A3 Domain Region 1803–1818 Contributes to the Stability of Activated Factor VIII and Includes a Binding Site for Activated Factor IX

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Esther Bloem1, Henriet Meems3, Maartje van den Biggelaar1, Koen Mertens4,5, and Alexander B. Meijer1

From the 1Department of Plasma Proteins, Sanquin Research, 1066 CX Amsterdam, The Netherlands and 4Pharmaceutical Sciences, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 1066 CX Amsterdam, The Netherlands

A recent chemical footprinting study in our laboratory suggested that region 1803–1818 might contribute to A2 domain retention in activated factor VIII (FVIIIa). This site has also been implicated to interact with activated factor IX (FIXa). Asn-1810 further comprises an N-linked glycan, which seems incompatible with a role of the amino acids 1803–1818 for FIXa or A2 domain binding. In the present study, FVIIIa stability and FIXa binding were evaluated in a FVIII-N1810C variant, and two FVIII variants in which residues 1803–1810 and 1811–1818 are replaced by the corresponding residues of factor V (FV). Enzyme kinetic studies showed that only FVIIIa/FV 1803–1810 or 1811–1818 has a decreased apparent binding affinity for FIXa. Flow cytometry analysis indicated that fluorescent FIXa exhibits impaired complex formation with only FVIII/FV 1803–1818 on lipospheres. Site-directed mutagenesis revealed that Phe-1816 contributes to the interaction with FIXa. To evaluate FVIIIa stability, the FVIII/FV chimeras were activated by thrombin, and the decline in cofactor function was followed over time. FVIII/FV 1803–1818 showed a decreased FVIIIa half-life. However, when the FVIII variants were activated in presence of FIXa, only FVIII/FV 1811–1818 demonstrated an enhanced decline in cofactor function. Surface plasmon resonance analysis revealed that the FVIII variants K1813A/K1818A, E1811A, and F1816A exhibit enhanced dissociation after activation. The results together demonstrate that the glycan at 1810 is not involved in FVIIIa cofactor function, and that Phe-1816 of region 1811–1818 contributes to FIXa binding. Both regions 1803–1810 and 1811–1818 contribute to FVIIIa stability.

The function of FVIII is to markedly enhance the proteolytic activity of activated factor IX (FIXa) in the activated factor X (FXa)-generating membrane-bound complex (1). Circulating FVIII consists of a light chain that is covalently linked to a heterogeneous heavy chain (2). The light chain comprises the domains a3-A2-C1-C2 and the heavy chain the domains A1-a1-A2-a2-B. a1, a2 and a3 represent short spacer regions that are rich in acidic amino acid residues. The heterogeneity of the heavy chain is the consequence of proteolysis of the B domain (3).

FVIII is homologous to factor V (FV), which also comprises three A domains, a B domain, and two C domains (3). FV serves its role in the coagulation cascade as a cofactor for FXa during the proteolytic conversion of prothrombin to thrombin. From the crystal structures of FVIII and inactivated FX can be deduced that both proteins have a highly similar three-dimensional structure (4–6). A functional relationship between these proteins is demonstrated by the observation that the A domains seem to comprise the main binding regions for the proteases and that the C domains are critical for the direct interaction of the proteins with the phospholipid membrane. Taking maximum advantage of the homology between FV and FVIII, amino acids of FVIII have been replaced in previous studies for the corresponding residues of FV to gain insight into the role of the exchanged regions for cofactor function (7).

FVIII requires activation by thrombin to perform its role as a cofactor. Upon activation, the B domain and the acidic a3 region are removed from FVIII, and the A1 and A2 domains are bisected. The resulting activated FVIII (FVIIIa) is a trimeric protein which binds with high affinity to phospholipid membranes comprising phosphatidylserine in the outer leaflet. Phospholipid membrane binding of FVIIIa is the initial event in the formation of the FXa-generating complex (8–10).

After activation, FVIIIa rapidly loses its activity to prevent unlimited FXa generation. Loss of activity is mediated by spontaneous dissociation of the A2 domain from FVIIIa (11–13). In addition, it has been suggested that activated protein C, FIXa, and FXa inactivate FVIIIa by proteolytic cleavages in the A1 and

1 To whom correspondence should be addressed: Dept. of Plasma Proteins, Sanquin Research, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands. Tel.: 31-20-5123151; Fax: 31-20-5123310; E-mail: s.mmeijer@sanquin.nl.

2 The abbreviations used are: FVIII, factor VIII; FX, factor X; FVIIIa, activated FVIII; FIXa, activated factor IX; FX, factor X; FXa, activated FX; MFI, mean fluorescence intensity; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SPR, surface plasmon resonance.

3 Hemophilia A is a bleeding disorder that is associated with a functional absence of blood coagulation factor VIII (FVIII).
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A2 domains. These cleavages have been suggested to induce an accelerated dissociation of the A2 domain from FVIIIa (14–18). In a recent study, we employed a novel chemical footprinting approach to identify amino acid regions that contribute to the retention of the A2 domain in FVIIIa. With this approach, we established that lysine residues 1967 and 1968 are more surface-exposed in dissociated FVIIIa than in intact FVIII. Site-directed mutagenesis revealed that these residues have an opposite contribution to the stability of FVIIIa (19).

Activation of FVIII is also crucial for the exposure of interaction sites for FIXa (9). It has been suggested that regions 484–509 and 558–565 in the A2 domain contribute to enhancing the proteolytic activity of FIXa (20–22). Within the FVIII light chain, our laboratory has previously established that two peptides including the amino acid residues 1804–1818 and 1811–1820 effectively compete with FVIIIa for binding to FIXa (23). From this finding, it has been concluded that the overlapping region, i.e. residues 1811–1818, constitutes a FIXa binding site.

The chemical footprinting approach has also revealed that the lysine residues in region 1803–1818 are more surface-exposed in dissociated FVIIIa than in intact FVIII (19). This suggests that this region might contribute to the direct interaction with the A2 domain in FVIIIa. An N-linked glycan has further been identified at position 1810, which is close to this putative FIXa or A2 domain interactive region. The presence of a bulky glycan at this site might seem incompatible with a role of this region for effective FIXa or A2 binding. These observations show that the function of amino acid residues 1803–1818 is completely unclear.

We now assessed the role of region 1803–1818 and the glycan at position 1810 for the cofactor function of FVIII. To this end, we employed an FVIII/FV chimera in which either residues 1803–1810 or 1811–1818 are replaced by the corresponding residues of FV. Alkaline substitution FVIII variants were prepared to further dissect the role of amino acids within region 1811–1818 for cofactor function and FVIIIa stability. In addition, we utilized a FVIII-N1810C variant that lacks the glycan at position 1810. Our findings show that the function of amino acid residues 1803–1818 is completely unclear.

EXPERIMENTAL PROCEDURES

Materials—All fine chemicals were from Merck, unless stated otherwise. HEPES was from SERVA (Heidelberg, Germany), NaCl was from FAGRON (Rotterdam, The Netherlands), and Tris-HCl was from Invitrogen. Glass beads (1.6 μm) were from Duke Scientific (Palo Alto, CA). Fluorescein EGR-ck was from Hematologic Technologies Inc. (Essex Junction, VT). Phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) were from Avanti Polar Lipids.

Proteins—B domain-deleted FVIII (WT-FVIII) and variants thereof have been constructed and purified as described (19, 24, 25). For the FVIII/FV 1803–1810 chimera, region 1803–1810 has been replaced by region 1668–1675 of FV. For FVIII/FV 1811–1818, region 1811–1818 has been replaced by FV region 1676–1683. To assess cofactor function of the alanine substitution FVIII variants K1813A/K1818A, E1811A, and F1816A, these variants were purified from concentrated culture media as described (24) with the exception that the final Q-Sepharose step was not utilized to concentrate the proteins. The purified proteins were dialyzed against 50 mM HEPES (pH 7.4), 0.8 M NaCl, 5 mM CaCl2, and 50% glycerol and stored at −20 °C. The purification of FXa, FIXa, and thrombin are described by Mertens and co-workers (26–28). FXa was from Enzyme Research (South Bend, IN). Bovine serum albumin was from Merck. Antibodies KM33 and EL14 have been described before (29, 30).

Fluorescein Labeling of FIXa—FIXa was dialyzed against 50 mM Tris-HCl (pH 7.5), 150 mM NaCl. Labeling of the active site in FIXa was performed in the dark by incubating FIXa with a 3-fold molar excess of fluorescein EGR-ck for 6 h at room temperature followed by an overnight incubation at 4 °C. Unbound label was removed employing extensive dialysis against 50 mM Tris-HCl (pH 7.5), 150 mM NaCl. The 100% labeling efficiency of FIXa was verified by measuring the residual proteolytic activity of FIXa toward the substrate FX in the presence of FVIIIa (19).

Cofactor Function of the FVIII Variants—Cofactor function was determined by an enzyme-linked immunosorbent assay as described by van den Biggelaar and co-workers with the exception that anti-C1 domain antibody KM33 was employed as capturing antibody instead of CLB-Cag12 (24, 25). Factor Xase activity of the FVIII variants was assessed as described before (19). Briefly, FXa generation was followed in time at 25 °C in 1.5 mM CaCl2, 40 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.2% (w/v) bovine serum albumin (BSA) employing 0.3 nM FVII and 25 μM phospholipid vesicles comprising 15% PS/20% PE/65% PC. The mixture further contained either 16 nM FIXa and varying concentrations (0–900 nM) of FX, or 200 nM FXa and varying concentrations (0–16 nM) of FIXa. FVIII was activated by 1 nM thrombin. The amount of generated FX was assessed as described (19).

FVIIa-FIXa Assembly on Lipospheres—Glass beads coated with phospholipids (lipospheres) were obtained as described (29, 31). 250,000 lipospheres/ml were incubated with 6 nM fluorescein-labeled FIXa and 0–3 nM WT-FVIII or its variants in a buffer comprising TBS, 0.1% fatty acid free BSA, and 1.5 mM CaCl2. FVIII was activated by the addition of 2 nM thrombin, and complex formation was allowed for 2 min at room temperature. Mean fluorescence intensity (MFI) present on the lipospheres was measured by flow cytometry employing a FACSLSR II (BD Biosciences) as indicated by Meems et al. (29).

Stability of the FVIIIa Variants—Assessment of the decline in FVIIIa cofactor function over time has been described by Bloem et al. (19). Briefly, 0.3 nM FVIII was activated by 2 nM thrombin in the presence of 25 μM phospholipid vesicles comprising 15% PS/20% PE/65% PC, 1.5 mM CaCl2, 40 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 0.2% (w/v) BSA. At increasing time intervals, FXa generation was allowed for 1 min by the addition of 16 nM FIXa and 200 nM FX to FVIIIa. To test the influence of FIXa on the activity decay, FVIII was incubated with 16 nM FIXa during the activation of FVIII. FXa generation was measured as described before (19).
**Surface Plasmon Resonance (SPR) Analysis**—SPR analysis was performed as described before (19) with the exception that WT-FVIII, E1811A, F1816A, and K1813A/K1818A were directly captured from the culture media via anti-C2 domain antibody EL14 that was immobilized on a CM5 sensor chip.

**RESULTS**

The FVIII/FV Chimeras and FVIII-N1810C Support FXa Generation—The FVIII variants FVIII/FV 1803–1810, FVIII/FV 1811–1818, and FVIII-N1810C were purified and analyzed by SDS-PAGE (Fig. 1). The light chains of the FVIII/FV chimera did not show an increased mobility on the SDS-gel compared with that of the light chain of WT-FVIII. This implies that the light chains of both chimeras have retained the glycan at position 1810. Compatible with the absence of the glycan in FVIII-N1810C, the light chain of this FVIII variant revealed an increased mobility on the SDS-gel. FVIII activity was assessed to establish whether the variants support FXa generation by FIXa (Fig. 2). The results showed effective FXa generation by all FVIII variants. This demonstrates that the FVIII variants effectively support FXa generation by FIXa up to the physiological FX concentration of approximately 136 nM.

The FVIII/FV Chimeras but Not FVIII-N1810C Show an Opposite Effect on Apparent FIXa Binding Affinity—To assess the putative role of regions 1803–1810 and 1811–1818 for FIXa binding, FXa generation should be assessed employing limiting concentrations of FIXa. To this end, the cofactor activity of the FVIII variants was assessed employing increasing concentrations of FIXa (Fig. 3). FXa generation as a function of the employed FIXa concentration was indistinguishable for WT-FVIII, E1811A, F1816A, and K1813A/K1818A (apparent $K_D$ of 1.3 ± 0.1 nM and 1.2 ± 0.1 nM, respectively). This implies that the glycan at position 1810 or the introduced cysteine at this position does not affect FIXa binding at all. Activity analysis of the FVIII/FV 1803–1810 chimera revealed a slight decrease in apparent $K_D$ (0.8 ± 0.2 nM) for FIXa, whereas the FVIII/FV 1811–1818 chimera showed an increase in apparent $K_D$ (2.4 ± 0.1 nM). As the apparent $K_D$ reflects the binding affinity of FVIIIa for FIXa, these findings imply a modest but significant FIXa binding defect of the FVIII/FV 1811–1818 chimera.

FVIII/FV 1811–1818 Displays Reduced Interaction with FIXa on Lipospheres—To evaluate FIXa binding to the FVIII variants, we assessed FIXa-FVIIIa complex assembly on lipospheres employing a flow cytometry study. To this end, increasing concentrations of FVIII and variants thereof were incubated with fluorescent FIXa and lipospheres. No fluorescent FIXa was detected on the lipospheres when FIXa was incubated with
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**FIGURE 4.** FVIIIa-FIXa complex assembly on lipospheres is markedly reduced for FVIII/FV 1811–1818. Lipospheres comprising 15% PS/20% PE/65% PC were incubated with 0–3 nM indicated FVIII variant and 6 nM fluorescently labeled FIXa. The FVIII variants were activated by thrombin, and the MFI of FIXa bound to the lipospheres was assessed by flow cytometry as described under “Experimental Procedures.” Data represent mean of two representative experiments. Error bars represent the spread between the obtained values.

FVIIIa for more than 15 min prior to flow cytometry analysis. This observation may be related to the notion that FVIIIa rapidly dissociates after its activation (11–13). FIXa may be unable to effectively bind dissociated FVIIIa. Therefore, the MFI of FIXa bound to these lipospheres was assessed after a 2-min activation time of FVIII by thrombin. The results revealed an almost indistinguishable increase in MFI as a function of the employed concentration of WT-FVIII, FVIII/FV 1803–1810, and FVIII-N1810C (Fig. 4). This finding suggests that there is no change in FIXa binding for these FVIII variants. The data further showed an reduced increase in MFI upon incubation of the fluorescently labeled FIXa with increasing concentrations of the activated FVIII/FV 1803–1818 chimera (Fig. 4). This suggests that there is a defect in the capacity of FVIII/FV 1811–1818 to pull FIXa to the surface of phospholipid membranes. Alternatively, the activated FVIII/FV 1811–1818 variant exhibits a markedly enhanced dissociation rate compared with that of activated WT-FVIII and FVIII/FV 1803–1810 variant.

**Region 1803–1818 but Not the N-Linked Glycan at 1810 Is Involved in FVIIIa Stability—**To investigate the stability of FVIIIa and derivatives thereof, we followed the activity of these variants with thrombin. To this end, the FVIII variants were activated with thrombin in the presence of 25 variants over time. To this end, the FVIII variants were activated with thrombin in the presence of 25 shadows over time. The results showed that the half-life of FVIIIa activity for all FVIII variants compared with the condition where FVIII was activated in the absence of FIXa (Fig. 5A). Intriguingly, next to FVIII-N1810C, the FVIII/FV 1803–1810 chimera now also showed a WT-FVIII-like decline in activity over time. However, the FVIII/FV 1811–1818 chimera still exhibited an increased loss of activity compared with the other FVIII variants. This finding is compatible with the observation that replacement of region 1811–1818 results in a FIXa binding defect. This impairs the ability of FIXa to stabilize FVIIIa optimally.

**Identification of Residues within Region 1811–1818 That Contribute to FIXa Interaction and/or FVIIIa Stability—**To further dissect the function of residues within region 1811–1818, we replaced the amino acid residues with alanine which are not shared between FVIII and FV with the exception of Thr-1812. The latter residue comprises only a small polar side chain and is therefore less likely to contribute to FIXa binding. FXa generation as a function of the FIXa concentration was assessed in a FVIII-K1813A/K1818A double mutant, a FVIII-E1811A variant, and a FVIII-F1816A variant (Fig. 6A). The E1811A variant exhibited an apparent KD for FIXa (1.2 ± 0.2 nm) that was close to that of WT-FVIII (0.9 ± 0.2 nm). Replacing the two positively charged residues in region 1811–1818 revealed a small decrease in KD (0.5 ± 0.1 nm). Apparently, replacement of these residues puts region 1811–1818 in a more optimal position for interaction with FIXa. F1816A revealed, however, an apparent KD for FIXa of 2.1 ± 0.4 nm, which is comparable with that obtained for the FIXa/FVIII 1811–1818 chimera (Fig. 3). This implies that Phe-1816 within region 1811–1818 contributes to FIXa binding.

We also assessed the effect of the alanine substitution on the dissociation rate of activated FVIII. To this end, we employed SPR analysis to follow the decrease in response units in time of immobilized activated FVIII. We have previously demonstrated that this decrease corresponds to A2 domain dissociation from FVIIIa (19). The results show that all variants display enhanced dissociation compared with WT-FVIII (Fig. 6B). This implies that the charged residues play a role in the stability of FVIIIa. Phe-1816 exhibits, however, a dual role. It contributes to FIXa binding as well as to FVIIIa stability.

**DISCUSSION**

We have previously established that the FVIII peptides 1804–1818 and 1811–1820 effectively block the interaction between FIXa and FVIIIa (23). Employing FVIII/FV chimera and alanine substitution variants, we now show that FVIII region 1811–1818 and in particular residue Phe-1816 therein contributes to FIXa binding (Figs. 3 and 6A). Next to this finding, we have established that amino acid regions 1803–1810 and 1811–1818 both contribute to the stability of FVIIIa (Figs. 5 and 6B).

It has been shown that FIXa can increase the stability of FVIIIa by binding amino acid regions in the A2 and A3 domains (32, 33). Compatible with our finding that FIXa requires region 1811–1818 for optimal binding to the A3 domain, we found that FIXa was unable to effectively increase the stability of the
FVIII/FV 1811–1818 chimera (Fig. 5). This observation may also provide an explanation for the markedly impaired binding of FIXa to the FVIII/FV 1811–1818 variant on lipospheres (Fig. 4). It may be the consequence of a reduced FIXa binding in combination with an enhanced dissociation rate of the activated FVIII/FV 1811–1818 variant.

Previously, Steen et al. made use of N-linked glycans to identify binding regions in FVa for FXa (34). Introduction of a glycan at position His-1683 of FV resulted in a defective FXa binding. This suggests that the region surrounding His-1683 contributes to the interaction between FVa and FXa. Intriguingly, primary sequence comparison between FVIII and FV reveals that Lys-1818 of FVIII corresponds to His-1683 of FV (Fig. 1). This notion illustrates the strong structure-function relationship between FV and FVIII. Not only does the A3 domain of FVIII and FV comprise the interactive sites for FIXa and FXa, it appears that corresponding regions in the A3 domain of FVIII and FV interact with their serine protease counterparts. Yet, we did not introduce a new major independent FXa binding site in the FVIII/FV chimeras. The presence of a new FXa binding site would result in effective competition between FIXa and the newly generated FXa in the FVIII activity analysis of the FVIII/FV chimeras (Fig. 2). In contrast, a slight reduction FXa generation at elevated FX concentrations is displayed by WT-FVIII and FVIII-N1810C. It seems therefore that region 1803–1818 of FVIII itself affects the binding of FX or its product FXa to FVIIIa at supraphysiological concentrations of FX.

It does seem remarkable that an N-linked carbohydrate is positioned close to a region that is critical for the cofactor function of FVIII. Yet, the presence or absence of the glycan does not affect FIXa binding or the stability of FVIIIa at all (Figs. 3–5). As can be observed in the crystal structures of FVIII, Asn-1810 is located at the tip of a V-shaped loop, which comprises the residues 1803–1818 (Fig. 7) (4, 5). Apparently, the glycan moiety protrudes out of the protein surface away from the FIXa binding region. This provides an explanation for the complete lack of interference of the N-linked glycan for the cofactor function of FVIII. The biological role of the glycan, if any, remains therefore unclear. It has been suggested that the carbohydrates of the B domain of FVIII assist in an effective exit of FVIII from the endoplasmic reticulum during biosynthesis of FVIII (35). The glycan at position 1810 may play a similar role in the biosynthetic pathway of FVIII.

We have previously demonstrated that the FVIII light chain remains bound to the phospholipids after dissociation of the A2 domain from the A1/A3-C1-C2 dimer (36). Our observations imply that FIXa may be unable to bind dissociated FVIIIa on the lipospheres. This suggests that the presence of the A2 domain is

FIGURE 5. FVIII regions 1803–1810 and 1811–1818 contribute to FVIIIa stability. A, 0.3 nM FVIII is activated by 2 nM thrombin in the presence of 25 μM phospholipids comprising 15% PS/20% PE/65% PC. At the indicated time points, FXa generation was allowed for 1 min by the addition of 16 nM FIXa and 200 nM FX. B, 0.3 nM FVIII is activated by 2 nM thrombin in the presence of 16 nM FIXa and 25 μM phospholipids comprising 15% PS/20% PE/65% PC. At the indicated time points, FXa generation was allowed for 1 min by the addition of 200 nM FX. Data represent mean ± S.D. (error bars) of at least six experiments.

FIGURE 6. Residues Glu-1811, Lys-1813/1818, and Phe-1816 contribute to the stability of FVIIIa, and Phe-1816 is involved in FIXa binding. A, FXa generation of the indicated FVIII variants as a function of FIXa was evaluated by incubating 0.3 nM FVIII with 200 nM FX and varying concentrations of FIXa. The activity was assessed employing 25 μM phospholipid vesicles comprising 15% PS/20% PE/65% PC. Data represent mean ± S.D. (error bars) of at least three independent experiments and are shown as the percentage of maximum FXa generation. B, WT-FVIII and its variants, E1811A, K1813A/K1818A, and F1816A were immobilized via the anti-C2 domain antibody EL14 to a density of 1500 response units (RU). 2 nM thrombin was perfused over the immobilized FVIII variants for 60 s in 20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM CaCl2, 0.005% Tween 20. The decline in response units is displayed in time. This decline reflects A2 domain dissociation as has been described previously (19).
required for effective binding of FIXa to FVIIIa. However, it has been shown that FIXa binds the isolated A2 domain with moderate affinity and the FVIII light chain with high affinity (1). Apparently, effective FIXa binding requires the presence of the complete FIXa binding interface on FVIII.

The results of the present study show that the previously published chemical footprinting approach is of great value for the identification of important regions within FVIII. We found that the lysine residue couple with the highest increase in surface exposure (i.e., residues 1967 and 1968) upon A2 domain dissociation from FVIII contributes to A2 domain retention (19). We now show that the regions 1803–1810 and 1811–1818, which have a less prominent increase in surface exposure, are also involved in retention of the A2 domain in FVIIIa (Fig. 5). For region 1803–1810, this can easily be understood as the crystal structure reveals that this region is in close proximity of A2 domain whereas region 1811–1818 contributes indirectly to the interaction of region 1803–1810 with the A2 domain. This mechanism would then contribute to the increased stability of FVIIIa in the presence of FIXa.

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