Hydrogen–Deuterium Exchange Mass Spectrometry Identifies Activated Factor IX-Induced molecular Changes in Activated Factor VIII

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Abstract

Hydrogen–deuterium exchange mass spectrometry (HDX-MS) was employed to gain insight into the changes in factor VIII (FVIII) that occur upon its activation and assembly with activated factor IX (FIXa) on phospholipid membranes. HDX-MS analysis of thrombin-activated FVIII (FVIIIa) revealed a marked increase in deuterium incorporation of amino acid residues along the A1–A2 and A2–A3 interface. Rapid dissociation of the A2 domain from FVIIIa can explain this observation. In the presence of FIXa, enhanced deuterium incorporation at the interface of FVIIIa was similar to that of FVIII. This is compatible with the previous finding that FIXa contributes to A2 domain retention in FVIIIa. A2 domain region Leu631-Tyr637, which is not part of the interface between the A domains, also showed a marked increase in deuterium incorporation in FVIIIa compared with FVIII. Deuterium uptake of this region was decreased in the presence of FIXa beyond that observed in FVIII. This implies that FIXa alters the conformation or directly interacts with this region in FVIIIa. Replacement of Val634 in FVIII by alanine using site-directed mutagenesis almost completely impaired the ability of the activated cofactor to enhance the activity of FIXa. Surface plasmon resonance analysis revealed that the rates of A2 domain dissociation from FVIIIa and FVIIIa-Val634Ala were indistinguishable. HDX-MS analysis showed, however, that FIXa was unable to retain the A2 domain in FVIIIa-Val634Ala. The combined results of this study suggest that the local structure of Leu631-Tyr637 is altered by FIXa and that this region contributes to the cofactor function of FVIII.

Keywords
- hydrogen–deuterium exchange
- factor VIII
- factor IXa
- A2 domain retention
- interactive regions

Introduction

Factor VIII (FVIII) is essential for the proper functioning of the coagulation cascade. Within the cascade, FVIII acts as a cofactor for factor IX (FIX) in the activated factor X (FXa)-generating complex.[1] Functional absence of FVIII is linked to the bleeding disorder hemophilia A stressing the critical role of FVIII for effective bleeding arrest at sites of vessel injury.[2] FVIII consists of A, B, and C domains organized as: A1-*a1*-A2-*a2*-B-*a3*-A3-C1-C2.[3,4] *a1*, *a2*, and *a3* represent short acidic amino acid regions comprising sulfated tyrosine residues. During synthesis, FVIII is cleaved between the B domain and the *a3* region. As a consequence, FVIII circulates in blood as a heterodimeric protein comprising a heavy chain (A1-*a1*-A2-*a2*-B) that is

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noncovalently linked to a light chain (*a3*-A3-C1-C2). In plasma, FVIII forms a tight complex with von Willebrand factor (VWF).[1] In this complex, FVIII is protected from premature binding to its ligands, proteolytic degradation, and fast clearance.[4]

FVIII requires activation by thrombin to perform its role in the coagulation cascade. It has also been shown that FXa can activate FVIII.[5,6] Proteolytic cleavage of FVIII occurs after Arg372 (between *a1* and A2 domain), Arg740 (between *a2* and the B domain), and Arg1689 (between *a3* and A3 domain). This leads to the release from FVIII of the B domain and the *a3* region as well as dissociation of the FVIII–VWF complex.[1,4] The resulting activated FVIII (FVIIIa) is a heterotrimer that consists of three noncovalently linked units: A1-*a1*, A2-*a2*, and A3-C1-C2. Once activated, FVIIIa can assemble with activated FIX (FIXa) on phosphatidylserine-containing membranes. Within the complex, FVIIIa enhances the catalytic activity of FIXa approximately 200,000-fold.[7] To prevent uncontrolled activation of FX, FVIIIa can be inactivated via proteolytic cleavage by activated protein C or via spontaneous dissociation of the A2 domain from FVIIIa.[4,8–10] It has been shown that the rate of A2 domain dissociation is reduced when FVIIIa is in complex with FIXa.[1]

Crystallographic and electron microscopy studies on FVIII have provided insights into the structure of FVIII.[11–13] These structures reveal that the A domains form a propeller-shaped structure on top of two parallelly aligned C domains. Molecular modeling studies combined with previous site-directed mutagenesis studies have shown that the C domains mainly interact with phospholipid membranes, whereas the A domains interact with FIXa and FX.[14–17] Despite the progress that has been made, detailed information is still lacking concerning molecular rearrangements in FVIII that take place upon its activation and its subsequent assembly with FIXa on phospholipid membranes.

Hydrogen–deuterium exchange mass spectrometry (HDX-MS) has been successfully employed to identify protein-binding sites in protein complexes. In addition, it has provided insight into putative local conformational changes in proteins upon complex formation or protein activation.[18–20] HDX-MS takes advantage from the fact that hydrogens, including those at the protein backbone, naturally exchange for deuterium atoms when a protein is transferred from H_2O to D_2O. Comparing the bound and unbound proteins, the rate of deuterium exchange can be altered at sites where proteins interact or conformational changes occur.[18–22] In the present study, we employed HDX-MS to assess changes in FVIII upon activation and binding to FIXa in the presence of procoagulant phospholipid membranes.

Methods

Materials

DMEM/F12 was from Lonza (Breda, The Netherlands) and fetal calf serum from Bodinco (Alkmaar, The Netherlands). L-α-Phosphatidylethanolamine transphosphatidylated (Egg, Chicken), L-α-phosphatidylserine (Brain, Porcine), and L-α-phosphatidylcholine (Egg, Chicken) were from Avanti Polar Lipids (Alabaster, Alabama, United States). Geneticin, Ultrapure urea, Molecular Biology grade 5M NaCl solution, and Tris-HCl, Precast SDS/PAGE gels, Coomassie Brilliant Blue, and DMRIE-C reagent were from Invitrogen (Breda, The Netherlands). PageRuler Unstained Broad Range Protein Ladder and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) were obtained from Thermo Fisher Scientific (Breda, The Netherlands). NaCl was purchased from Fagron (Rotterdam, The Netherlands) and HEPES was from Serva (Heidelberg, Germany). Formic acid, acetonitrile, and ultrapure water for mass spectrometry were from Biosolve (Valkenswaard, The Netherlands). Deuterium oxide 99.9% and leech-derived hirudin was from Sigma-Aldrich (Saint Louis, Missouri, United States). A BigDye Terminator Sequencing kit was obtained from Applied Biosystems (Foster City, United States). A QuikChange site-directed mutagenesis kit was purchased from Agilent Technologies (Santa Clara, California, United States). All other chemicals were from Merck (Darmstadt, Germany).

Proteins

Plasma-derived thrombin and FIXa were obtained as described previously.[23,24] Active site titration combined with Bradford analysis revealed more than 90% purity of the employed FIXa batch.[25,26] CLB-CAg9, CLB-EL14, CLB-VK34, and CLB-CAg117 antibodies have been described in previous studies.[27,28] B-domain-deleted FVIII (GenBank accession number ABV90867.1) was codon-optimized and purchased from Thermo Fisher Scientific (United States) in pcDNA3.1(+) expression vector using *Nhe*I and *Not*I restriction. FVIII-V634A mutant (legacy numbering is used throughout this article) was generated using the QuikChange site-directed mutagenesis kit. Mutagenesis was confirmed using BigDye Terminator. B-domain-deleted FVIII expressing stable HEK293 cells were generated by transfection of FVIII expression vectors using DMRIE-C reagent, after which cells were cultured in the presence of 1 mg/mL G-418. Clones were selected and expressing clones were identified using enzyme-linked immunosorbent assay (CAg117-CAg9^{PO}). Expressed proteins were purified using CLB-VK34 antibody as described.[28] Protein concentrations were determined as described in van den Biggelaar et al.[29] Although SDS-PAGE revealed that FVIII-V634A was more than 95% pure (►**Supplementary Fig. S1A**, available in the online version), the specific activity was only 140 U/mg. This implies a poor activity of the variant. SDS-PAGE and activation characteristics of FVIII, FVIII-V634A, and FIXa are shown in ►**Supplementary Fig. S1** (available in the online version).

Liposome Preparation

L-α-Phosphatidylethanolamine, L-α-phosphatidylserine, and L-α-phosphatidylcholine were mixed in a 4:3:13 molar ratio. After evaporation of organic solvents, lipids were resuspended in Tris-buffered saline (TBS, pH 7.4) and aliquots were stored at −80°C. Liposomes were prepared by sonication and centrifugation at 16,000 × g for 30 minutes at 4°C. Phosphate content was determined by phosphate measurement according to Rouser.[30] Liposomes were stored maximum 1 week at 4°C.

Hydrogen–Deuterium Exchange Mass Spectrometry

22.5 µL of FVIII (final concentration 2.4 µM) was preincubated with 6.7 µL lipids (final concentration 0.16 mM) at room temperature (RT) for 5 minutes in a buffer containing 460 mM NaCl, 10 mM $CaCl_2$, and 20 mM imidazole pH 7.3. Subsequently, 10 µL of preincubated FVIII was mixed with 4.9 µL of FIXa (13.8 µM in 50 mM NaCl, 25 mM Tris-HCl, pH 7.5, 50% glycerol) or with buffer alone and incubated for 90 seconds at RT. Then, this was mixed with 2.5 µL thrombin (1.35 µM in TBS with 10 mM $CaCl_2$ and 12.5% glycerol) or with buffer alone and was incubated for 60 seconds at RT. The final concentration of FVIII after thrombin addition was 1.4 µM, 92 µM for the lipids, 3.9 µM for FIXa, and 0.2 µM for thrombin. Immediately after this incubation the sample was placed in a LEAP PAL system (LEAP Technologies, Morrisville, North Carolina, United States), cooled at 4°C, and the sample was further processed for deuterium exchange. Briefly, 3 µL sample was diluted in 27 µL buffer (130 mM NaCl, 20 mM imidazole pH 7.3, 10 mM $CaCl_2$ in H2O or 98% D2O) for 100 seconds at 24°C. By this dilution the final concentration of NaCl in the sample was 150 mM. Then 25 µL of the diluted sample was quenched in 25 µL quenching solution (1 g TCEP dissolved in 2 mL 2M urea, 1M NaOH) for 1 minute at 4°C. Further processing of in-line digestion into peptides and liquid chromatography of the sample were performed in a cooled (4°C) environment under an isocratic flow of 5% acetonitrile, 0.1% formic acid at a flow of 100 µL/min. Samples were digested on a pepsin column (Poroszyme Immobilized Pepsin Cartridge) and peptides were bound to a trap (Acclaim Guard Column 120 C18, 5 µm, 2.0 × 10 mm, Thermo Fisher). Peptides were eluted from the trap and resolved over a 30 mm length, 1 mm diameter C18 column, particle size 3 µm (Hypersil Gold C18, Thermo Cat no 25003–031030) using a 12 minute gradient from 0 to 64% acetonitrile at 50 µL/min. Peptides in the mobile phase were measured by an LTQ Orbitrap-XL (Thermo). Using an electrospray ionization source, an electrospray was created at 4.5 kV with 30, 10, and 10 arbitrary units of sheath, auxiliary, and sweep gas, respectively. Signal was obtained in the positive mode, in the mass range of 300 to 2,000 m/z by resolution of 30,000.

A reference peptide list was generated by fragmenting FVIII-DB wild type (WT) using collision-induced dissociation fragmentation on an LTQ Orbitrap-XL (Thermo) and by HCD on an Orbitrap-Fusion (Thermo) and analysis using Peaks Studio 7.0 (Bioinformatics Solutions Inc.). Deuterium uptake was calculated using HDExaminer (Sierra Analytics) for peptides within 1 minute retention time. Then, the identified peptides were investigated manually for misidentifications or other errors. Peptides of which the deuterium incorporation could not accurately be calculated due to low intensity or high complexity were discarded. The percentage of deuterium uptake was calculated for each peptide relative to the theoretical maximum deuterium incorporation. Differences in uptake of more than 5% compared with the theoretical maximum were modeled in the FVIII structure using PyMol software (Schrodinger, LLC).

Surface Plasmon Resonance Analysis

Surface plasmon resonance (SPR) using a Biacore T200 (GE Life Sciences) was performed as described in Bloem et al.[31] Briefly, human anti-FVIII C2 domain antibody CLB-EL14 IgG4 was coupled to a CM5 chip to a density of 5,000 response units (RU) according to manufacturer's suggestions. FVIII (WT or V634A) was bound to the anti-FVIII C2 antibody on the chip to 2,500 RU in a buffer of 150 mM NaCl, 5 mM $CaCl_2$, 0.05% (v/v) Tween 20, 20 mM HEPES, pH 7.4. 2 nM thrombin was passed over FVIII for 1 minute and dissociation of the FVIII-A2 domain was monitored.

FXa Generation Assays

FXa generation was performed as described in Meems et al.[14] Briefly, FVIII (0.3 nM) or FVIII-V634A (0.3 nM) was incubated with FX (200 nM) and various concentrations of FIXa (0–64 nM) in the presence thrombin (1 nM), 25 µM of phospholipids, and 1.5 mM $CaCl_2$ in a buffer containing 150 mM NaCl, 50 mM Tris-HCl plus 0.2% (w/v) fatty acid free bovine serum albumin, pH 7.8, in a final volume of 40 µL at 25°C. The reaction was stopped after 2 minutes by adding 50 µL of 16 mM EDTA in assay buffer. FXa activity was determined in a microtiter plate reader (Spectramax, Molecular Devices) at 25°C using S-2765 chromogenic substrate. The effect of FIXa on the decline of FVIIIa cofactor function over time has been determined as described in Bloem et al.[31] Briefly, 0.3 nM FVIII or FVIII-V634A was activated by 2 nM thrombin in the presence of 25 µM phospholipid vesicles, 1.5 mM $CaCl_2$, 40 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 0.2% (w/v) BSA. At nine time intervals after activation, FXa generation was allowed for 1 minute by the addition of 16 nM FIXa and 200 nM FX. In a parallel experiment, the FVIII and FVIII-V634A were activated under the same conditions and then in the presence of 16 nM FIXa. At nine time intervals after activation, FXa generation was allowed by the addition of 200 nM FX. FXa generation was measured as described before.[14]

Results

HDX-MS of Thrombin-Activated FVIII in the Presence and Absence of FIXa

FVIII was activated by thrombin with and without FIXa to assess the molecular changes in FVIII using HDX-MS. Activation was performed in the presence of procoagulant phospholipids to facilitate the interaction with FIXa.[4] To obtain a global overview of the molecular changes in FVIII, we incubated the protein mixtures for 100 seconds in D_2O after which the proteins were processed for MS analysis. In total, we obtained 663 peptides covering 89% of FVIII (►**Supplementary Fig. S2** and ►**Supplementary Table S1**, available in the online version). Compared with FVIII, a marked increase in deuterium incorporation was observed in FVIIIa mainly for A2 domain peptides located at the interface between the A2 and A3 domains. On the A3 domain, one peptide at the interface with the A2 domains also showed a marked increase in deuterium uptake (►**Fig. 1A, B**). The results suggest a conformational change at the involved FVIII regions, or that these

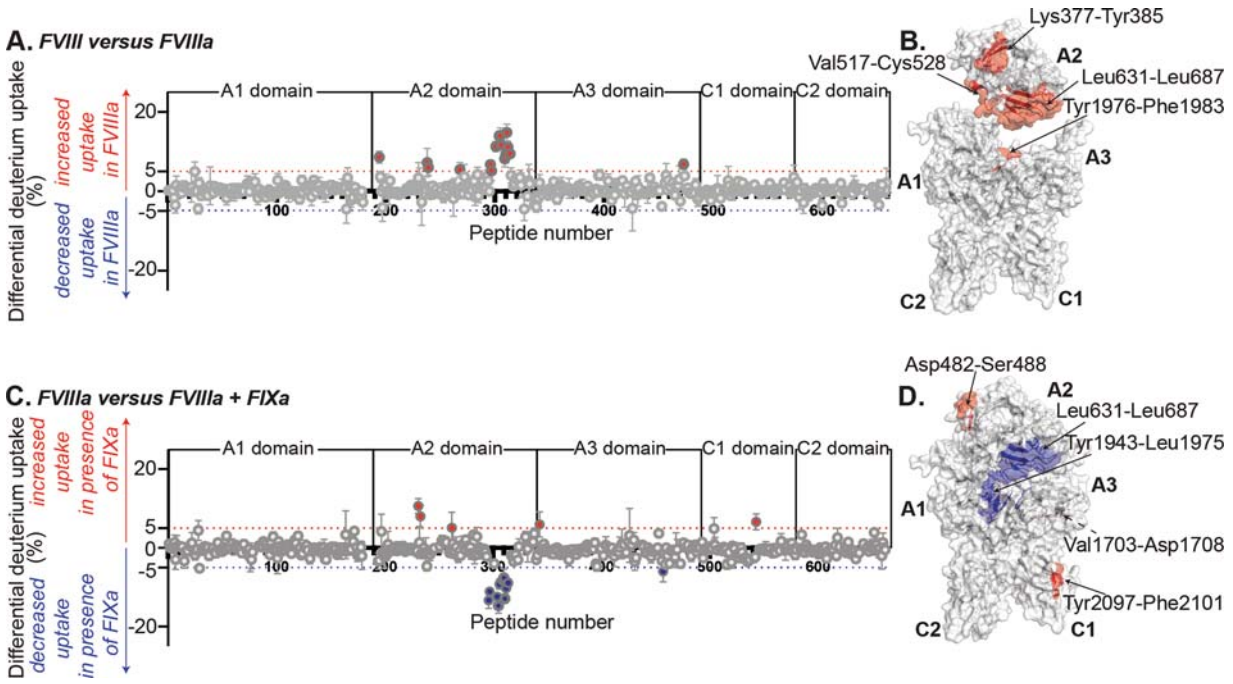

Fig. 1 HDX-MS on FVIII, FVIIIa, and FVIIIa in the presence of FIXa. FVIII, FVIIIa, or FVIIIa with FIXa was incubated in deuterated buffer for 100 seconds in the presence of procoagulant phospholipid membranes. The percentage of deuterium incorporation of a peptide was assessed relative to the theoretical maximum of incorporation in that peptide. The amino acid sequence of the peptides, shown by the ID number on the *x*-axis in panels (**A**) and (**C**), is provided in ►**Supplementary Table S1**. Panel (A) shows the differential deuterium incorporation between FVIII and FVIIIa. The *y*-axis displays the percentage of deuterium uptake of the peptides of FVIIIa subtracted with that of FVIII. The peptides revealing a difference of more than 5% or less than −5% are indicated on the structure of FVIII in panel (**B**) (PDB: 3cdz[11]). Panel (C) shows the differential deuterium incorporation of FVIIIa in the presence and absence of FIXa. The *y*-axis displays the percentage of deuterium uptake of peptides of FVIIIa subtracted with that of FVIIIa in the presence of FIXa. Peptides that showed a difference of more than 5% or less than −5% are indicated on the structure of FVIII in panel (**D**) (PDB: 3cdz[11]). The average of three independent experiments is shown in panels (A) and (C). Error bars represent the standard deviation. HDX-MS, hydrogen–deuterium exchange mass spectrometry.

regions become more exposed to the solvent after activation of FVIII. Intriguingly, in the presence of FIXa, peptides at the interface between the A domains of FVIIIa showed a decrease in deuterium incorporation compared with FVIIIa in the absence of FIXa (►**Fig. 1C, D**). Notably, two peptides covering region Leu631-Trp637, which is not part of the interface, also showed a prominent decrease in deuterium uptake in the presence of FIXa. Several identified peptides displayed an increased deuterium incorporation in A2, A3, and C1 domains (Asp482-Ser488, Val1703-Asp1708, and Tyr2097-Phe2101) (►**Fig. 1C, 1D**). The results together imply that multiple regions located at the interface or the surface of the A domains display differential deuterium incorporation upon activation of FVIII or upon interaction of FVIIIa with FIXa.

Time-Resolved HDX-MS of FVIII and FVIIIa in the Presence and Absence of FIXa

To gain more insight into the altered deuterium uptake of the peptides in FVIIIa in the presence or absence of FIXa, HDX was also assessed at different time intervals (see ►**Supplementary Fig. S3** (available in the online version) for the full set of deuterium uptake plots). To this end, FVIII, FVIIIa, and FVIIIa in the presence of FIXa were incubated with phospholipids in D_2O for 10, 30, and 100 seconds. In FVIII, the A2 domain interface peptides Leu668-Phe678, Met680-Leu687, and Tyr656-Thr667 showed a deuterium uptake of 6% of the theoretical maximum uptake at 10 seconds, which increased to 11% at 100 seconds (►**Fig. 2A**). Upon activation of FVIII by thrombin, deuterium uptake for these peptides was increased by 11 to 14% for all the time points. Remarkably, in the presence of FIXa, the levels of deuterium incorporation for the peptides at the domain interface were almost indistinguishable from that of FVIII prior to thrombin activation (►**Fig. 2A**). These findings together may be explained by the rapid dissociation of the A2 domain from FVIIIa, which leads to the increased deuterium uptake of peptides at the A2 interface. Within the FVIIIa–FIXa complex, FIXa is known to reduce the rate of A2 domain dissociation for FVIIIa thereby stabilizing FVIIIa.[1] This phenomenon may explain the reduced deuterium incorporation of the interface peptides when FVIII is activated in the presence of FIXa.

A2 Domain Region Leu631-Trp637 Contributes to Cofactor Function

The trend of deuterium incorporation obtained for peptides overlapping the region Leu631-Trp637 was distinct from that of the peptides at the A2–A3 domain interface. In the presence of FIXa, deuterium uptake in this region of FVIIIa was reduced beyond that observed in FVIII prior to thrombin activation (►**Fig. 2B**). Intriguingly, substitution of Val634 by an alanine has been associated with mild to moderate hemophilia A.[32,33] A residual FVIII activity of 2 to 5% has been assessed in patients

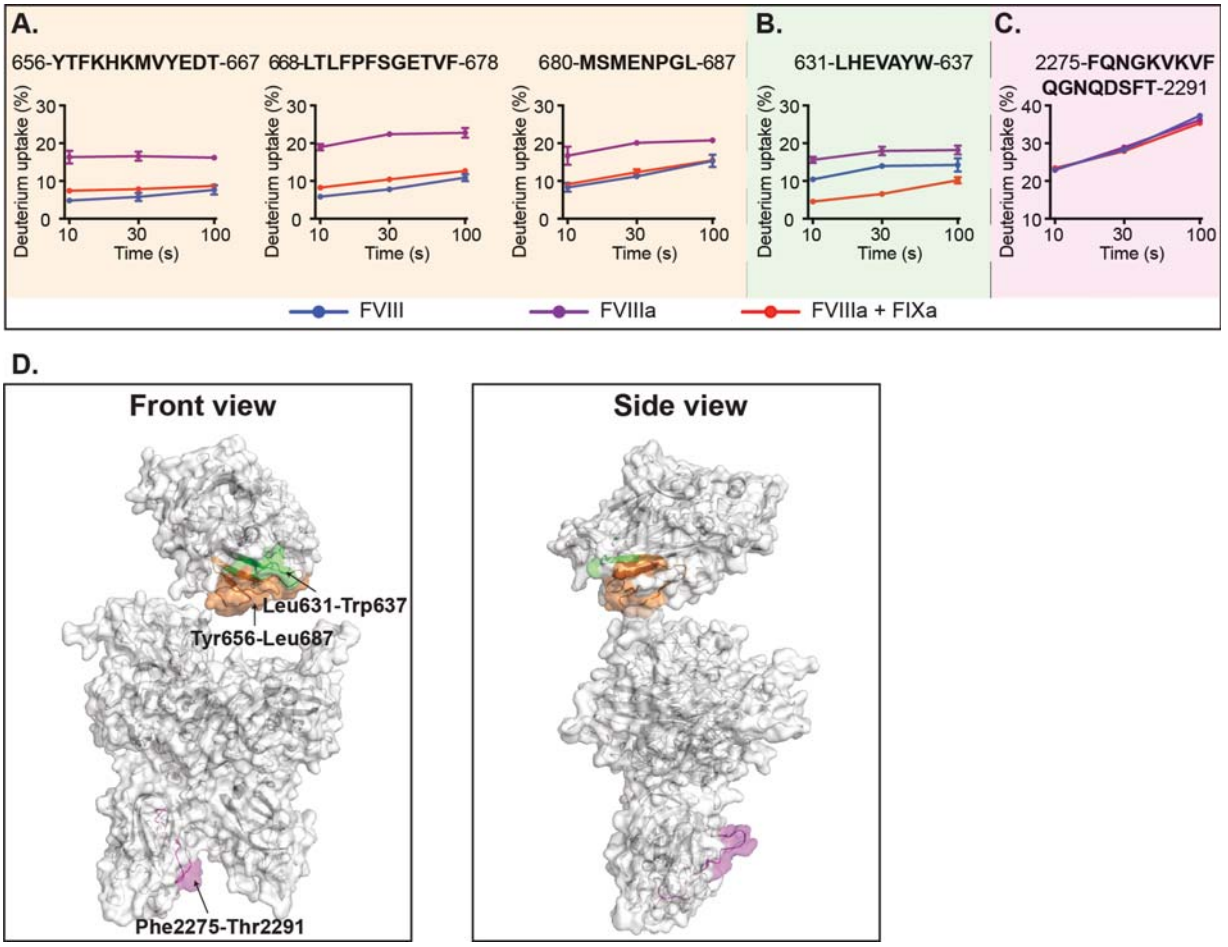

Fig. 2 Time-dependent deuterium incorporation of FVIII, FVIIIa, and FVIIIa in the presence of FIXa. FVIII, FVIIIa, and FVIIIa in the presence of FIXa were incubated with deuterium for 10, 30, or 100 seconds and the level of deuterium uptake was assessed by HDX-MS. The percentage of deuterium uptake relative to the maximum theoretical uptake is shown on the *y*-axis of the panels (**A**)–(**C**). Panel (A) shows the deuterium uptake of the interface peptides 656-YTFKHKMVYEDT-667, 668-LTLFPFSGETVF-678, and 680-MSMENPGL-687. Panel (B) displays deuterium uptake of A2 domain peptide 631-LHEVAYW-637, which is not part of the A domain interface. Panel (C) shows a representative peptide that displayed no change in deuterium incorporation, i.e., C2 domain peptide 2275-FQNGKVKVFQGNQDSFT-2291. The mean of two independent experiments is shown in panels (A)–(C). Error bars represent the spread between the actual data points. Panel (**D**) shows the regions that are covered by the peptides on the crystal structure of FVIIII (PDB: 3cdz[11]). HDX-MS, hydrogen–deuterium exchange mass spectrometry.

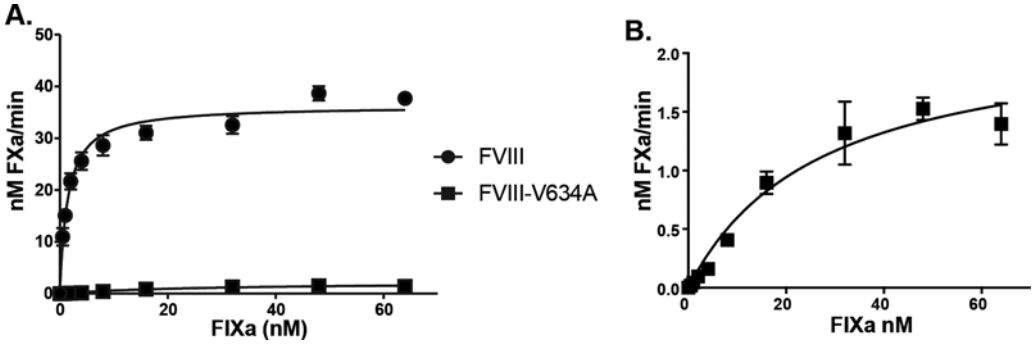

Fig. 3 FXa generation by FIXa in the presence of FVIII or FVIII-V634A. (**A**) Activation of FX was assessed by increasing concentration of FIXa (0–64 nM) in the presence of 0.3 nM FVIII or FVIII-V634A, 200 nM FX, 1 nM thrombin, 1.5 mM $CaCl_2$, and 25 µM phospholipids as described in the Materials and Methods section. FVIII is shown by circles and FVIII-V634A by squares (**B**). In panel (B), the *y*-axis is adjusted and shows FXa generation by FIXa in the presence of FVIII-V634A.

with mild hemophilia A.[32,33] To gain more insight into the role of Val634 for FVIII function, we constructed and purified a FVIII-V634A variant (►**Supplementary Fig. S1**, available in the online version). Results revealed a marked decrease of efficiency of FVIIIa-V634A in enhancing the activity of FIXa compared with FVIIIa (►**Fig. 3**). No defect in thrombin activation of the variant could be observed (►**Supplementary Fig. S1B**, available in the online version). The findings also

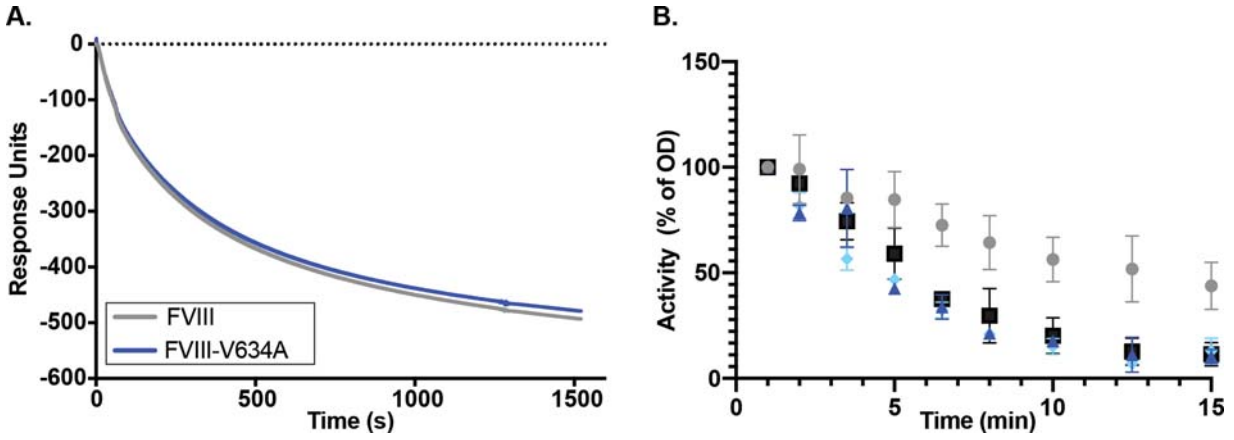

Fig. 4 A2 domain dissociation from activated FVIII and FVIII-V634A. (**A**) Stability of activated FVIII and FVIII-V634A assessed by SPR analysis. FVIII and FVIII-V634A were immobilized on a CM5 sensor chip to a density of 2,500 response units via anti-C2 domain antibody EL14. 2 nM thrombin in 150 mM NaCl, 5 mM $CaCl_2$, 0.05% (v/v) Tween 20, 20 mM HEPES, pH 7.4 was passed over the immobilized FVIII for 60 seconds. The decrease in response units, caused by A2 domain dissociation from activated FVIII (*gray line*) and FVIII-V634A (*blue line*), was monitored as a function of time. (**B**) Stability of FVIII and FVIII-V634A assessed by enzyme kinetics. 0.3 nM FVIII (*black squares*) or FVIII-V634A (*light blue diamonds*) was activated by 2 nM thrombin as described in the Materials and Methods section. At the indicated time points, 16 nM FIXa and 200 nM FX were added to allow FXa generation. 0.3 nM FVIII (*gray circles*) or FVIII-V634A (*dark blue triangles*) were activated by 2 nM thrombin in the presence of 16 nM FIXa. At the indicated time points, 200 nM FX was added to allow FXa generation. SPR, surface plasmon resonance.

show that the FIXa concentration required to reach half-maximum of FXa generation is approximately 18-fold higher for the FVIIIa-V634A compared with FVIIIa (►**Fig. 3A, B**). These observations suggest that FVIIIa-V634A may have an altered interaction with FIXa. It cannot be excluded, however, that the Val634Ala mutation leads to an accelerated rate of A2 domain dissociation upon activation of the FVIIIa-V634A variant.

A2 Domain Retention Studies in FVIII and FVIII-V634A

SPR analysis was performed to assess whether activated FVIII-V634A exhibits an increased rate of A2 domain dissociation compared with FVIIIa. To this end, FVIII and the FVIII-V634A variant were immobilized via an anti-C2 domain antibody to the same density on the surface of a CM5 sensor chip. The FVIII variants were subsequently activated by thrombin. The decrease in RU reflects the dissociation rate of the A2 domain from FVIIIa as described in previous studies.[31,34] Results revealed no difference in A2 dissociation between FVIIIa and FVIIIa-V634A (►**Fig. 4A**). This suggests that an increased instability of FVIIIa-V634A does not explain the impaired activity of this variant. The result also confirms that the variant exhibits no activation defect.

The stability of FVIIIa and FVIIIa-V634A was also evaluated using an enzyme kinetics assay. To this end, FVIII and FVIIIa-V634A were activated in the presence and absence of FIXa on phospholipid membranes. The ability of FVIIIa and FVIIIa-V634A to enhance the activity of FIXa was followed in time (►**Fig. 4**). Both FVIIIa and FVIIIa-V634A showed the same decrease in enhancing the activity of FIXa when the cofactors were activated in the absence of FIXa. Notably, only FVIIIa showed a delayed decrease in enhancing the FIXa activity when both cofactors were activated in the presence of FIXa. This implies that FIXa is unable to stabilize the activated FVIII-V634A variant.

HDX-MS was employed to establish whether there are major conformational changes between FVIII and FVIII-V634A. Comparison of FVIIIa and FVIIIa-V634A revealed, however, no difference in deuterium uptake between the two proteins (►**Fig. 5A**). This implies that there is no major structural change, if any at all, in the FVIIIa-V634A variant compared with FVIIIa. In the presence of FIXa, however, deuterium incorporation of thrombin-activated FVIII-V634A was almost indistinguishable from those of FVIIIa and FVIIIa-V634A in the absence of FIXa (►**Fig. 1C** vs. ►**Fig. 5B**). This confirms the result obtained by the enzyme kinetics assay that FIXa is unable to retain the A2 domain in FVIIIa-V634A. Taken together, the combined findings show that the region Leu631-Trp637 contributes to cofactor function. In addition, the FVIII-V634A variant exhibits an altered interaction with FIXa.

Discussion

In this study, we investigated the deuterium perturbations in coagulation FVIII upon thrombin activation and its assembly with FIXa on phospholipid membranes. FVIII activation resulted in a marked increase in deuterium incorporation mainly in A2 domain regions at the interface with the A3 domain (►**Fig. 1A, B**). This observation is compatible with the self-dampening of cofactor activity which is the result of the rapid A2 domain dissociation from FVIIIa.[35] The interactive regions are fully exposed to the solvent after dissociation of the A2 domain thereby facilitating deuterium incorporation at these sites. In this view, it remains remarkable, however, that no changes are observed in the A1 domain at the A1–A2 domain interface. Also, for the A3 domain, only a short stretch of amino acids revealed enhanced incorporation (►**Fig. 1A, B**). This suggests that there is only a limited interaction between amino acid residues at the interface between the A domains. Full contact between the A domains is, however, not to be expected. The affinity of the A2 domain for the A1 and A2 domains should be low to facilitate rapid dissociation from FVIIIa.[4] Although it remains a speculation,

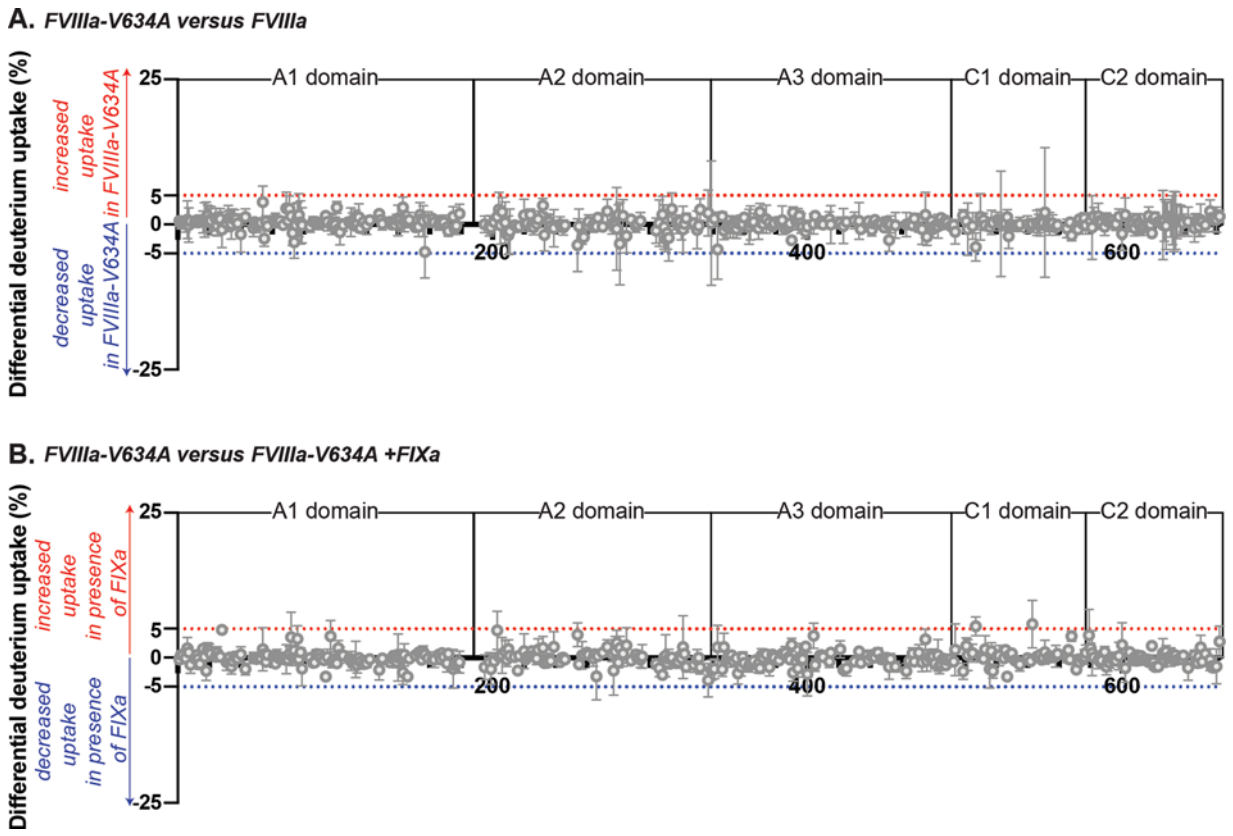

Fig. 5 HDX-MS on FVIII and FVIII-V634A in the presence and absence of FIXa. FVIII and FVIII-V634A in the presence and absence of FIXa were incubated for 100 seconds in deuterated buffer. The percentage of deuterium incorporation of a peptide was assessed relative to the theoretical maximum of incorporation in that peptide. The amino acid sequence of the peptides, shown by the ID number on the *x*-axis in the panels (**A**) and (**B**), is provided in Supplementary Table S1. Panel (**A**) shows the differential deuterium incorporation between FVIIIa and FVIIIa-V634A. The *y*-axis displays the percentage of deuterium uptake of the peptides of FVIIIa subtracted with that of FVIIIa-V634A. Panel (**B**) shows the differential deuterium incorporation between FVIIIa-V634A and FVIIIa-V634A in the presence of FIXa. The *y*-axis displays the percentage of deuterium uptake of the peptides of FVIIIa-V634A subtracted with that of FVIII-V634A in the presence of FIXa. The average of three independent experiments is shown in panels (**A**) and (**B**). Error bars represent the standard deviation. HDX-MS, hydrogen–deuterium exchange mass spectrometry.

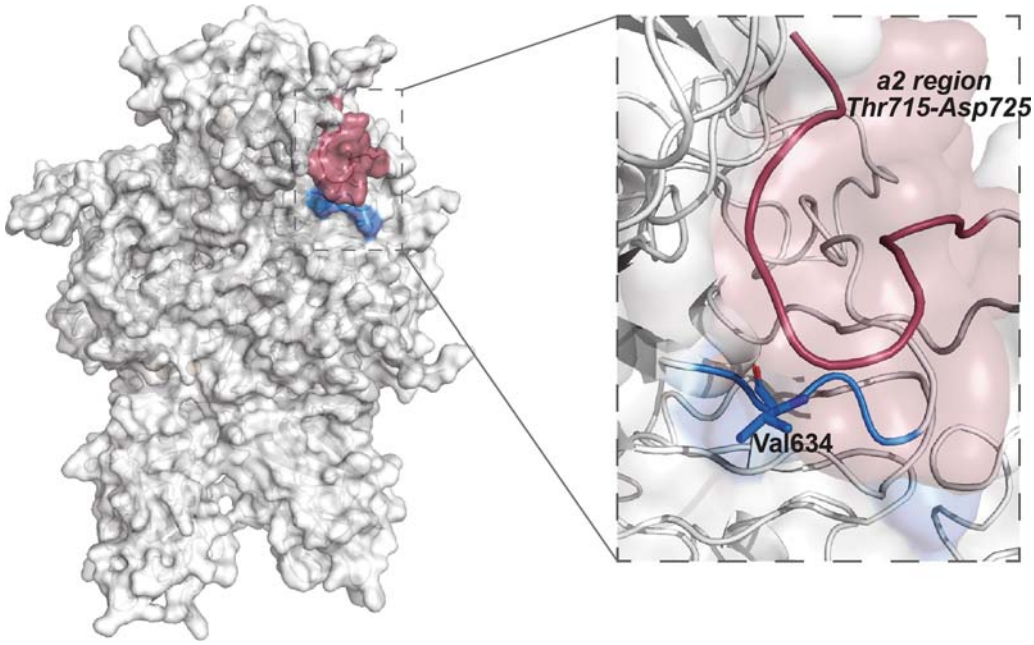

Fig. 6 FVIII *a2* region partially covers A2 region Leu631-Trp637. Part of the crystal structure of FVIII is shown in a surface representation (PDB code: 2r7e[13]). FVIII region Leu631-Trp637 is colored in *blue* while the *a2* region Thr715-Asp725 is displayed in *red*.

our data may imply that only the A3 domain peptide region Tyr1976-Phe1983 interacts with the A2 in FVIIIa. If so, this would then explain why the HDX of other regions in the A1 and A3 domains remains unchanged. This would also imply that the isolated A2 domain undergoes a major structural change at the interface region after its dissociation. This may also contribute to the poor ability of the isolated A2 domain to enhance the activity of FIXa.[1,36]

Changes were also observed for amino acid regions that are outside the interface between the A domains. The peptides overlapping Leu631-Trp637 showed an increased deuterium uptake upon activations of FVIII (►**Fig. 2B, D**). Interestingly, the crystal structure of FVIII published by Shen et al shows that the acidic *a2* regions partially cover Leu631-Trp637 (►**Fig. 6**).[13] Thrombin cleavage at the arginine residue that flanks the *a2* regions may fully expose the Leu631-Trp637 region to the protein surface thereby explaining the enhanced deuterium uptake. In the presence of FIXa, the deuterium uptake of Leu631-Trp637 was decreased beyond that of FVIII prior to its activation (►**Fig. 2B**). This suggests that FIXa either changes the local conformation or directly interacts with this region.

The functional importance of the region Leu631-Trp637 is demonstrated by the observation that the FVIII-V634A variant is almost unable to enhance the activity of FIXa (►**Fig. 3**). This is in line with previous observations that the natural Val634Ala variant of FVIII is linked to hemophilia A.[32] It is, however, surprising that this single substitution has such a major impact on the cofactor function. Although, the HDX of peptides including the Val634Ala variant cannot be directly compared with HDX-MS of its WT counterpart concerning the Leu631-Trp637 site; almost no difference in HDX was observed between FVIII-V634A and FVIII (►**Fig. 5A**). This suggests that the substitution has no major impact on the overall structure of FVIII. While HDX-MS did reveal changes compatible with A2 domain retention in FVIII in the presence of FIXa, these changes were absent for the activated Val634Ala variant (►**Fig. 1C** vs. ►**Fig. 5B**). Results from the enzyme kinetics assay were compatible with this observation. FIXa was able to stabilize the cofactor function of WT-FVIII and not that of FVIII-Val634Ala (►**Fig. 4B**). This together implies that there may be an altered interaction between FIXa and the A2 domain of FVIII-V634A.

Several putative FIXa-interactive regions have been identified on FVIII (►**Supplementary Fig. S4A**, available in the online version). A limitation of the present study is that no reliable HDX information could be obtained for regions 484–507[6,37,38] and 558–565[6,17] because a lack of sequence coverage or inconsistent results. For regions 2,228–2,240[39] and 1,811–1,818,[40] no changes were observed in HDX in the presence of FIXa. It should be noted, however, that HDX-MS provides only information about deuterium exchange of the amide hydrogens of the protein backbone. Hydrogens of the side chain residues exchange either too fast or not at all on the measured time scale. If mainly the charged lysine residues of, e.g., region 1,811–1,818 interact directly with FIXa without altering the local conformation of this region, no difference in deuterium incorporation would then be expected.

Irrespective of this limitation, the study did provide novel information about the FVIIIa–FIXa complex assembly. We found a previously undescribed role for region Leu631-Trp637 in enhancing the activity of FIXa. Notably, superposition of the structures of FIXa and FVIII on the homologous prothrombinase complex revealed that Val634 may be indeed located at the A2 domain/protease domain interface close to FIXa 330-helix (►**Supplementary Fig. S4D**, available in the online version). Although it remains somewhat speculative, it does support the conclusion that region Leu631-Trp637 may directly interact with FIXa.

What is known about this topic?

- Activated factor VIII (FVIIIa) enhances the catalytic activity of activated factor IX (FIXa).
- Upon activation and subsequent FIXa binding, FVIII undergoes unknown molecular changes.

What does this paper add?

- Hydrogen–deuterium exchange mass spectrometry (HDX-MS) analysis revealed molecular changes in FVIII upon its activation and binding to FIXa.
- Guided by HDX-MS analysis, a contribution of FVIII region Leu631-Tyr636 to the function of the cofactor was identified.
- The data explain the molecular mechanism behind the functional defect of the FVIII-V634A variant which is associated with hemophilia A.

Authors' Contributions
J.v.G., N.F., M.B.-S, and M.A.P. performed the experiments; C.v. d.Z. provided technical assistance; J.v.G., N.F., M.v.d.B., and A. B.M. designed the experiments; E.E. assisted with setting up of HDX-MS experiments; J.v.G., N.F., M.A.P., and M.v.d.B. analyzed the results; N.F. and J.v.G. made the figures; and J. v.G., N.F., M.v.d.B., and A.B.M. wrote the article.

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Conflict of Interest
None declared.

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References

1 Fay PJ. Activation of factor VIII and mechanisms of cofactor action. Blood Rev 2004;18(01):1–15
2 Mannucci PM, Tuddenham EG. The hemophilias–from royal genes to gene therapy. N Engl J Med 2001;344(23):1773–1779

3 Vehar GA, Keyt B, Eaton D, et al. Structure of human factor VIII. Nature 1984;312(5992):337–342
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 (11):3983–3996
5 Kamikubo Y, Mendolicchio GL, Zampolli A, et al. Selective factor VIII activation by the tissue factor-factor VIIa-factor Xa complex. Blood 2017;130(14):1661–1670
6 Fay PJ. Factor VIII structure and function. Int J Hematol 2006;83 (02):103–108
7 van Dieijen G, Tans G, Rosing J, Hemker HC. The role of phospholipid and factor VIIIa in the activation of bovine factor X. J Biol Chem 1981;256(07):3433–3442
8 Fay PJ. Subunit structure of thrombin-activated human factor VIIIa. Biochim Biophys Acta 1988;952(02):181–190
9 Lollar P, Parker CG. pH-dependent denaturation of thrombin-activated porcine factor VIII. J Biol Chem 1990;265(03):1688–1692
10 Lollar P, Parker ET. Structural basis for the decreased procoagulant activity of human factor VIII compared to the porcine homolog. J Biol Chem 1991;266(19):12481–12486
11 Ngo JC, 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(04):597–606
12 Stoilova-McPhie S, Villoutreix BO, Mertens K, Kemball-Cook G, Holzenburg A. 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography. Blood 2002;99(04):1215–1223
13 Shen BW, Spiegel PC, Chang CH, et al. The tertiary structure and domain organization of coagulation factor VIII. Blood 2008;111 (03):1240–1247
14 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(18):3938–3946
15 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(08):6374–6381
16 Bajaj SP, Schmidt AE, Mathur A, et al. Factor IXa:factor VIIIa interaction. helix 330-338 of factor ixa interacts with residues 558-565 and spatially adjacent regions of the a2 subunit of factor VIIIa. J Biol Chem 2001;276(19):16302–16309
17 Fay PJ, Beattie T, Huggins CF, Regan LM. Factor VIIIa A2 subunit residues 558-565 represent a factor IXa interactive site. J Biol Chem 1994;269(32):20522–20527
18 Pantazatos D, Gessner CR, Woods VL Jr, Gilbert GE. Changes in the factor VIII C2 domain upon membrane binding determined by hydrogen-deuterium exchange MS. Biochem J 2014;461(03):443–451
19 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(41):29670–29679
20 Przeradzka MA, van Galen J, Ebberink EHTM, et al. D' domain region Arg782-Cys799 of von Willebrand factor contributes to factor VIII binding. Haematologica 2020;105(06):1695–1703
21 van den Biggelaar M, Madsen JJ, Faber JH, et al. Factor VIII interacts with the endocytic receptor low-density lipoprotein receptor-related protein 1 via an extended surface comprising "hot-spot" lysine residues. J Biol Chem 2015;290(27):16463–16476
22 Song H, Olsen OH, Persson E, Rand KD. Sites involved in intra- and interdomain allostery associated with the activation of factor VIIa pinpointed by hydrogen-deuterium exchange and electron transfer dissociation mass spectrometry. J Biol Chem 2014;289(51): 35388–35396
23 Hendrix H, Lindhout T, Mertens K, Engels W, Hemker HC. Activation of human prothrombin by stoichiometric levels of staphylocoagulase. J Biol Chem 1983;258(06):3637–3644
24 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(02):96–100
25 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254
26 Lenting PJ, ter Maat H, Clijsters PP, Donath MJ, van Mourik JA, Mertens K. Cleavage at arginine 145 in human blood coagulation factor IX converts the zymogen into a factor VIII binding enzyme. J Biol Chem 1995;270(25):14884–14890
27 Foster PA, Fulcher CA, Marti T, Titani K, Zimmerman TS. A major factor VIII binding domain resides within the amino-terminal 272 amino acid residues of von Willebrand factor. J Biol Chem 1987; 262(18):8443–8446
28 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(08):1114–1121
29 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(11):2235–2242
30 Rouser G, Siakotos AN, Fleischer S. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. Lipids 1966;1(01):85–86
31 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(36):26105–26111
32 McGinniss MJ, Kazazian HH Jr, Hoyer LW, Bi L, Inaba H, Antonarakis SE. Spectrum of mutations in CRM-positive and CRM-reduced hemophilia A. Genomics 1993;15(02):392–398
33 Ahmed RPH, Ivaskevicius V, Kannan M, Seifried E, Oldenburg J, Saxena R. Identification of 32 novel mutations in the factor VIII gene in Indian patients with hemophilia A. Haematologica 2005; 90(02):283–284
34 Bloem E, Meems H, van den Biggelaar M, van der Zwaan C, Mertens K, Meijer AB. Mass spectrometry-assisted study reveals that lysine residues 1967 and 1968 have opposite contribution to stability of activated factor VIII. J Biol Chem 2012;287(08):5775–5783
35 Fay PJ, Jenkins PV. Mutating factor VIII: lessons from structure to function. Blood Rev 2005;19(01):15–27
36 Mertens K, Celie PH, Kolkman JA, Lenting PJ. Factor VIII-factor IX interactions: molecular sites involved in enzyme-cofactor complex assembly. Thromb Haemost 1999;82(02):209–217
37 Healey JF, Lubin IM, Nakai H, et al. Residues 484-508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. J Biol Chem 1995;270(24):14505–14509
38 Jenkins PV, Dill JL, Zhou Q, Fay PJ. Clustered basic residues within segment 484-510 of the factor VIIIa A2 subunit contribute to the catalytic efficiency for factor Xa generation. J Thromb Haemost 2004;2(03):452–458
39 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(06): 3379–3388
40 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(11):9370–9377