the second through fifth complement-type repeats of LDLR provide a binding site for both FVIII and RAP. These conclusions are based on results from testing the binding of these ligands to various fragments of LDLR in the presence or absence of an anti-FVIII antibody fragment and to the fragments containing mutations of selected conserved residues.

The overlap of FVIII- and RAP-binding sites on LDLR is in accordance with other studies. In LRP-deficient mice, adenovirus-mediated overexpression of RAP resulted in an increase of FVIII level (6). Most likely, this was due to blocking of LDLR by RAP, and not other RAP-sensitive determinants. Among them, VLDLR (expressed in endothelium) does not contribute to FVIII clearance in mice (15); also, such a role of megalin cannot be justified due to its location in the renal proximal tubule, accessible only to small plasma ligands (16). In turn, upon injection of [^{125}I]FVIII in mice, most of the radioactivity was accumulated in the liver (5), which further supports the role of LDLR in the above study (6).

Our data support the general model of ligand recognition by the LDLR family, previously defined in a number of studies (17, 21, 22, 24, 29, 52–54). According to this model, a CR consensus sequence contains a conserved tryptophan at position 1 and acidic residues at positions 2, 6, 7, and either 4 or 5, and predominantly an acidic residue at position 3 (Fig. 2). These residues coordinate Ca^{2+} via backbone carbonyls (positions 1 and 3) and side-chain carboxyls (positions 2, 6, 7, and either 4 or 5). The side chain of the conserved tryptophan also interacts with the aliphatic moiety of the lysine of the ligand. In our mutagenic study, the conserved tryptophans of CR.2, CR.4, and CR.5 were found to be important for binding RAP and FVIII. Moreover, our study indicated that the less conserved homologous phenylalanine (Phe-105) of CR.3 for both ligands serves the same role as the tryptophan. This observation supports the data of Fisher *et al.* (22), which proposed the same role of this phenylalanine (in CR.3) for the interaction with RAP.

In accordance with previous studies (17–21, 50, 55, 56), our CD study showed that all seven CR domains of LDLR coordinate Ca^{2+}. Importantly, this study showed that the folding of all expressed CR doublets and their mutants was similar, thus supporting the validity of our mapping results. At the same time, the folding of the purified LDLR cluster was less efficient based on its low binding activity. This was most likely due to more complexity of the cluster as compared with a CR doublet. Upon refolding of the preparation of the cluster, its binding activity was restored to the level comparable with the LDLR exodomain (expressed in mammalian cells).

In regard to the affinity of RAP for LDLR, their K_D was previously assessed as 50–250 nM in solid phase- and cell culture-based assays (57). In our study, based on real-time binding, the assessed K_D values for both the LDLR exodomain and the LDLR cluster (~1 nM) were comparable with RAP K_D values for other receptors from the LDLR family: VLDLR (K_D = 0.7 nM), megalin (K_D = 8 nM), and LRP (K_D = 18 nM) (58–60). The discrepancy between our result and that of Medh *et al.* (57) was likely due to differences in the experimental conditions. More importantly, the

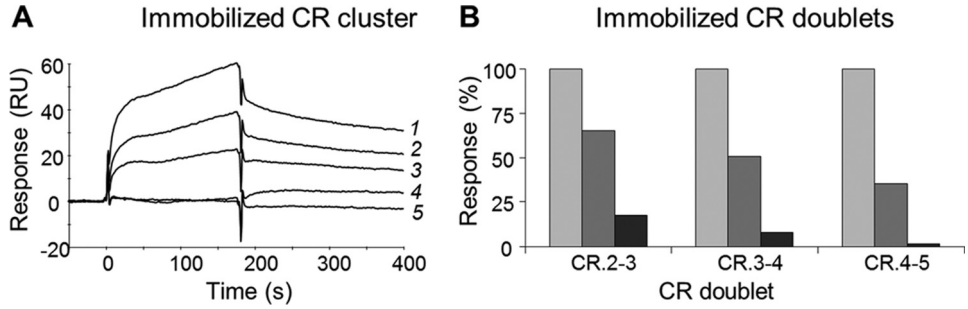

FIGURE 6. **Binding of FVIII to LDLR fragments in the presence of ScFv iKM33.** *A*, in SPR, immobilized LDLR cluster was tested for binding with FVIII (full size recombinant FVIII, 275 nM) in the absence of iKM33 (*1*) or in its presence at 275 nM (*2*), 550 nM (*3*), or 1375 nM (*4*). *Curve 5* corresponds to injection of buffer. *RU*, resonance units. *B*, in a similar way, the immobilized CR doublets 2-3, 3-4, and 4-5 were tested for binding with FVIII (275 nM) in the absence of iKM33 (*light gray bars*) or in its presence at 275 nM (*dark gray bars*) or 1375 nM (*black bars*). The resulting signals were recorded after 3 min of the association and are expressed in percentages of those obtained in the absence of iKM33.

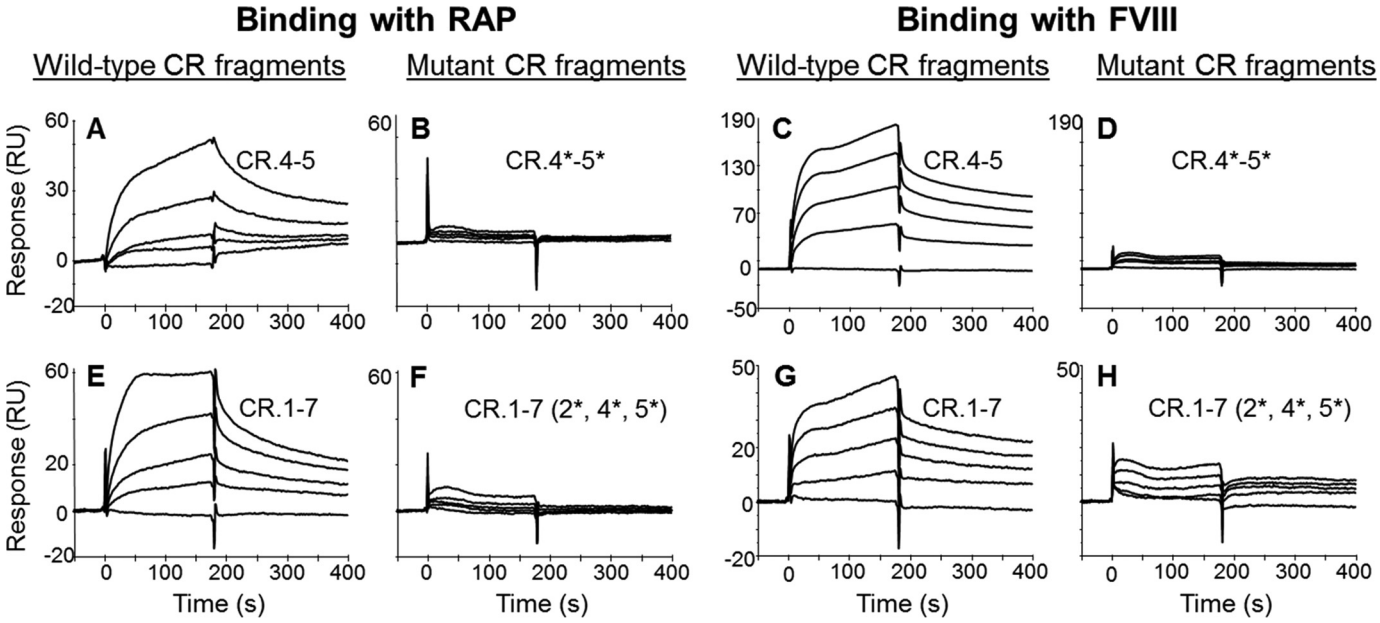

FIGURE 7. **Binding of RAP and FVIII to the mutants of CR.4-5 and LDLR cluster.** In SPR, CR.4-5 (*A* and *C*), CR.4-5 W144S/W193S (*B* and *D*), LDLR cluster (*E* and *G*), and its triple-mutant W66S/W144S/W193S (*F* and *H*) were immobilized and tested for binding with RAP (0.6, 1.3, 2.5, and 5 nM) (*A*, *B*, *E*, and *F*) and FVIII (full size recombinant FVIII, 50, 100, 150, and 200 nM) (*C*, *D*, *G*, and *H*). Injections of the buffer only were used as controls. For each pair of a particular LDLR fragment and its mutant tested *versus* a given ligand, the signals are shown in the same scale. CR domains affected by mutagenesis are marked by *. *RU*, resonance units.

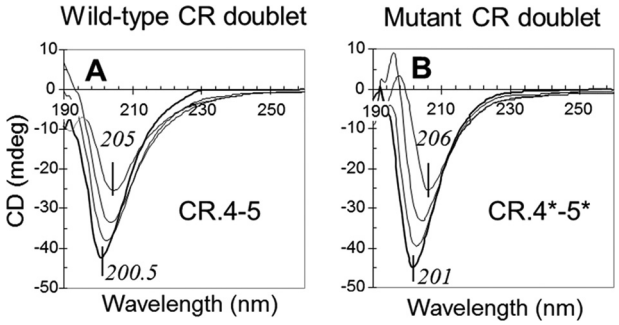

FIGURE 8. **Far-UV CD spectra of CR.4-5 and its mutant upon the titration by EDTA.** CR.4-5 (*A*) and CR.4-5 W144S/W193S (*B*) were taken at concentrations of 30 μM. The spectra correspond to the absence of EDTA (*bottom curves* in *bold*) and its increase as 1×, 2×, and 3× over molar equivalent of Ca^{2+} in the solutions, shown as respective increase in the signals (*upper curves*). *mdeg*, millidegrees.

similarity of the affinities of both LDLR fragments in our study showed that the RAP-binding site of LDLR is located within its cluster.

Our mapping of the RAP-binding site of LDLR is in agreement with previous studies, which showed that the isolated CR.3-4 and CR.4-5 bind RAP (22, 56). Adding CR.2-3 to this set defines the whole RAP-binding region of LDLR as CR.2-5 with a possible preference for CR.3-5. As RAP is a common ligand of the LDLR family, it is worthwhile to compare its binding sites between the receptors. In LRP cluster II, the RAP-binding region is presented by seven adjacent CRs and comprises the sites for other ligands (30, 31, 37, 61). In LDLR, the RAP-binding region comprises the sites for FVIII (present study) and apoE (CR.4-5) (27), whereas a longer region involving CR.3-7 and an EGF-like repeat is required for apoB-100 (28, 62). In contrast, proprotein convertase subtilisin/kexin type 9 (PCSK9) has an atypical site on the receptor, which is formed by the EGF-like domain (63). Thus, at least for LDLR and LRP, the binding sites for RAP typically comprise or overlap sites for other ligands.

The comparison of the LDLR-binding sites for its ligands shows that CR.4-5 is involved in the majority of these interactions. Notably, this doublet has an unusually long linker between the domains that provides more flexibility to adapt to the ligands, and the module CR.5 has significantly higher affin-

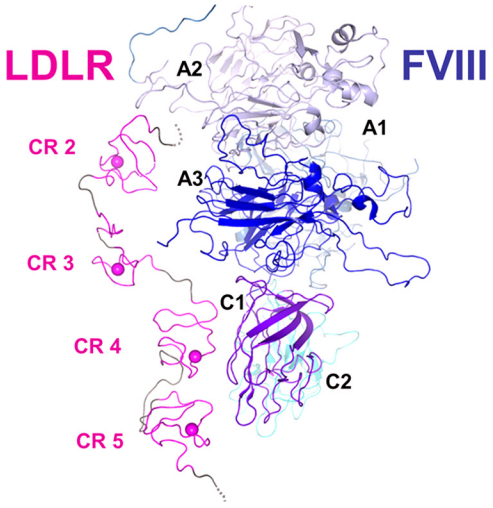

FIGURE 9. **Proposed interaction of LDLR with FVIII.** The model is based on atomic coordinates of crystal structures of the LDLR exodomain (Protein Data Bank (PDB) ID 1N7D) and FVIII (PDB ID 2R7E). For the LDLR part, only the CR.2-5 region is shown (*magenta* and *gray*). In each CR domain, the coordinated Ca^{2+} is depicted as a *sphere*. The model was constructed using PyMOL.

ity for Ca^{2+} than any other CR of the receptor (56). In contrast to other CR doublets forming the known binding sites of LDLR, CR.4-5 has the conserved tryptophans in both CRs, and our study required mutation of both to decrease the binding activity of the doublet.

The following summarizes the major data for the interactions of FVIII with LDLR and LRP. In a series of studies by Mertens and co-workers (2, 64, 65), it was shown that the CR clusters of LDLR and LRP compete for binding to FVIII. Within each of the LRP clusters II and IV, approximately four adjacent CRs form a binding site for FVIII (31, 33), whereas the matching epitope of FVIII involves multiple lysines on all three domains of its light chain (65–68). Consistent with this, the anti-FVIII ScFv KM33, which recognizes the C1-domain of the light chain, interferes with FVIII binding to both LDLR and LRP (46, 48, 69). Our present study shows that similarly to LRP, four adjacent CRs of LDLR form a site for FVIII, and on the FVIII side, the contact interface involves the C1-domain. Altogether, these data indicate that the FVIII-binding sites for both receptors are similar and involve the A3, C1, and C2 domains. Based on these considerations, we propose a model of interaction of LDLR and FVIII in which the CR.2-5 region of the receptor matches the respective site of FVIII, as shown for the particular orientation of these molecules on Fig. 9.

Future studies would involve more detailed characterization of the molecular interface between LDLR and FVIII and investigation of a possible role of heparan sulfate proteoglycans (HSPGs) or other intermediates in this interaction. These directions could facilitate generation of new FVIII products with better sustainability in the circulation.

Acknowledgments—We are thankful to Dr. Gabby Rudenko for providing us with a construct for LDLR expression and to our colleagues from FDA/CBER, Dr. Michael Kennedy and Malgorzata Norton, for support in the SPR, and to Dr. Pei Zhang, Laura Wood, and Samuel Woodle for help in the manuscript preparation.

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