Factor VIII Interacts with the Endocytic Receptor Low-density Lipoprotein Receptor-related Protein 1 via an Extended Surface Comprising “Hot-Spot” Lysine Residues

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Background: It is unclear how the LDL receptor family binds large protein ligands.

Results: HDX and lysine scanning identified factor (F)VIII regions and specific lysine residues binding low-density lipoprotein receptor-related protein 1 (LRP1).

Conclusion: FVIII-LRP1 interaction involves multiple “hot-spot” lysine residues in the A3C1 domains.

Significance: Our study sheds light on interactions of complex ligands with the LDL receptor family.

Lysine residues are implicated in driving the ligand binding to the LDL receptor family. However, it has remained unclear how specificity is regulated. Using coagulation factor VIII as a model ligand, we now study the contribution of individual lysine residues in the interaction with the largest member of the LDL receptor family, low-density lipoprotein receptor-related protein (LRP1). Using hydrogen-deuterium exchange mass spectrometry (HDX-MS) and SPR interaction analysis on a library of lysine replacement variants as two independent approaches, we demonstrate that the interaction between factor VIII (FVIII) and LRP1 occurs over an extended surface containing multiple lysine residues. None of the individual lysine residues account completely for LRP1 binding, suggesting an additive binding model. Together with structural docking studies, our data suggest that FVIII interacts with LRP1 via an extended surface of multiple lysine residues that starts at the bottom of the C1 domain and winds around the FVIII molecule.

The low-density lipoprotein receptor family is an ancient family of transmembrane receptors regulating diverse biological processes ranging from lipoprotein metabolism to blood coagulation (1–3). Members of the LDL receptor family are able to interact with an unusual number of ligands; e.g. more than 30 ligands have been reported for the largest member, LRP1, including lipoproteins, viruses, bacteria toxins, proteinase-inhibitor complexes, and coagulation proteins (4, 5). Given the importance of the LDL receptor family in human physiology, much research has been focused on the questions of how the various members of the LDL receptor family can interact with such a large variety of structurally unrelated ligands and which molecular mechanisms regulate specificity of the interaction.

The LDL receptor family is characterized by the presence of a varying number of distinct domains: the YWTD β-propeller and EGF domains, a transmembrane region, an intracellular cytoplasmic tail, and ligand-binding domains or complement-type repeats (CR domains). Ligand binding is mediated by clusters of structured CR domains that consist of ~40 amino acids folding into a compact structure. Each CR domain contains three disulfide bonds, a highly conserved octahedral calcium-binding cage, and a short β-hairpin near the N-terminal end (6). The calcium cage is required both for structural integrity of the CR domain as well as for direct ligand binding and is formed by six oxygen atoms derived from four conserved acidic residues and two backbone carbonyl groups (6–9). The CR domains are connected by flexible linker sequences, thereby enabling the receptor family to bind to a wide range of proteins.

Most of our knowledge on ligand-receptor interaction is based on the intracellular chaperone receptor-associated protein (RAP), which binds with high affinity to members of the LDL receptor family (10, 11). Based on a co-crystallization study of two CR domains from the LDL receptor with the third domain of RAP (RAP-D3), a general mode for ligand recognition by lipoprotein receptors has been proposed (12). In this “acidic necklace” model, each CR domain encircles an ε-amino group of a lysine residue in a tripartite salt bridge (Lys256 and Lys270 in RAP-D3) via the three remaining oxygen atoms from the acidic residues forming the octahedral calcium cage (12). This model was supported by a previous study in which Lys256 and Lys270 were identified as being critical for the interaction of RAP-D3 to complete LRP1 using random mutagenesis (13). Further support for the lysine binding model was provided by the NMR structure of the complex of complement-type repeat 5 and 6 of LRP1 and the first domain of RAP (RAP-D1) (14), although the models differ in terms of the additional contribution of hydrophobic interactions. Recently, we further experi-

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2 The abbreviations used are: LRP, low-density lipoprotein receptor-related protein; HDX, hydrogen-deuterium exchange; RAP, receptor-associated protein; FVIII, factor VIII; CR, complement-type repeat; LDLR, low density lipoprotein receptor.
mental demonstrated that positively charged arginines cannot substitute for the two dominant lysine residues (Lys<sup>256</sup> and Lys<sup>270</sup>) in the interaction with the RAP-D3 domain and LRP1 (15). In agreement with these data, arginine residues also do not take over lysine residues in the high-affinity interactions of RAP-D1 (Lys<sup>143</sup> and Lys<sup>146</sup>) with the LDL receptor (16). In a recent elegant study, Dolmer et al. (17) established that lysine residues are the sole contributors to binding of RAP-D3 to an LRP1 fragment containing two CR domains and that pairs of lysine residues ensure high-affinity interaction in an additive rather than a synergistic manner.

RAP prevents premature ligand binding during biosynthesis of the LDL receptor family members by efficiently competing with other ligands (18–20). It therefore seems very possible that the affinity and molecular mechanism of interaction have been optimized during evolution. Although the acidic necklace model is consistent with previous observations that lysine residues are implicated in the binding of ligands other than RAP to the LDL receptor family (21–28), it has remained unclear how large ligands containing multiple lysine residues interact with the LDL receptor family members. This is particularly relevant because experiments using single CR domains and simple (modified) amino acids have also shown that arginine and protonated histidine, especially pairs of proximal charges, can interact well with CR domains (29). Taken together, it has also remained unclear whether lysine residues make a dominant contribution to the interaction with the LDL receptor family for low-affinity ligands. Another important question is how specificity of the interaction is attained. It has been suggested that high-affinity interaction requires the engagement of at least two separate lysine residues located at an appropriate distance from each other (12, 30). In addition, it has been proposed that there are modest requirements for being a coordinating lysine residue and that specificity may be regulated by a second, immediately adjacent positive charge (17, 29). We therefore set out to study in detail the interaction of LRPI with its low-affinity ligand FVIII using two independent approaches with particular emphasis on lysine residues. Hydrogen-deuterium mass spectrometry was used to obtain insight in the contact areas on B-domain-deleted FVIII for LRPI cluster II. In addition, we used a lysine mutagenesis approach to study the specific role of lysine residues in the FVIII light chain in the interaction with full-length LRPI and LRPI cluster II. These combined studies reveal important novel aspects of the interaction between FVIII and LRPI.

**Experimental Procedures**

*Plasmid Mutagenesis—* FVIII light chain in pcDNA3.1 was created from wild type FVIII-GFP in pcDNA3.1 (31) by fusing the signal peptide to the acidic a<sub>3</sub> domain (glutamic acid at position 1649) by QuikChange<sup>TM</sup> mutagenesis (Stratagene, La Jolla, CA) using forward primer 5’-TTGGATCCCATCCTACATTCGTGAAATACCTGATGACTAC-3’ and reverse primer 5’-AGTGAATCCGATTTTTCACAAAGCAGAATC6GCAA-3’. B domain-deleted (del 746–1639) factor VIII (FVIIIIdB) in pcDNA3.1 has been described before (32). Point mutations in the FVIII light chain and FVIIIIdB were introduced by QuikChange<sup>TM</sup> mutagenesis (Stratagene) using the appropriate primers. The coding regions of all constructs were verified by sequence analysis. Sequence reactions were performed with BigDye terminator sequencing kit (Applied Biosystems, Foster City, CA).

*Expression of Recombinant Proteins—* Recombinant factor VIII (FVIII) Turoctocog alfa (previously named N8) was produced in CHO cells as described previously (33). The molecule consists of a heavy chain of 88 kDa including a 21-amino acid residue truncated B-domain and a light chain of 79 kDa; it contains four N-glycosylation sites of which two are complex bi-antennary glycans and two are high-mannose structures. FVIII light chain variants were expressed under serum-free conditions using 293-F cells (Invitrogen, R790-07) and FreeStyle 293 expression medium (Invitrogen). FreeStyle 293 cells were transfected using 293fectin (Invitrogen) according to the manufacturer’s instructions for small scale expression and using linear PEI M<sub>r</sub> ~25,000 (Polysciences Inc.) for large scale expression. Cells were harvested 4–5 days after transfection. Cells were centrifuged for 8 min at 4600 × g, and the supernatant was collected. The cell pellet was subsequently washed with 0.55 M NaCl in FreeStyle 293 expression medium and centrifuged for 8 min at 4600 × g. The conditioned medium was combined with the 0.55 M NaCl wash and filtered using a 0.22-μm disposable filter unit. FVIII light chain variants were purified using CLB-Cag117 coupled to CNBr-Sepharose 4B as an affinity matrix followed by concentration using Q-Sepharose (31) (Amersham Biosciences, Roosendaal, The Netherlands). HEK293 cell lines stably expressing FVIIIIdB variants were produced as described (34) and grown in DMEM-F12 medium supplemented with 10% FCS. Recombinant FVIIIIdB variants were purified and analyzed as described using CLB-VK34 IgG1 coupled to CNBr-Sepharose 4B as an affinity matrix followed by concentration using Q-Sepharose (21). LRPI cluster II was expressed in baby hamster kidney cells and purified as described (35) using glutathione S-transferase fused RAP coupled to CNBr-Sepharose 4B as an affinity matrix. Subsequently, purified LRPI cluster II was concentrated using Q-Sepharose.

*HDX-MS Experiments—* 189 μM FVIII and 256 μM LRPI cluster II were dialyzed to a buffer comprising 20 mM imidazole, pH 7.3, 10 mM CaCl<sub>2</sub>, 500 mM NaCl, and complex formation was allowed for 30 min at room temperature. In-exchange of deuterium was initiated by a 10-fold dilution into either 20 mM imidazole, pH 7.3, 10 mM CaCl<sub>2</sub>, 111 mM NaCl in 100% H<sub>2</sub>O for the undeuterated experiments or 20 mM imidazole, pH 7.3, 10 mM CaCl<sub>2</sub>, 111 mM NaCl in 100% D<sub>2</sub>O for the deuterated experiments. All sample deuterium labeling, quenching, injection to MS, and timing of samples were handled by an automated setup (Leap Technologies Inc.) as described (36) with minor adjustments. Minor modifications included exchange times ranging from 10 s to 256 min, the use of a 7-min linear acetonitrile gradient of 8–45% containing 0.1% formic acid, and the use of a SYNAPT G2 high definition mass spectrometer (Waters). Four undeuterated and two complete HDX experiments were performed for FVIII and FVIII in complex with LRPI cluster II within 2 consecutive days. Blank injections were performed between each sample injection to confirm the absence of peptide carryover from previous runs. Peptidic peptides were identified in separate experiments using MS<sup>2</sup> methods. MS<sup>2</sup> data...
were processed using ProteinLynx Global Server software (Waters version 2.4). Searches of MS² data were conducted as non-specific digest with N-terminal acetylation of FVIII as modification. The sequence coverage map of FVIII was plotted using the online tool MSTools (37). The relative deuteration incorporation levels for each peptic peptide were automatically calculated using DynamX software (Waters) as described (36). The data were expressed in either mass units (Da) or relative fractional exchange, which was calculated by dividing the deuteration level (in Da) by the total number of backbone amide hydrogens that could have become deuterated (equal to the sum of amino acids, minus proline residues minus 1 for the N-terminal amide). The experimental uncertainty of measuring a deuteration level was found to be ±0.15 Da. Using this value of experimental uncertainty and a 98% confidence interval, a significant difference value of 0.5 Da was calculated for individual time points, and a value of 1.5 kDa was calculated for summed HDX differences across all time points.

**Surface Plasmon Resonance Analysis**—Association and dissociation of LRP1 cluster II to FVIII light chain and FVIIIßD variants were assessed by SPR analysis employing a Biacore 3000 biosensor (Biacore AB, Uppsala, Sweden). The anti-C2 antibody CLB-EL14 IgG4 (38) (26 fmol/mm⁻² for FVIII light chain variants and 39 fmol/mm⁻² for FVIIIßD variants) was immobilized onto a CM5 sensor chip using the amine coupling method according to the manufacturer’s instructions. Subsequently, FVIII light chain variants were bound directly from medium to the anti-C2 antibody at a density of 17 fmol/mm⁻², and purified FVIIIßD variants were bound at a density of 9 fmol/mm⁻². LRP1 cluster II (0.2–200 nM) was passed over the FVIII light chain or FVIIIßD variants in a buffer containing 150 mM NaCl, 5 mM CaCl₂, 0.005% (v/v) Tween 20, and 20 mM Hepes (pH 7.4) at 25 °C with a flow rate of 20 μl/min. The sensor chip surface was regenerated three times after each concentration of LRP1 cluster II using the same buffer containing 1 M NaCl.

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25 °C with a flow rate of 20 μl/min. The sensor chip surface was regenerated three times after each concentration of FVIII using the same buffer containing 1 M NaCl. Binding to LRP1 was corrected for binding in the absence of LRP1. The response units at time point 235 s were plotted as a function of the concentration.

**Structural Modeling**—The illustrative model of the triplet canonical binding mode of LRP to the light chain of FVIII is constructed using the Rosetta modeling suite (40) and the NAMD molecular dynamics software package (41). Based on structural alignment with the x-ray crystallographic structure of the LDLR-RAP complex (Protein Data Bank code 4BDV) (12), a homology model of the calcium-loaded LRP double CR 56 was created using the Rosetta comparative modeling protocol (42) by generating 30,000 decoys of the LRP1 CR56 double module. Calcium ions, one per complementary repeat, were inserted based on the structural alignments and adjusted slightly by hand to remove atomistic overlap. The resulting calcium-loaded LRP double complementary repeat 56 (LRP1 CR56) model was positioned relative to the light chain of FVIII (PDB: 4BDV, chain B) (43) to reconstruct the interaction motif of the LDLR-RAP complex in which CR5 interacts with Lys2065 and CR6 interacts with Lys2092. The lysine residues of the FVIII light chain, Lys2065 and Lys2092, were overlaid with the NZ atoms of the two interacting lysine residues (Lys2065 and Lys2070) of RAP. To complete the triplet binding mode, a further CR5 module was positioned to interact with Lys2136 and connected to its adjacent CR56 module by the loop modeling application in Rosetta, making 1,000 loop decoys. The specific sequence to be remodeled in each fragment was identified using the domain...
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Chemical modification of lysine residues in the FVIII light chain abolishes the interaction with LRP1 cluster II—To address the role of lysine residues in the interaction between LRP1 cluster II and the FVIII light chain, we chemically modified lysine residues of the FVIII light chain on an SPR sensor.

Results

HDX-MS of LRP1 Interaction with Cofactor FVIII—We have previously established that the main LRP1 binding on FVIII is localized in the FVIII light chain (45). HDX mass spectrometry was now employed to elucidate the surface areas on FVIII that show reduced HDX in the presence of LRP1 cluster II. These areas either are covered by complement-type repeats or exhibit a local change in conformation caused by cluster II binding. HDX of FVIII in the presence and absence of LRP1 cluster II was followed at various time intervals ranging from 10 s to 256 min. Inline pepsin protein digest conditions were established that afforded peptides of reasonably small length (typically 5–20 amino acids). These conditions enabled detection of a total of 277 peptides covering 76% of the primary sequence of FVIII, with redundant coverage of all subunits, i.e. A1, A2, A3, C1, and C2 (Figs. 1 and 2). The majority of the peptides displayed an exchange pattern that is unaffected by the binding of LRP1 cluster II (Figs. 1 and 2, blue color). However, several overlapping peptic peptide fragments encompassing residues 114–132, 369–387, 399–428, 481–506, 1669–1689, 1735–1746, 1817–1834, 2054–2069, 2078–2097, 2213–2234, 2264–2273, and 2292–2296 showed reduced HDX in the presence of LRP1 cluster II (Figs. 1 and 2, red color). The raw data of some representative HDX profiles for these peptides are shown in Fig. 1. This analysis revealed that the regions that demonstrate reduced HDX are predominantly on the FVIII light chain and are scattered throughout the a3, A3, C1, and C2 domains (Figs. 1 and 2).

Chemical Modification of Lysine Residues in the FVIII Light Chain Abolishes the Interaction with LRP1 Cluster II—We chemically modified lysine residues in the FVIII light chain and followed the response at various time intervals (10 s to 256 min) after association with LRP1 cluster II. The response at 235 s after association is plotted as a function of the concentration.
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FIGURE 4. Library of lysine to arginine replacements in the FVIII light chain identifies the contribution of multiple lysine residues in the interaction with LRP1 cluster II. Association and dissociation of LRP1 cluster II to FVIII light chain variants were assessed by SPR analysis employing a BIAcore 3000 biosensor (Biacore AB). The anti-C2 antibody CLB-EL14 IgG4 (26 fmol/mm²) was immobilized onto a CMS sensor chip using the amine coupling method according to the manufacturer’s instructions. Subsequently, FVIII light chain variants were bound to the anti-C2 antibody at a density of 17 fmol/mm². LRP1 cluster II (0.2–200 nM) was passed over the FVIII light chain variants in a buffer containing 150 mM NaCl, 5 mM CaCl₂, 0.005% (v/v) Tween 20, and 20 mM Hepes (pH 7.4) at 25 °C with a flow rate of 20 μl/min. The sensor chip surface was regenerated three times after each concentration of LRP1 cluster II using the same buffer containing 1M NaCl. Binding to FVIII light chain variants was corrected for binding in the absence of FVIII. Binding data during the association phase were fitted in a one-phase exponential association model. A, representative experiment for the interaction between LRP1 cluster II and wild type FVIII light chain. B, on each SPR sensor chip, we included a control channel (only CLB-EL14 IgG4), wild type FVIII light chain, and two FVIII light chain variants. Therefore, wild type FVIII light chain was analyzed multiple times (n = 14) C and D, representative experiments for variants. E, the Kₐ for LRP1 cluster II for the FVIII light chain variants was compared with the Kₐ of wild type FVIII light chain (n = 14, degrees of freedom = 13, *, p < 0.10, t value = 2.16, ***, p < 0.001, t value = 4.22) using a two-tailed Student's t test. Error bars indicate ± S.D.
chip using the compounds sulfo-NHS-acetate and sulfo-NHS-biotin. Both these compounds form stable, covalent amide bonds with primary amines, resulting in the replacement of the positively charged \( \text{H}^+ \)-amino group of the lysine residue by an uncharged acetate group (sulfo-NHS-acetate) or biotin group (sulfo-NHS-biotin). Subsequently, LRP1 cluster II and a control antibody CLB-CAg69 directed against the \( \alpha_3 \) domain of FVIII were passed over the unmodified and chemically modified FVIII light chain. Although LRP1 cluster II readily bound to the unmodified FVIII light chain, no detectable binding was observed for the FVIII light chain in which the lysine residues were chemically modified (Fig. 3). As a control, the CLB-CAg69 antibody bound to a similar extent to the unmodified as well as to the chemically modified FVIII light chain (Fig. 3). These data suggest that lysine-containing surface patches mediate the interaction between LRP1 cluster II and the FVIII light chain.

Multiple Lysine Residues in the FVIII Light Chain Contribute to the Interaction with LRP1 Cluster II—To systematically address which lysine residues in the FVIII light chain contribute to LRP1 cluster II interaction, we constructed a library of FVIII variants carrying lysine to arginine (KR) replacements. All of these compounds form stable, covalent amide bonds with primary amines, resulting in the replacement of the positively charged \( \text{H}^+ \)-amino group of the lysine residue by an uncharged acetate group (sulfo-NHS-acetate) or biotin group (sulfo-NHS-biotin). Subsequently, LRP1 cluster II and a control antibody CLB-CAg69 directed against the \( \alpha_3 \) domain of FVIII were passed over the unmodified and chemically modified FVIII light chain. Although LRP1 cluster II readily bound to the unmodified FVIII light chain, no detectable binding was observed for the FVIII light chain in which the lysine residues were chemically modified (Fig. 3). As a control, the CLB-CAg69 antibody bound to a similar extent to the unmodified as well as to the chemically modified FVIII light chain (Fig. 3). These data suggest that lysine-containing surface patches mediate the interaction between LRP1 cluster II and the FVIII light chain.

| Lysine position | Replacement | Arginine (K_D) | Alanine (K_D) | Glutamic acid (K_D) |
|----------------|-------------|---------------|---------------|---------------------|
| 1693           | **K1693A**  | 63 ± 5        | 170 ± 19      | 154 ± 17            |
| 1827           | **K1827A**  | 80 ± 7        | 157 ± 16      | 130 ± 13            |
| 1967           | **K1967A**  | 73 ± 6        | 91 ± 5        | ND                  |
| 2065           | **K2065A**  | 168 ± 14      | 202 ± 17      | 210 ± 22            |
| 2092           | **K2092A**  | 72 ± 6        | 45 ± 4        | 98 ± 10             |

**TABLE 1**

Effect of replacement of lysine residues by positively charged arginine, uncharged alanine, or negatively charged glutamic acid on the interaction between the FVIII light chain and LRP1 cluster II

As shown in Fig. 5, lysine to alanine and lysine to glutamic acid replacements at position 1693, 1827, 1967, 2065, and 2092 were assessed by SPR analysis employing a BIAcore 3000 biosensor (Biacore AB). The anti-C2 antibody CLB-EL14 IgG4 (26 fmol/mm^2) was immobilized onto a CMS sensor chip using the amine coupling method according to the manufacturer’s instructions. Subsequently, FVIII light chain variants were bound to the anti-C2 antibody at a density of 17 fmol/mm^2. LRP1 cluster II (0.2–200 nM) was passed over the FVIII light chain variants in a buffer containing 150 mM NaCl, 5 mM CaCl_2, 0.005% (v/v) Tween 20, and 20 mM Heps (pH 7.4) at 25 °C with a flow rate of 20 µl/min. The sensor chip surface was regenerated three times after each concentration of LRP1 cluster II using the same buffer containing 1 M NaCl. Binding to FVIII light chain variants was corrected for binding in the absence of FVIII. Binding data during the association phase were fitted in a one-phase exponential association model.

**Figure 5.** Lysine to alanine and lysine to glutamic acid replacements at position 1693, 1827, 1967, 2065, and 2092. Association and dissociation of LRP1 cluster II to FVIII light chain variants Lys1693, Lys1827, Lys1967, Lys2065, and Lys2092 were assessed by SPR analysis employing a BIAcore 3000 biosensor (Biacore AB). The anti-C2 antibody CLB-EL14 IgG4 (26 fmol/mm^2) was immobilized onto a CMS sensor chip using the amine coupling method according to the manufacturer’s instructions. Subsequently, FVIII light chain variants were bound to the anti-C2 antibody at a density of 17 fmol/mm^2. LRP1 cluster II (0.2–200 nM) was passed over the FVIII light chain variants in a buffer containing 150 mM NaCl, 5 mM CaCl_2, 0.005% (v/v) Tween 20, and 20 mM Heps (pH 7.4) at 25 °C with a flow rate of 20 µl/min. The sensor chip surface was regenerated three times after each concentration of LRP1 cluster II using the same buffer containing 1 M NaCl. Binding to FVIII light chain variants was corrected for binding in the absence of FVIII. Binding data during the association phase were fitted in a one-phase exponential association model.

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these variants were tested for LRP1 cluster II interaction using SPR analysis. On each SPR sensor chip, we included a control channel (only CLB-EL14 IgG4), wild type FVIII light chain, and two FVIII light chain variants. Therefore, wild type FVIII light chain was analyzed multiple times (n = 14). For each channel, binding data during the association phase were fitted in a one-phase exponential association model. A typical example of the binding curves as well as the fit of the one-phase exponential association model (dotted line) for wild type FVIII light chain is represented in Fig. 4A. Responses at equilibrium were plotted as a function of the LRP1 cluster II concentration and fitted by non-linear regression using a standard hyperbola to generate $K_D$ values. The $K_D$ value for wild type FVIII light chain was $30 \pm 4$ nM (Fig. 4B). Initially, we constructed FVIII light chain variants based on the domain structure of FVIII carrying 2 (a3), 17 (A3), 8 (C1), or 9 (C2) replacements. However, KR variants of the A3 domain (17 replacements) and the C1 domain (8 replacements) were poorly expressed. We therefore constructed KR variants carrying single, double, or triple lysine to arginine replacements in the A3 and C1 domain. Unfortunately, the K1732R FVIII light chain variant was poorly expressed and could not be assessed using SPR analysis. For all
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of the other variants, binding to LRP1 cluster II was analyzed, and the $K_D$ was calculated as described above. Representative experiments are shown in Fig. 4, C and D. The $K_D$, for LRP1 cluster II for these variants was compared with the $K_D$ of wild type (30 ± 4 ns) using a two-tailed Student’s $t$ test (Fig. 4E). Our SPR analysis showed that lysine residues in the C2 domain of the FVIII light chain do not contribute to the interaction with LRP1 cluster II. In contrast, multiple lysine residues in the a3A3C1 domains contributed to the interaction of FVIII to LRP1, although none of the individual substitutions completely abolished LRP1 cluster II binding. Lysine residues contributing to the interaction included Lys1673, Lys1674, Lys1693, Lys1813, Lys1818, Lys1827, Lys1967, Lys1972, Lys2065, Lys2092, and Lys2136 (Fig. 4E). Of these, Lys1693, Lys1827, and Lys1967 ranked the highest in the A3 domain, and Lys2065 and Lys2092 ranked the highest in the C1 domain. These residues were selected for further studies.

Single and Multiple Replacements of Selected Lysine Residues in the A3 Domain Lys1693, Lys1827, and Lys1967 and in the C1 Domain Lys2065 and Lys2092 by Arginine, Alanine, or Glutamic Acid—From the SPR data of our library of FVIII light chain variants, we concluded that multiple lysine residues contribute to the interaction with LRP1 cluster II. However, there are some lysine residues in the A3 domain (Lys1693, Lys1827, and Lys1967) and in the C1 domain (Lys2065 and Lys2092) that contribute most to the interaction. To study the effect of charge, these lysine residues were replaced by positively charged arginine, uncharged alanine, or negatively charged glutamic acid. The variant carrying a K1967E replacement was poorly expressed and could not be analyzed. All other FVIII light chain variants demonstrated a reduced interaction with LRP1 cluster II (Fig. 5 and Table 1), confirming the contribution of lysine residues at positions 1693, 1827, 1967, 2065, and 2092 to the interaction with LRP1 cluster II. There were no major differences in the interaction with LRP1 cluster II for FVIII light chain variants carrying lysine to arginine or lysine to alanine replacements for the positions 1967, 2065, and 2092. This confirms our previous observation that, despite their positive charge, arginine cannot replace lysine in the interaction with LRP1. However, for lysine residues at positions 1693 and 1827, alanine replacements demonstrated a higher $K_D$ as compared with arginine replacements (Table 1), suggesting that although for these specific residues a lysine residue is preferred, the charge of the arginine can maintain some interaction with LRP1. In addition, we have reversed the positive charge of the lysine residue by replacing the lysine with a negatively charged glutamic acid residue. Alanine and glutamic acid variants demonstrated a similar $K_D$, indicating that there was no additive effect of neutralizing the charge (Table 1). To study the additive effect of combining lysine replacements, we constructed FVIII variants carrying 3 (Lys1693, Lys1827, and Lys1967), 2 (Lys2065 and Lys2097), and 5 lysine replacements. All FVIII light chain variants carrying the K1967E replacement were poorly expressed and could not be analyzed. All the other variants demonstrated a reduced interaction with LRP1 cluster II (Fig. 6 and Table 2). FVIII light chain variants containing 3 lysine replacements in the A3 domain demonstrated a slightly increased $K_D$ as compared with the variants containing only 1 lysine replacement in the A3 domain. There was no major difference between the lysine to arginine and lysine to alanine variants (Table 2). For the C1 domain, the $K_D$ for variants in which both Lys2065 and Lys2092 were replaced was increased as compared with the C1 variants carrying only 1 lysine replacement. For the C1 domain, alanine or glutamic acid replacements did result in a higher $K_D$ as compared with arginine replacements (Table 2). The combined variants in which the 5 lysine residues in the A3 and C1 were replaced by arginine or alanine demonstrated a $K_D$ that was ~20-fold (arginine) or 40-fold (alanine) reduced as compared with wild type FVIII light chain (Table 2). In addition to interaction studies by capturing FVIII light chain variants directly from medium via the C2 domain on an SPR chip, interaction studies were performed using purified FVIII light chain variants and full-length LRP1 (Fig. 7). These studies showed that, although the FVIII light chain variant carrying 5 lysine to arginine replacements demonstrated a 20-fold reduced interaction with LRP1 cluster II, binding to full-length LRP1 was not affected. However, replacements at the same position by alanine or glutamic acid did result in a reduced interaction with full-length LRP1 (Fig. 7).

Binding of FVIIIaB Variants to LRP1 Cluster II and Full-length LRP1 from Light Chain to Heterodimer—To verify the contribution of the 5 lysine residues in the FVIII cofactor, we purified FVIIIaB variants carrying 5 lysine to arginine, alanine, or glutamic acid replacements. FVIII variants were bound via the C2 domain to antibody CLB-EL14 IgG4, and a concentration range of LRP1 cluster II was passed over the immobilized FVIII variants. These SPR binding studies indicated that all FVIIIaB variants showed a dramatically reduced interaction with LRP1 cluster II in the order wild type $> 5 \times \text{KR} > 5 \times \text{KA} > 5 \times \text{KE}$ (Fig. 8). Although for individual lysine residues, there was only a minor difference between KR, KA, and KE variants, the combined KE replacements proved to be more detrimental for the interaction with LRP1 cluster II than KA and KE replacements. In addition, we studied the contribution of the 5 lysine residues in the interaction with full-length LRP1. Therefore, we directly coupled full-length LRP1 on the CM5 chip and passed over the FVIIIaB variants. Binding studies indicated that the binding was reduced in the order $5 \times \text{KR} > 5 \times \text{KA} > 5 \times \text{KE}$. The binding defect of the

TABLE 2

| Domain | Arginine ($K_D$) | Alanine ($K_D$) | Glutamic acid ($K_D$) |
|--------|-----------------|-----------------|-----------------------|
| A3     | 201 ± 18        | 222 ± 20        | ND                    |
| C1     | 220 ± 21        | 620 ± 59        | 534 ± 52              |
| A3C1   | 567 ± 48        | 1339 ± 144      | ND                    |

As compared with arginine antibody CLB-EL14 IgG4 (26 fmol/mm²) was immobilized onto a CM5 sensor chip using the amine coupling method according to the manufacturer’s instructions. Subsequently, FVIII light chain variants were bound to the anti-C2 antibody at a density of 17 fmol/mm². LRP1 cluster II (0.2–200 nM) was passed over the FVIII light chain variants in a buffer containing 150 mM NaCl, 5 mM CaCl₂, 0.005% (v/v) Tween 20, and 20 mM Hepes (pH 7.4) at 25°C with a flow rate of 20 µl/min. The sensor chip surface was regenerated three times after each concentration of LRP1 cluster II using the same buffer containing 1 mM NaCl. Binding to FVIII light chain variants was corrected for binding in absence of FVIII. Binding data during the association phase were fitted in a one-phase exponential association model.
5xKR variant was remarkably minor, whereas the binding defects of the 5xKA and 5xKE were more severe (Fig. 9).

**Discussion**

This study demonstrates that lysine residues indeed make a dominant contribution to the LRP1-FVIII interaction. When we chemically modified all surface-exposed lysine residues of FVIII using NHS-biotin or NHS-sulfo acetate, the binding to LRP1 cluster II is completely abrogated. Our combined experimental approach using HDX-MS and site-directed mutagenesis further suggests that the interaction of LRP1 with FVIII occurs over an extended surface containing multiple lysine residues in FVIII. Hydrogen deuterium exchange–mass spectrometry (HDX-MS) experiments identified regions 114–132, 369–
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![Graph showing binding of FVIII to LRP1 cluster II.](image)

**FIGURE 8. Binding of FVIIIdB variants to LRP1 cluster II.** A, association and dissociation of LRP1 cluster II to FVIIIdB variants were assessed by SPR analysis employing a BiACore 3000 biosensor (Blacore AB). The anti-C2 antibody CLB-EL14 IgG4 (39 fmol/mM^2^ for FVIIIdB variants) was immobilized onto a CM5 sensor chip using the amine coupling method according to the manufacturer’s instructions. Subsequently, FVIIIdB variants were bound to the anti-C2 antibody at a density of 9 fmol/mM^-2_. LRP1 cluster II (0–200 nM) was passed over the FVIIIdB variants in a buffer containing 150 mM NaCl, 5 mM CaCl₂, 0.005% (v/v) Tween 20, and 20 mM Hepes (pH 7.4) at 25 °C with a flow rate of 20 μL/min. The sensor chip surface was regenerated three times after each concentration of LRP1 cluster II using the same buffer containing 1 M NaCl. Binding to FVIIIdB variants was corrected for binding in the absence of FVIII. Binding data during the association phase were fitted in a one-phase exponential association model. B, the responses at equilibrium were fitted by non-linear regression using a standard hyperbola to generate K_d values (GraphPad Prism 4 software). Error bars indicate ± S.D.

The majority of the lysine residues that were identified in the mutagenesis study are located within those regions that were also identified using the HDX-MS study and are therefore considered “hot spots.” These include 1673/1674, 1813–1818, 1827, 1967, 1972, 2065, and 2092 to be involved in the binding of FVIII to LRP1 cluster II. As expected (45), these regions are mainly located in the FVIII light chain and scattered throughout the a3, A3, C1, and C2 domains of FVIII. Subsequent SPR analysis of a library of FVIII light chain variants carrying lysine to arginine revealed that multiple (11) lysine residues within the FVIII light chain including 1673/1674, 1693, 1813/1818, 1827, 1967, 1972, 2065, 2092, and 2136 contribute to the interaction with LRP1 cluster II. However, none of the individual lysine residues accounts completely for LRP1 cluster II binding. We did observe an additive effect of combining multiple lysine replacements, and further mutagenesis studies using positively charged arginine, uncharged alanine, or negatively charged glutamic acid residues indicated that reversing the positive charge had the strongest effect on the interaction.

The question remains why certain lysine residues contribute more to the interaction than others. Based on a study on the specificity of binding of the serpins PAI-1 and PN-1 to LRP1, Jensen et al. (30) have suggested that there may be modest requirements to be a coordinating lysine residue. However, inspection of the X-ray structure of the LDLR-RAP complex suggests that members of the LDL receptor family interact with clusters of lysines being 18–20 Å apart (12). In addition, it has been suggested that hydrophobic interactions may play an additional role in the binding of ligands to the complement-type repeats (14). Although the acidic necklace model as proposed by Fisher et al. (12) suggests that hydrophobic interactions are mediated by the side chain of the lysine residue of the ligand, Jensen et al. (14) propose that an additional hydrophobic residue of the ligand, typically a leucine or an isoleucine, is important for the interaction. Therefore, hydrophobic residues located in the vicinity of the lysine residue may determine whether a lysine residue is a hot spot or not. Alternatively, the presence of a second proximal charge near the lysine residue, or perhaps even repulsive charges from amino acid residues surrounding the lysine residue, may determine specificity (17, 29).

No consensus for either hydrophobic or charged residues in the vicinity of our identified lysine residues could be identified. We therefore took a close look at the spatial distribution of the identified interactive regions and lysine residues within FVIII. Crystal structure analysis revealed that all hot-spot lysine residues that we identified may spatially align with the acidic binding pockets of the extracellular part of the LDL receptor. The distance from the bottom of the C1 domain to the top of the A3 domain spans 69 Å, a distance that LRP1 cluster II can easily encompass using just 3–4 complement-type repeats. We therefore propose that LRP1 cluster II interacts with the FVIII light chain via an extended surface that starts at the bottom of the C1 domain and extends to the top of the A3 domain. This prompted us to perform structural modeling with the goal to...
both exemplify and substantiate likely interactions between LRP1 cluster II and the FVIII light chain. Initially, we focused on the FVIII C1 domain in which both HDX-MS and mutagenesis studies indicated the contribution of 2 lysine residues in the C1 domain, Lys2065 and Lys2092. Homology modeling was performed in consensus with the canonical binding mode for members of the LDL receptor family using the x-ray crystallographic structure of the LDLR-RAP complex as a template (12) (Fig. 10). Hence, each CR module contains three acidic aspartic acid residues placed in a concave surface region that interacts with lysine clusters. The proposed canonical interaction mode between LRP1 triple module and the FVIII light chain allows two principal binding modes oppositely oriented relative to each other, i.e. LRP1 N terminus to C terminus aligns along the Lys2065-Lys2092-Lys2136 as opposed to Lys2136-Lys2092-Lys2065 surface patch. Two key features contribute to a favorable interaction between lysine regions and the LRP triple module. Shape complementarity is eminently achievable due to the flexible

FIGURE 9. Binding of FVIIIdB variants to full-length LRP1. A, association and dissociation of FVIIIdB variants to full-length LRP1 were assessed by SPR analysis employing a BIAcore 3000 biosensor (Biacore AB). LRP1 (Biomac) was coupled directly on a CM5 chip according to the manufacturer’s instructions at three different densities (15, 18, and 21 fmol/mm²). FVIIIdB variants (5–1000 units/ml based on chromogenic activity) were passed over the immobilized LRP1 in a buffer containing 150 mM NaCl, 5 mM CaCl₂, 0.005% (v/v) Tween 20, and 20 mM Hepes (pH 7.4) at 25 °C with a flow rate of 20 μl/min. The sensor chip surface was regenerated three times after each concentration of FVIII using the same buffer containing 1 M NaCl. Binding to LRP1 was corrected for binding in the absence of LRP1. Shown are the SPR curves for the channel on which 21 fmol/mm² LRP1 was coupled. B, the response units at time point 235 s were plotted as a function of the concentration for all three LRP1 surface densities.
nature of the chain of complementary repeats, allowing the receptor to adapt to the ligands' surface topology and exploit hydrophobic interaction in between repeats. Also, the ionic interactions between the positively charged lysine residues and the negatively charged aspartic acid residues resulted in close packing of the partners, suggesting that these features are the driving forces behind the interaction. The widespread imprint of LRP1 binding on FVIII identified by HDX-MS analysis points to the notion that during LRP1 binding, CR repeats switch between different lysine clusters. This is reflected in the designed LRP1 model where several interacting modes are equally likely (Fig. 10).

In addition to the overlap of HDX and the lysine mutagenesis study, there were also some differences. Residues 1693, 1967, 1972, and 2136 were identified only in the lysine mutagenesis study, whereas regions 1735–1746, 2213–2234, 2264–2273, and 2292–2296 were only identified using the HDX-MS approach. We therefore consider these regions and lysine residues “soft spots.” The effect of the soft-spot lysine residues may be explained by conformational changes; lysine residue 1693 is directly facing the hot-spot lysine at position 1818, and the soft-spot lysine residue 1967 has been shown to interact with the A2 domain of FVIII (46). As for the soft-spot regions identified by HDX-MS, region 1735–1746 is located within the A3 region and consists of the amino acids FQEFTDGSFTQP. The remaining 3 regions are located within the C2 domain. The C2 domain has previously been reported to bind to LRP1 with low affinity ($K_D \approx 3.6 \mu M$) (45). The region 2292–2296 (PVVNS) contains no positively charged residues, the region 2264–2273 (SSQDHGWQTL) contains 1 histidine residue, and the region 2213–2234 (QGRSNAWRPQVNNPKEWLQVDF) contains 2 arginine residues and 1 lysine residue. We did not observe any contribution of lysine residues in the C2 domain of FVIII to the interaction with LRP1 in our mutagenesis study (Fig. 4E), suggesting that the reported low affinity of the C2 domain may be mediated by residues other than lysine.

In recent years, the importance of the C domains in the life cycle of FVIII has been appreciated because they are involved in lipid binding, von Willebrand factor (VWF) interaction, and cellular uptake (47–52). The C2 domain is loosely attached to the C1 and A1 domains, and it is likely that the domain has a high degree of rotational freedom. We believe that the positioning of the FVIII C domains relative to each other may therefore be an important aspect in the biology of FVIII. The regions in the C2 domain that were identified by HDX-MS may reflect conformational changes within these areas as opposed to a direct interaction with LRP1 cluster II. The dynamics of the FVIII C domains in response to interaction with various ligands and lipids remains a subject for further studies.

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