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ORIGINAL ARTICLE

Unique surface-exposed hydrophobic residues in the C1 domain of factor VIII contribute to cofactor function and von Willebrand factor binding

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Abstract

Background: The identity of the amino acid regions of factor VIII (FVIII) that contribute to factor IXa (FIXa) and von Willebrand factor (VWF) binding has not been fully resolved. Previously, we observed that replacing the FVIII C1 domain for the one of factor V (FV) markedly reduces VWF binding and cofactor function. Compared to the FV C1 domain, this implies that the FVIII C1 domain comprises unique surface-exposed elements involved in VWF and FIXa interaction.

Objective: The aim of this study is to identify residues in the FVIII C1 domain that contribute to VWF and FIXa binding.

Methods: Structures and primary sequences of FVIII and FV were compared to identify surface-exposed residues unique to the FVIII C1 domain. The identified residues were replaced with alanine residues to identify their role in FIXa and VWF interaction. This role was assessed employing surface plasmon resonance analysis studies and enzyme kinetic assays.

Results: Five surface-exposed hydrophobic residues unique to the FVIII C1 domain, ie, F2035, F2068, F2127, V2130, I2139 were identified. Functional analysis indicated that residues F2068, V2130, and especially F2127 contribute to VWF and/or FIXa interaction. Substitution into alanine of the also surface-exposed V2125, which is spatially next to F2127, affected only VWF binding.

Conclusion: The surface-exposed hydrophobic residues in C1 domain contribute to cofactor function and VWF binding. These findings provide novel information on the fundamental role of the C1 domain in FVIII life cycle.

KEYWORDS

factor VIII, factor IXa, kinetics, surface plasmon resonance, Von Willebrand factor

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Przeradzka and Freato contributed equally to this study.

1 | INTRODUCTION

Coagulation factor VIII (FVIII) is a large heterodimeric protein that serves its role in the coagulation cascade as a cofactor for activated factor IX (FIXa) during the proteolytic conversion of factor X (FX) into activated FX (FXa). FVIII is essential for proper functioning of the coagulation cascade as its functional absence has been associated with the X-linked bleeding disorder hemophilia A.[1]

FVIII is synthesized as a single chain protein of 2332 amino acids, which are organized into three homologous A domains, a B domain, and two homologous C domains. Short acidic amino acid regions comprising sulphated tyrosine amino acid residues are at the C-terminal side of A1 and A2 domains and at the N-terminal side of the A3 domain.[2,3] Prior to secretion, FVIII is processed into a heavy chain (domains: A1-A2-B) and a light chain (domains: A3-C1-C2), which remain associated via electrostatic and metal ion-dependent interactions.[3] Because of limited proteolysis of the B domain, the molecular weight of FVIII in plasma ranges between 170 and 300 kDa.[2,4-7] The crystal structures of FVIII show that the A domains are ordered in a triangular shape stacked on top of two C domains aligned in parallel.[8-10]

FVIII circulates in plasma in a tight complex with von Willebrand factor (VWF). In this complex, FVIII is protected from premature ligand binding, proteolytic degradation, and rapid plasma clearance.[11,12] Upon initiation of the coagulation cascade, FVIII is activated by thrombin, which cleaves specific sites next to the acidic regions of FVIII.[3] This leads to the release of the B domain, disconnection of the A1 and A2 domains, and dissociation of the FVIII:VWF complex. After activation, FVIII can bind with high affinity to procoagulant phospholipid membranes that expose phosphatidylserine (PS) in the outer leaflet. Activated FVIII (FVIIIa) that is bound to the phospholipid surface provides a platform for effective interaction with FIXa resulting in the activated FX-generating complex.

Because of the high structural similarity between FVIII and factor V (FV), chimeric variants of FVIII and FV have been utilized in a number of studies to gain insight into the structure and function of FVIII and FV.[13-16] Factor V (FV) is the cofactor for FXa in the coagulation cascade and can form together with FXa and procoagulant phospholipid membranes the prothrombinase complex that efficiently converts prothrombin into thrombin.[8,17] Using FVIII/FV chimeric proteins, the phospholipid binding role of the hydrophobic surface loops at the bottom of the C domains has, for instance, been addressed.[8,13,18-21] Another example is that contribution of the C domains to the intracellular trafficking of FVIII to the Weibel-Palade bodies has been studied in endothelial cells using chimeric FVIII/FV variants.[15]

Several FIXa and VWF interaction sites have been identified in FVIII. Competition studies with FVIII-derived peptides or isolated subunits indicated that the C2 domain may comprise a binding site for FIXa.[22] Using site-directed mutagenesis studies, interaction sites for FIXa have further been identified in the A2 and A3 domain.[23,24] For the interaction with VWF, convincing evidence has been provided that the acidic region at the N-terminal side of the A3 domain is critical.[25-27] It has also been proposed that the C1 and C2 domains contribute to VWF binding as well.[18,28,29] However, a FVIII variant in which the C2 domain was replaced by the C2 domain of FV ($FVIII_{C2FVC2}$) displayed only a small reduction in VWF binding and nearly normal cofactor function.[15] This shows that the main interaction sites for VWF and FIXa are, most likely, outside the C2 domain.

In a previous study, we established that replacing the C1 domain of FVIII with that of FV ($FVIII_{C1FVC1}$) has a major impact on VWF binding and FVIII cofactor function.[15] This suggests that the C1 domain of FV lacks surface-exposed structural elements that can support the interaction with FIXa and VWF. In the present study, we now compare the C1 domains of FVIII and FV to identify the unique surface-exposed elements on the FVIII C1 domain that contribute to cofactor function and VWF binding. Results revealed five surface-exposed hydrophobic residues that were either more polar or more buried in the C1 domain core of FV, ie, F2035, F2068, F2127, V2130, I2139. Site-directed mutagenesis of FVIII followed by functional studies showed that these residues differentially contribute to FIXa and/or VWF binding. In particular F2127 proved to be important for both the interaction with VWF and FIXa.

Essentials

- Factor VIII C1 domain comprises unresolved factor IXa and von Willebrand factor binding sites.
- A factor VIII/factor V C1 chimera has decreased affinity for factor IXa and von Willebrand factor.
- In contrast to factor V, factor VIII C1 domain has unique surface-exposed hydrophobic residues.
- The exposed hydrophobic residues contribute to factor IXa and von Willebrand factor binding.

2 | MATERIALS AND METHODS

2.1 | Materials

Fine chemicals were from Merck, unless otherwise stated. DMEM-F12 medium was from Lonza, Foetal Calf Serum (FCS) was from Bodinco. DMRIE-C reagent and Opti-MEM medium were from Thermo Fisher Scientific. Chicken egg L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), and porcine brain L-α-phosphatidylserine (PS) were from Avanti Polar Lipids Inc. Geneticin G-418 sulphate, precast SDS/PAGE gels, and Brilliant Blue Coomassie were from Invitrogen. Tris-HCl was from Invitrogen, NaCl was obtained from Fagron, and HEPES was from Serva. Human serum albumin (HSA) was from the Division of Products of Sanquin. The FXa substrate S-2765 with the thrombin inhibitor I-2581 was from Chiralix.

TABLE 1 Primers used in this study to obtain single substitutions of the C1 domain

Variant	Primer sequence
F2035A	
Sense	5′-AGCGGCCACATCCGGGACGCCCAGATCACCGCCTCCGGC-3′
Anti-sense	5′-GCCGGAGGCGGTGATCTGGGCGTCCCGGATGTGGCCGCT-3′
F2068A	
Sense	5′-TGGTCCACCAAAGAGCCCGCCAGCTGGATCAAGGTGGAC-3′
Anti-sense	5′-GTCCACCTTGATCCAGCTGGCGGGCTCTTTGGTGGACCA-3′
V2125A	
Sense	5′-AGCACCGGCACCCTGATGGCCTTCTTCGGCAACGTGGAC-3′
Anti-sense	5′-GTCCACGTTGCCGAAGAAGGCCATCAGGGTGCCGGTGCT-3′
F2127A	
Sense	5′-GGCACCCTGATGGTGTTCGCCGGCAACGTGGACAGCAGC-3′
Anti-sense	5′-GCTGCTGTCCACGTTGCCGGCGAACACCATCAGGGTGCC-3′
V2130A	
Sense	5′-ATGGTGTTCTTCGGCAACGCCGACAGCAGCGGCATCAAG-3′
Anti-sense	5′-CTTGATGCCGCTGCTGTCGGCGTTGCCGAAGAACACCAT-3′
I2139A	
Sense	5′-AGCGGCATCAAGCACAACGCCTTCAACCCCCCCATCATT-3′
Anti-sense	5′-AATGATGGGGGGGTTGAAGGCGTTGTGCTTGATGCCGCT-3′

2.2 | Alignment of the FVIII and FV models

The crystal structure of the human B-domain deleted FVIII was aligned with the one of the bovine inactivated FVa with Pymol (PyMOL, Molecular Graphics System, v1.3, Schrödinger, LLC). Values of the accessible surface areas (ASA, Å^2) were obtained from the "Protein Interfaces, Surfaces and Assemblies Service" (PISA) at the European Bioinformatics Institute (EBI, http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) using the PDB codes 2r7e for the FVIII crystal structure and 1sdd for the FVai crystal structure and then compared with each other. Hydrophobicity of amino acid residues residing on the C1 domain was evaluated according to the hydrophobicity scale.[9,30-32] Alignments of the human FVIII to the bovine and human FV were taken from Liu et al.[33]

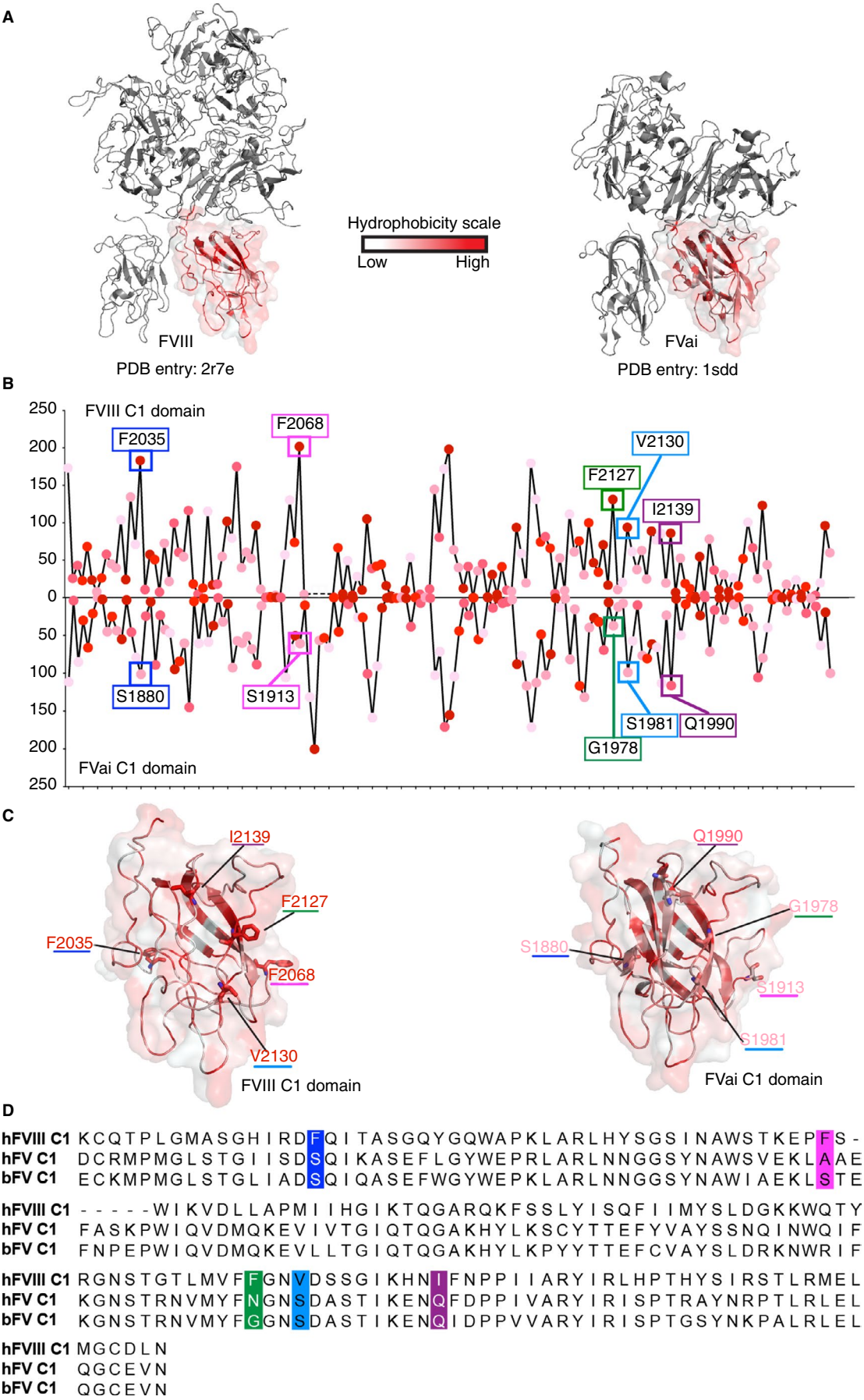
2.3 | Proteins

B-domain deleted FVIII (GenBank accession number ABV90867.1) was codon optimized and purchased from Thermo Fisher Scientific (USA) in a pcDNA3.1(+) expression vector using NheI and NotI restriction. FVIII B domain-deleted variants with single amino acid substitutions F2035A, F2068A, V2125A, F2127A, V2130A, and I2139A were obtained by site-directed mutagenesis. Site-directed mutagenesis was performed using the QuikChange kit (Agilent Technologies) using appropriate primers (Table 1). DNA sequences were verified by sequencing analysis of the FVIII encoding parts on the mutated plasmid using BigDye Terminator Sequencing kit (Applied Biosystems). Stable transfection of Human Embryonic Kidney 293 cells (HEK293) and protein production was performed as described.[34] Recombinant FVIII mutants and wild-type were purified using VK34 monoclonal antibody[35] as described by Meems et al.[36] SDS-PAGE of the purified FVIII variants is shown in Figure S1 in supporting information. Recombinant VWF was prepared as described previously.[37] Human plasma derived FIXa, FX, and α-thrombin were purified as indicated.[38-40]

2.4 | Surface plasmon resonance

Binding studies were performed by surface plasmon resonance analysis employing Biacore T-200 biosensor system (GE Healthcare) as described.[41] For assessment of the FVIII-VWF interaction, recombinant VWF was immobilized at the density of 900 response units (RU) onto a CM5 sensor chip using the amine coupling method according to the manufacturer's instructions. Varying concentrations of FVIII and mutations thereof were passed over immobilized VWF at a flow rate of 30 μL/min in a buffer containing 20 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 0.05% Tween 20 at 25°C.

FIGURE 1 Comparison between C1 domains of FV and FVIII. A, On the left is displayed the crystal structure of FVIII [PDB entry: 2r7e] and on the right the structure of inactivated bovine FVa (FVai) [PDB entry: 1sdd].[9,32] The transparent surfaces of the C1 domains are colored according to the local level of hydrophobicity.[31] B, The solvent accessible surface area is plotted as a function of the amino acid position in the C1 domain of FVIII (top) and FVai (bottom). The white to red color code represents the hydrophobicity of the amino acids.[31] C, Selected hydrophobic residues from panel B are indicated in the structures of the FVIII C1 domain and FVai C1 domain. D, Sequence alignment of human FVIII C1 domain (hFVIII C1), human FV C1 domain (hFV C1), and bovine FV C1 domain (bFV C1). Selected hydrophobic residues from panel B are indicated in the sequence


A
FVIII
PDB entry: 2r7e
Hydrophobicity scale
Low
High
FVai
PDB entry: 1sdd
B
FVIII C1 domain
F2035
F2068
V2130
F2127
I2139
S1880
S1913
S1981
Q1990
G1978
FVai C1 domain
C
I2139
F2127
F2035
F2068
V2130
FVIII C1 domain
Q1990
G1978
S1880
S1913
S1981
FVai C1 domain
D
hFVIII C1 KCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFS-
hFV C1 DCRMPMGLSTGIISDSQIKASEFLGYWEPRLARLNNGGSYNAWSVEKLAAE
bFV C1 ECKMPMGLSTGLIADSQIQASEFWGYWEPKLARLNNGGSYNAWIAEKLSTE
hFVIII C1 -----WIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTY
hFV C1 FASKPWIQVDMQKEVIVTGIQTQGAKHYLKSCYTTEFYVAYSSNQINWQIF
bFV C1 FNPEPWIQVDMQKEVLLTGIQTQGAKHYLKPYYTTEFCVAYSLDRKNWRIF
hFVIII C1 RGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMEL
hFV C1 KGNSTRNVMYFNGNSDASTIKENQFDPPIVARYIRISPTRAYNRPTLRLEL
bFV C1 KGNSTRNVMYFGGNSDASTIKENQIDPPVVARYIRISPTGSYNKPALRLEL
hFVIII C1 MGCDLN
hFV C1 QGCEVN
bFV C1 QGCEVN

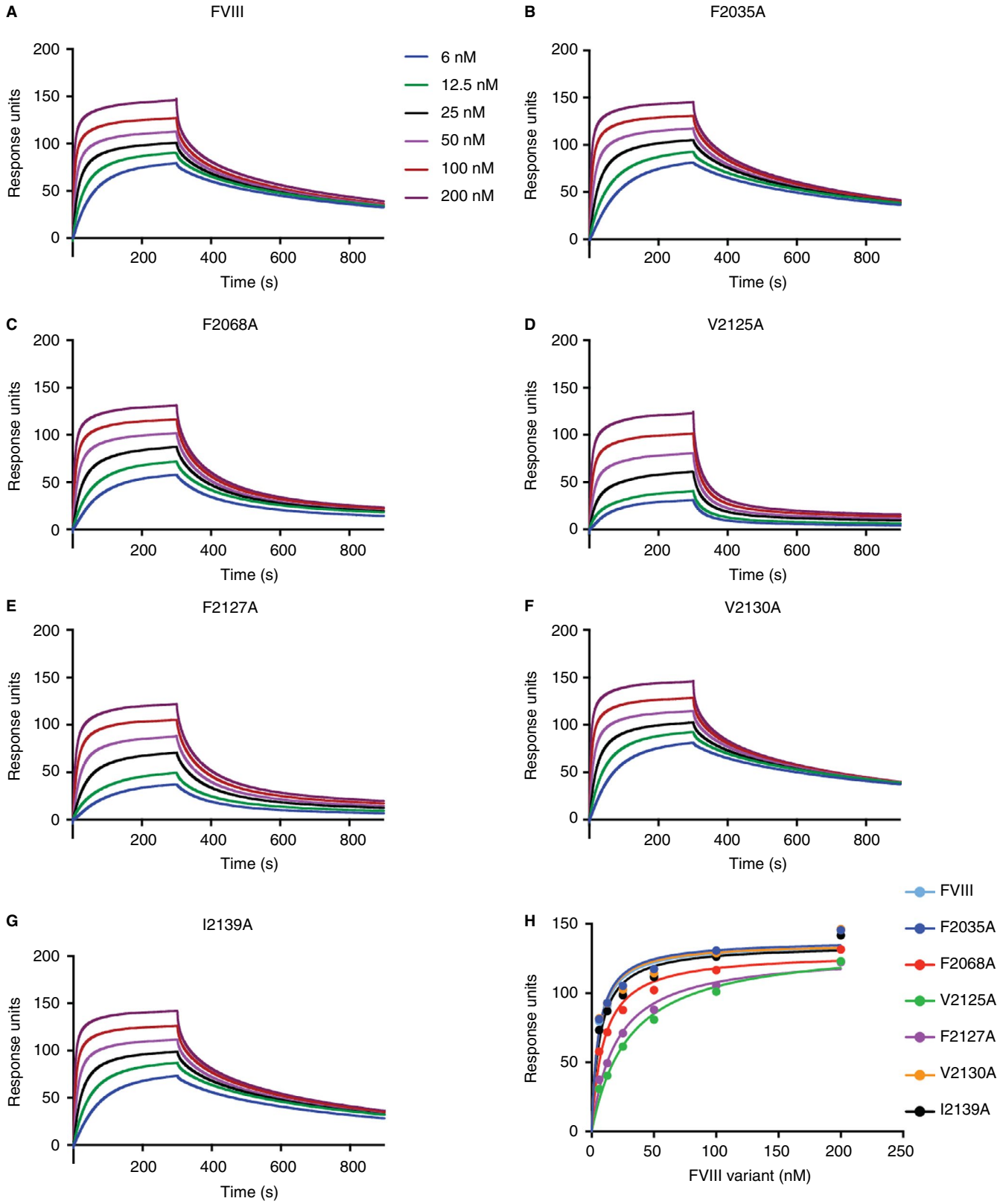


FIGURE 2 Binding of FVIII variants to VWF using SPR analysis. Panels A to G, Various concentrations (6-200 nmol/L) of the indicated FVIII variants were passed over VWF that was immobilized onto a CM5 sensor chip. The binding response is indicated as response units (RU) and was assessed in 20 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 5 mmol/L CaCl2, 0.05% (v/v) Tween 20 at a flow rate of 30 µL/min at 25°C. Binding to VWF was corrected for the binding to a channel without VWF. H, The maximum RU plotted as a function of employed concentration of the FVIII variants

The sensor chip was regenerated after each protein injection using a regeneration buffer containing 20 mmol/L HEPES (pH 7.4), 1 mol/L NaCl, 20 mmol/L EDTA. FVIII binding to VWF was corrected for binding to an empty channel. Responses at equilibrium were plotted as a function of the FVIII concentration to estimate the K_D values.

2.5 | Factor X activation studies

FXa generation assays were performed in the presence of sonicated lipids as described.[21] Briefly, 25 μmol/L of 15% PS, 20% PE, and 65% PC phospholipid vesicles were added to a mix containing 0.3 nmol/L FVIII and 0.2 μmol/L FX. FIXa was mixed in a concentration range from 0 to 16 nmol/L in a buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl 0.2% (w/v) Bovine Serum Albumin (BSA), pH 7.8. Complex formation was allowed as the result of the addition of 1 nmol/L of thrombin and 1.5 mmol/L of $CaCl_2$ at 25°C. The reaction was terminated by addition of 8.8 mmol/L EDTA. Quantity of FXa generated was assessed as described.[21]

3 | RESULTS

3.1 | The FVIII C1 domain comprises unique hydrophobic residues that are exposed to the surface

To identify the surface-exposed structural elements on the C1 domain that are unique to FVIII relative to FV, we compared structures of the C1 domain of human FVIII and FV. Unfortunately, no structure of human FV is available that comprises the complete light chain of human FV. Consequently, detailed information about intra- and inter-domain contacts is lacking. However, there is a crystal structure of inactivated bovine FV (FVai) in which the C1 domain shares more than 80% primary sequence identity with the C1 domain of human FV. We therefore compared the surface exposure of the amino acid residues of the human and bovine C1 domain in the crystal structure of human FVIII and bovine FVai (Figure 1A,B). Results revealed a unique set of five surface-exposed hydrophobic residues, ie, F2035, F2068, F2127, V2130, I2139 that are either more buried or more polar in FVai than in FVIII (Figure 1C). Sequence alignment of the bovine FV C1 domain with human FV C1 domain showed that the polar nature of these residues is maintained in human FV C1 domain (Figure 1D). The findings together imply that the FVIII C1 domain comprises unique hydrophobic residues that are potentially in contact with the solvent rather than with the interior of the C1 domain core.

3.2 | Amino acid residues V2125 and F2127 are crucial for high affinity binding to VWF

To assess the putative role of the surface-exposed hydrophobic residues for VWF binding, five new recombinant FVIII variants were constructed and purified. In these variants, each one of the identified hydrophobic residues was replaced by an alanine residue, ie, F2035A, F2068A, F2127A, V2130A, I2139A. Surface plasmon resonance (SPR) analysis was employed to evaluate the effect of the substitutions on VWF binding. To this end, increasing concentration of the FVIII variants were passed over VWF that was immobilized on a CM5 sensor chip (Figure 2). Results showed that the interaction between FVIII and VWF could not be accurately described by a single site binding model. This may be compatible with the observation that multiple contact sites have been identified between FVIII and VWF.[18,25-29,42] To still gain insight into the binding efficiency, we plotted the maximum binding response as a function of the employed FVIII concentration. The concentration at which half-maximum is reached is used as a measure for this binding efficiency. Results showed that replacement of F2035, V2130, and I2139 by an alanine residue had little, or no effect at all, on the interaction with VWF. In contrast, the substitution at position 2068 resulted in reduced VWF binding. A marked reduction in VWF binding was observed for the F2127A variant. This was reflected by a ~4-fold increase in FVIII concentration that was required to reach half-maximum binding (Figure 2H and Table 2). Notably, V2125 is in close proximity to F2127 (Cα-Cα distance = 6.9 Å according to the crystal structure by Shen et al[9]) and is also exposed to the solvent. We therefore decided to assess whether a FVIII V2125A variant exhibits reduced VWF binding as well. SPR analysis showed that replacement of V2125 to alanine indeed markedly reduces the binding to VWF. The FVIII concentration, at which half maximum binding is reached, was increased more than 6-fold compared to that required for FVIII-WT (Figure 2H and Table 2). These data suggest that F2068 and especially F2127 and V2125 contribute to the interaction with VWF.

3.3 | Replacement of surface-exposed hydrophobic residues by alanine affects cofactor function

The FXa generation efficiency of the FVIII variants was assessed to verify the role of the exposed hydrophobic residues for cofactor function. FXa generation was evaluated at increasing concentrations of FIXa in presence of the thrombin-activated FVIII variants, phospholipids, and calcium ions (Figure 3). The concentration of FIXa at which the half-maximum rate (half-Vmax) of FXa generation is reached can be used as an estimation of the apparent FIXa binding affinity of the FVIII variant (Table 2). The results show that V2125A and I2139A substitutions did not affect the efficiency of FX conversion in the presence of increasing concentrations of FIXa. F2035A presented only a minor reduction in its ability to stimulate FIXa compared to FVIII-WT, whereas for the F2068A and V2130A variant FXa generation was moderately impaired. The F2068A variant mainly displayed a reduced maximum rate of FXa generation. The variant F2127A showed the strongest defect in cofactor function. For this variant, the FIXa concentration to reach half-Vmax was more than 6-fold higher than required for FVIII-WT. These results suggest that the residues F2068 and V2130 and especially phenylalanine at position 2127 contribute to the interaction with FIXa.

TABLE 2 Apparent equilibrium dissociation constants (K_D) of FVIII variants in interaction with FIXa (Figure 3) and VWF (Figure 2)

	Apparent K_d (nmol/L)	
FVIII Variant	**VWF binding**	**FIXa interaction**
FVIII	5.7 ± 1.8	0.6 ± 0.1
F2035A	5.5 ± 1.4	0.8 ± 0.1
F2068A	9.7 ± 1.9	1.2 ± 0.2
V2125A	29.9 ± 4.9	0.4 ± 0.1
F2127A	19.6 ± 2.9	3.9 ± 0.4
V2130A	5.4 ± 1.6	1.5 ± 0.1
I2139A	6.6 ± 1.7	0.6 ± 0.1

Abbreviations: FIXa, activated factor IX; FVIII, factor VIII; VWF, von Willebrand factor

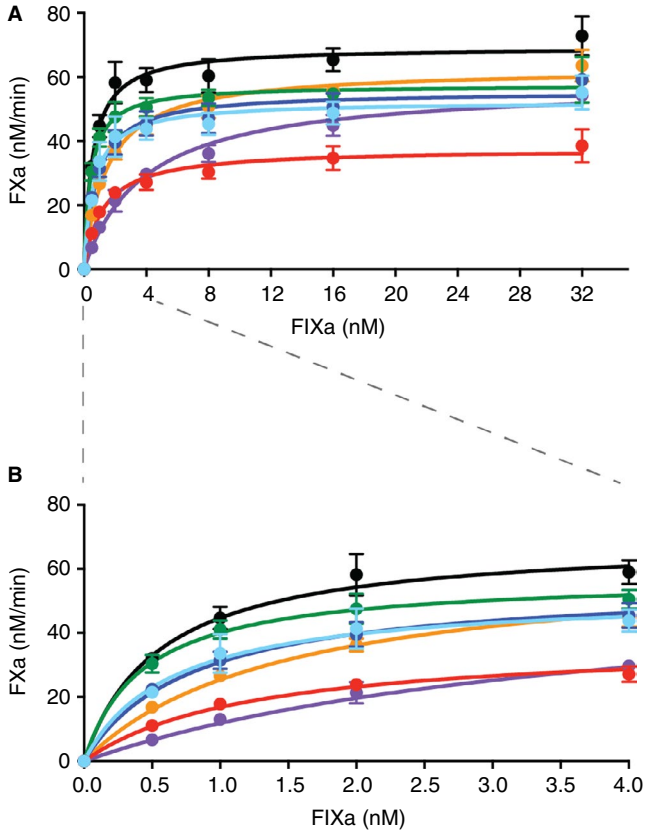


FIGURE 3 FXa generation by FIXa in presence of FVIII variants. A, B, FX was converted into FXa in presence of increasing concentrations of FIXa (0.5-16 nmol/L), phospholipids, Ca^{2+} ions, and 0.3 nmol/L of the FVIII variants as described in the Materials and Methods section. FVIII wild-type is represented in light blue, F2035A in blue, F2068A in red, V2125A in green, F2127A in purple, V2130A in orange, and I2139A in black. Data represent the mean value ± SD of three independent experiments

4 | DISCUSSION

There is a strong structure and function relationship between FVIII and FV. This aspect has provided the unique opportunity in this and other studies to gain insight into the ligand-interactive regions of FVIII and FV.[13,15-17] In the present study, we built upon the previous observation that replacement of the C1 domain of FVIII with that of FV ($FVIII_{C1FVC1}$) affects cofactor function.[15] These findings together suggest that the role of the C1 domain of FVIII is more important and complex than previously assumed. Wakabayashi et al[43] have come to a similar conclusion employing a FVIII variant comprising two FVIII C2 domains ($FVIII_{C2FVIIIC2}$). Intriguingly, the impaired cofactor function as a function of the FIXa concentration of the $FVIII_{C2FVIIIC2}$ variant was remarkably similar to that of the $FVIII_{C1FVC1}$ variant.[15,43] The partial functional defect of both chimeras may be explained by a putative misalignment of the non-native FVIII A3 - FV C1 and FVIII A3 - FVIII C2 domain interface.[43] Although we do not exclude this possibility, we assessed in this study whether there may be unique structural elements in the FVIII C1 domain that mediate the interaction with FIXa and VWF.

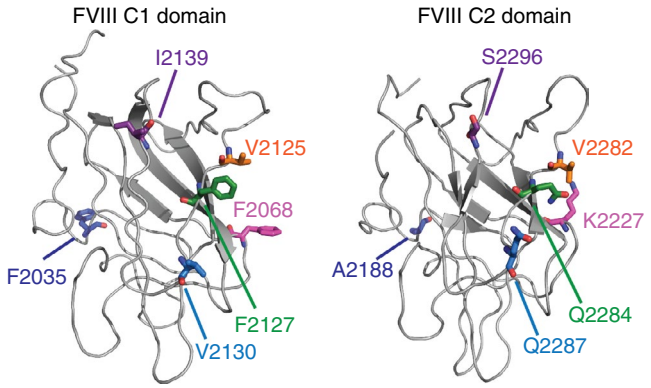


FIGURE 4 Comparison of the C1 and C2 domain of FVIII. On the left is displayed the crystal structure of the FVIII C1 domain [PDB entry: 2r7e[9]] and on the right the FVIII C2 domain. The analyzed surface exposed hydrophobic residues of the C1 residues and the corresponding residues in the C2 domain are indicated in the figure

The identification of the five surface-exposed hydrophobic amino acids in the FVIII C1 domain is remarkable in that hydrophobic residues are usually not interacting with the surrounding solvent (Figure 1).[44] Like in the FV C1 domain, structure and primary sequence comparison between the C1 and C2 domain of FVIII shows that the corresponding residues are also polar in the FVIII C2 domain (Figure 4). This further demonstrates the uniqueness of the surface-exposed hydrophobic residues in the FVIII C1 domain. Our study now reveals that the residues F2068, F2127, V2130 are of functional importance for FVIII biology as these residues contribute to VWF and/or FIXa interaction (Figures 2 and 3). No apparent role for FVIII cofactor function or VWF binding could be attributed to the other surface-exposed residues. Why the FVIII C1 domain comprises these hydrophobic residues remains therefore a topic for further investigation.

A previous Hydrogen Deuterium eXchange Mass Spectrometry (HDX-MS) study on FVIII in complex with the FVIII-binding D'D3 fragment of VWF revealed altered hydrogen-deuterium exchange in three FVIII regions in presence of D'D3, ie, W2062-S2069, T2086-S2095, and S2157-L2166.[28] Based on this observation, the authors concluded that these regions contribute to VWF binding. The VWF binding

residue F2068 is indeed part of one of these regions, which is compatible with this conclusion (Table 2, Figure 2). Although the authors did find reduced deuterium incorporation in region M2124-Y2148, it was excluded from the analysis because of inconsistent results. Yet, residue F2127, which is part of this region, contributes the most to VWF binding among the identified residues in this study (Table 2, Figure 2). We propose that region M2124-Y2148 comprises a VWF binding region after all. It should be considered, however, that HDX-MS mainly records perturbations in deuterium uptake at the amide backbone level and does not provide any information on the side chains of amino acid residues. A direct interaction between the side chain of F2127 of FVIII and VWF without minimal alterations of the hydrogen bonding network of the protein backbone may, therefore, remain undetected by HDX-MS. This may provide an alternative explanation about why HDX-MS did not consistently identify M2124-Y2148 as a region that comprises VWF binding residues. Irrespective of these notions, our study and the HDX-MS study provide complementary information about the role of the C1 domain in VWF binding.

Multiple binding sites have been identified in FVIII for FIXa and VWF in this and other studies.[18,25-29] The relative contribution of each of the identified residues for the overall interaction between FVIII and FIXa/VWF remains to be established. F2127, however, proves to be important for the interaction with both FIXa as well as for VWF binding. This observation is compatible with the protective role of VWF in preventing premature FIXa binding to FVIII. Most likely, VWF not only sterically hinders the interaction between FVIII and FIXa, it may partially share the binding regions on FVIII with FIXa. The proximity of F2127 to the hydrophobic V2125 suggests the involvement of the latter in VWF binding and cofactor function as well. Surprisingly, the variant V2125A displayed a marked reduction in VWF binding, but had no effect in FIXa interaction (Figures 2 and 3). As displayed in Figure 4, the counterpart of C1 V2125, in C2 is also a valine residue (V2282) suggesting that hydrophobicity at this level may be a conserved feature among the C domains. Therefore, while F2127 appears to be specific for VWF and FIXa interaction, V2125 could be rather of support in the interaction with VWF. The importance of F2127 for FVIII function is also demonstrated by the notion that substitution of this residue into a serine residue is associated with hemophilia A.[45-49] The results of our study now provide a possible mechanistic explanation for the cause of the bleeding observed in the patients with a F2127S variant of FVIII. VWF and/or FIXa binding to this FVIII variant may be affected in the patients.

Taken together, our findings provide novel information about the role of the C1 domain in supporting both VWF and FIXa binding. In this view, these findings also stress the importance of the FVIII C1 domain for enhancing the enzymatic activity of FIXa. We propose that activation of FVIII, followed by FVIII-VWF complex dissociation, liberates the now surface-exposed hydrophobic residues for optimal interaction with FIXa.

AUTHOR CONTRIBUTIONS

M.A.P., M.B.S., N.F., C.v.d.Z., and J.v.G. performed experiments; M.A.P., N.F., M.v.d.B, K.M., and A.B.M designed the research; M.A.P., M.B.S, N.F., and A.B.M. analyzed results; M.A.P. and N.F. made the figures; M.A.P., N.F., and A.B.M. wrote the paper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING INFORMATION

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REFERENCES

1. Mannucci PM, Tuddenham EG. The hemophilias–from royal genes to gene therapy. *N Engl J Med*. 2001;344:1773–1779.
2. Vehar GA, Keyt B, Eaton D, *et al*. Structure of human factor VIII. *Nature*. 1984;312:337–342.
3. Fay PJ. Factor VIII structure and function. *Int J Hematol*. 2006;83:103–108.
4. Lenting PJ, van Mourik JA, Mertens K. The life cycle of coagulation factor VIII in view of its structure and function. *Blood*. 1998;92:3983–3996.
5. Andersson LO, Forsman N, Huang K, *et al*. Isolation and characterization of human factor VIII: molecular forms in commercial factor VIII concentrate, cryoprecipitate, and plasma. *Proc Natl Acad Sci USA*. 1986;83:2979–2983.
6. Fass DN, Knutson GJ, Katzmann JA. Monoclonal antibodies to porcine factor VIII coagulant and their use in the isolation of active coagulant protein. *Blood*. 1982;59:594–600.
7. Truett MA, Blacher R, Burke RL, *et al*. Characterization of the polypeptide composition of human factor VIII: C and the nucleotide sequence and expression of the human kidney cDNA. *DNA Mary Ann Liebert Inc*. 1985;4:333–349.
8. Ngo JCK, Huang M, Roth DA, Furie BC, Furie B. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. *Structure*. 2008;16:597–606.
9. Shen BW, Spiegel PC, Chang C, *et al*. The tertiary structure and domain organization of coagulation factor VIII. *Blood*. 2008;111:1240–1247.
10. Svensson LA, Thim L, Olsen OH, Nicolaisen EM. Evaluation of the metal binding sites in a recombinant coagulation factor VIII identifies two sites with unique metal binding properties. *Biol. Chem*. 2013;394(6):761–765. https://www.degruyter.com/view/j/bchm.2013.394.issue-6/hsz-2012-0298/hsz-2012-0298.xml
11. Pipe SW, Montgomery RR, Pratt KP, Lenting PJ, Lillicrap D. Life in the shadow of a dominant partner: the FVIII-VWF association and its clinical implications for hemophilia A. *Blood*. 2016;128:2007–2016.
12. Takeyama M, Nogami K, Okuda M, Shima M. von Willebrand factor protects the Ca2+-dependent structure of the factor VIII light chain. *Br J Haematol*. 2009;146:531–537.
13. Gilbert GE, Novakovic VA, Kaufman RJ, Miao H, Pipe SW. Conservative mutations in the C2 domains of factor VIII and factor V alter phospholipid binding and cofactor activity. *Blood*. 2012;120:1923–1932.
14. Bloem E, Meems H, van den Biggelaar M, Mertens K, Meijer AB. A3 domain region 1803–1818 contributes to the stability of activated factor VIII and includes a binding site for activated factor IX. *J Biol Chem*. 2013;288:26105–26111.
15. Ebberink EH, Bouwens EA, Bloem E, *et al*. Factor VIII/V C-domain swaps reveal discrete C-domain roles in factor VIII function and intracellular trafficking. *Haematologica*. 2017;102:686–694.
16. Bovenschen N, Boertjes RC, van Stempvoort G, *et al*. Low density lipoprotein receptor-related protein and factor IXa share structural requirements for binding to the A3 domain of coagulation factor VIII. *J Biol Chem*. 2003;278:9370–9377.

17. Bos MHA, Camire RM. Blood coagulation factors V and VIII: molecular mechanisms of procofactor activation. *J Coagul Disord*. 2010;2:19–27.
18. Gilbert GE, Kaufman RJ, Arena AA, Miao H, Pipe SW. Four hydrophobic amino acids of the factor VIII C2 domain are constituents of both the membrane-binding and von Willebrand factor-binding motifs. *J Biol Chem*. 2002;277:6374–6381.
19. Lu J, Pipe SW, Miao H, Jacquemin M, Gilbert GE. A membrane-interactive surface on the factor VIII C1 domain cooperates with the C2 domain for cofactor function. *Blood*. 2011;117:3181–3189.
20. Novakovic VA, Cullinan DB, Wakabayashi H, Fay PJ, Baleja JD, Gilbert GE. Membrane-binding properties of the Factor VIII C2 domain. *Biochem J*. 2011;435:187–196.
21. Meems H, Meijer AB, Cullinan DB, Mertens K, Gilbert GE. Factor VIII C1 domain residues Lys 2092 and Phe 2093 contribute to membrane binding and cofactor activity. *Blood*. 2009;114:3938–3946.
22. Soeda T, Nogami K, Nishiya K, *et al*. The factor VIIIa C2 domain (residues 2228–2240) interacts with the factor IXa Gla domain in the factor Xase complex. *J Biol Chem*. 2009;284:3379–3388.
23. Vincent Jenkins P, Freas J, Schmidt KM, Zhou Q, Fay PJ. Mutations associated with hemophilia A in the 558–565 loop of the factor VIIIa A2 subunit alter the catalytic activity of the factor Xase complex. *Blood*. 2002;100:501–508.
24. Lenting PJ, Donath MJ, van Mourik JA, Mertens K. Identification of a binding site for blood coagulation factor IXa on the light chain of human factor VIII. *J Biol Chem*. 1994;269:7150–7155.
25. Leyte A, van Schijndel HB, Niehrs C, *et al*. Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. *J Biol Chem*. 1991;266:740–746.
26. Saenko EL, Scandella D. The acidic region of the factor VIII light chain and the C2 domain together form the high affinity binding site for von willebrand factor. *J Biol Chem*. 1997;272:18007–18014.
27. Lollar P, Hill-Eubanks DC, Parker CG. Association of the factor VIII light chain with von Willebrand factor. *J Biol Chem*. 1988;263:10451–10455.
28. Chiu PL, Bou-Assaf GM, Chhabra ES, *et al*. Mapping the interaction between factor VIII and von Willebrand factor by electron microscopy and mass spectrometry. *Blood*. 2015;126:935–938.
29. Jacquemin M, Lavend'homme R, Benhida A, *et al*. A novel cause of mild/moderate hemophilia A: mutations scattered in the factor VIII C1 domain reduce factor VIII binding to von Willebrand factor. *Blood*. 2000;96:958–965.
30. Krissinel E, Henrick K. Inference of macromolecular assemblies from crystalline state. *J Mol Biol*. 2007;372:774–797.
31. Eisenberg D, Schwarz E, Komaromy M, Wall R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J Mol Biol*. 1984;179:125–142.
32. Adams TE, Hockin MF, Mann KG, Everse SJ. The crystal structure of activated protein C-inactivated bovine factor Va: Implications for cofactor function. *Proc Natl Acad Sci USA*. 2004;101:8918–8923.
33. Liu ML, Shen BW, Nakaya S, *et al*. Hemophilic factor VIII C1- and C2-domain missense mutations and their modeling to the 1.5-angstrom human C2-domain crystal structure. *Blood*. 2000;96:979–987.
34. Fribourg C, Meijer AB, Mertens K. The interface between the EGF2 domain and the protease domain in blood coagulation factor IX contributes to factor VIII binding and factor X activation. *Biochemistry*. 2006;45:10777–10785.
35. van den Brink EN, Turenhout EA, Bank CM, Fijnvandraat K, Peters M, Voorberg J. Molecular analysis of human anti-factor VIII antibodies by V gene phage display identifies a new epitope in the acidic region following the A2 domain. *Blood*. 2000;96:540–545.
36. Meems H, Van Den Biggelaar M, Rondaij M, van der Zwaan C, Mertens K, Meijer AB. C1 domain residues Lys 2092 and Phe 2093 are of major importance for the endocytic uptake of coagulation factor VIII. *Int J Biochem Cell Biol*. 2011;43:1114–1121.
37. Van Den Biggelaar M, Bierings R, Storm G, Voorberg J, Mertens K. Requirements for cellular co-trafficking of factor VIII and von Willebrand factor to Weibel-Palade bodies. *J Thromb Haemost*. 2007;5:2235–2242.
38. Mertens K, Bertina RM. Activation of human coagulation factor VIII by activated factor X, the common product of the intrinsic and the extrinsic pathway of blood coagulation. *Thromb Haemost*. 1982;47:96–100.
39. Mertens K, Bertina RM. Pathways in the activation of human coagulation factor X. *Biochem J*. 1980;185:647–658.
40. Hendrix H, Lindhout T, Mertens K, Engels W, Hemker HC. Activation of human prothrombin by stoichiometric levels of staphylocoagulase. *J Biol Chem*. 1983;258:3637–3644.
41. Bloem E, van den Biggelaar M, Wroblewska A, *et al*. Factor VIII C1 domain spikes 2092–2093 and 2158–2159 comprise regions that modulate cofactor function and cellular uptake. *J Biol Chem*. 2013;288:29670–29679.
42. van den Biggelaar M, Meijer AB, Voorberg J, Mertens K. Intracellular cotrafficking of factor VIII and von Willebrand factor type 2N variants to storage organelles. *Blood*. 2009;113:3102–3109.
43. Wakabayashi H, Fay PJ. Replacing the factor VIII C1 domain with a second C2 domain reduces factor VIII stability and affinity for factor IXa. *J Biol Chem*. 2013;288:31289–31297.
44. Dyson HJ, Wright PE, Scheraga HA. The role of hydrophobic interactions in initiation and propagation of protein folding. *Proc Natl Acad Sci USA*. 2006;103:13057–13061.
45. Trossaërt M, Regnault V, Sigaud M, Boisseau P, Fressinaud E, Lecompte T. Mild hemophilia A with factor VIII assay discrepancy: using thrombin generation assay to assess the bleeding phenotype. *J Thromb Haemost*. 2008;6:486–493.
46. Castaman G, Mancuso ME, Giacomelli SH, *et al*. Molecular and phenotypic determinants of the response to desmopressin in adult patients with mild hemophilia A. *J Thromb Haemost*. 2009;7:1824–1831.
47. Green PM, Bagnall RD, Waseem NH, Giannelli F. Haemophilia A mutations in the UK: results of screening one-third of the population. *Br J Haematol*. 2008;143:115–128.
48. Martín-Salces M, Venceslá A, Alvárez-Román MT, *et al*. Clinical and genetic findings in five female patients with haemophilia A: Identification of a novel missense mutation, p.Phe2127Ser. *Thromb Haemost*. 2010;104:718–723.
49. Johnsen JM, Fletcher SN, Huston H, *et al*. Novel approach to genetic analysis and results in 3000 hemophilia patients enrolled in the my life. Our future initiative. *Blood Adv*. 2017;1:824–834.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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