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Combining mutations that modulate inter-subunit interactions and proteolytic inactivation enhance the stability of factor VIIIa¹

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Summary

FVIIIa is labile due to the dissociation of A2 subunit. Previously, we introduced hydrophobic mutations at select A1/A2/A3 subunit interfaces yielding more stable FVIII(a) variants. Separately we showed that altering the sequence flanking the primary FXa cleavage site in FVIIIa (Arg336) yielded reduced rates of proteolytic inactivation of FVIIIa. In this study we prepared the FXa-cleavage resistant mutant (336(P4-P3')562) combined with mutations of Ala108Ile, Asp519Val/Glu665Val or Ala108Ile/Asp519Val/Glu665Val and examined the effects of these combinations relative to FVIII thermal stability, rates of FVIIIa decay and proteolytic inactivation of FVIIIa by FXa. Thermal decay rates for 336(P4-P3')562/Ala108Ile, 336(P4-P3')562/Asp519Val/Glu665Val, and 336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val variants were reduced by ~2–5-fold as compared with WT primarily reflecting the effects of the A domain interface mutations. FVIIIa decay rates for 336(P4-P3')562/Asp519Val/Glu665Val and 336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val variants were reduced by ~25 fold, indicating greater stability than the control Asp519Val/Glu665Val variant (~14-fold). Interestingly, 336(P4-P3')562/Asp519Val/Glu665Val and 336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val variants showed reduced FXa-inactivation rates compared with the 336(P4-P3')562 control (~4-fold), suggesting A2 subunit destabilization is a component of proteolytic inactivation. Thrombin generation assays using the combination variants were similar to the Asp519Val/Glu665Val control. These results indicate that combining multiple gain-of-function FVIII mutations yields FVIII variants with increased stability relative to a single type of mutation.

Keywords

factor VIII; factor VIIIa; factor Xa; protein stability; thrombin generation assay

Introduction

Factor (F)VIII², a plasma protein that is decreased or defective in individuals with hemophilia A, is expressed as both single chain and heterodimer forms. The FVIII

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Conflicts of interest:
None declared.

heterodimer consists of a heavy chain (HC) comprised of A1(a1)A2(a2)B domains and a light chain (LC) comprised of (a3)A3C1C2 domains, where the lower case *a* designates short (~30–40 residue) segments rich in acidic residues (see Ref. (1) for review). FVIII is activated by thrombin- or FXa-catalyzed cleavages at the a1A2, a2B and a3A3 junctions. The resulting product, FVIIIa, is a heterotrimer comprised of subunits designated A1, A2, and A3C1C2. FVIIIa functions as a cofactor for the serine protease FIXa in the conversion of zymogen FX to the serine protease, FXa (see Ref. (1) for review).

The instability of FVIIIa results from weak electrostatic interactions between the A2 subunit and the A1/A3C1C2 dimer (2, 3) and its dissociation leads to dampening of FXase activity (4, 5). Mutations to increase buried hydrophobic area and/or reduce the buried hydrophilic area often result in enhanced protein stability (6). Earlier studies showed that acidic residues localized to hydrophobic pockets at the A1-A2 interface (Asp519) and A2-A3 interface (Glu665 and Glu1984) when mutated to hydrophobic residues (Ala or Val), yielded favorable effects on FVIII/FVIIIa stability and /or activity (7, 8). In addition, replacing Ala108, that localized to a hydrophobic pocket at the A1-C2 domain interface, with the more bulky Ile also showed a marked increase in FVIII stability (9). Furthermore, introducing nascent disulfide bridges between FVIII subunits by double Cys mutation at A2-A3 or A1-C2 interfaces yielded selected FVIII variants with enhanced FVIII/FVIIIa stability (9–11).

The FXase complex is down regulated through two FVIIIa-dependent mechanisms (see Ref. (1) for review). A proteolytic pathway, catalyzed by both activated protein C (APC) (12, 13) and FXa (12, 14), inactivates FVIIIa by initial cleavage at Arg336 in the A1 subunit and subsequent cleavage at Arg562 in the A2 subunit. Cleavage at the former site, which correlates with loss of function, appears to alter the orientation of A2 subunit with the A1/A3C1C2 dimer (15), while cleavage at the latter site destroys a FXa-interactive site (16). These cleavages that inactivate FVIIIa occur at a slower rate than the activating cleavages catalyzed by FXa (12, 17, 18) and this property favors FXa as initially serving to activate FVIII. We have previously shown that P4-P3' residues flanking the Arg336 and Arg562 sites contribute towards determining rates of activated protein C (APC) or FXa cleavage at these sites (17–19). Furthermore, either slowing the rate of cleavage at Arg336 by replacement with the Arg562 flanking sequence or eliminating this cleavage by an Arg336Gln mutation yielded increased cofactor activity and enhanced thrombin generation parameters (19).

In this study we generated FVIII variants by combining mutations to increase FVIII/FVIIIa stability and reduce FVIIIa decay rates with mutations to reduce rates of FXa-catalyzed proteolytic inactivation. Results from this study show that it is possible to prepare FVIII variants with multiple mutations that possess the attributes of the individual mutations and with no detrimental effects to FVIII function.

²Abbreviations: FVIII, factor VIII; FIXa, factor IXa; FX, factor X; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Materials and methods

Nomenclature

The FVIII protein sequence in this manuscript is numbered from the mature N-terminus using reference sequence NP_000123.1. According to Human Genome Variation Society (HGVS) FVIII variants designated in the text as (336(P4-P3')562, Ala108Ile, Asp519Val, and Glu665Val) correspond to 355(P4-P3')581, p.Asp538Val, and p.Glu684Val (20).

Materials

Recombinant FVIII (Kogenate™) was a generous gift from Dr. Lisa Regan of Bayer Corporation (Berkeley, CA). Dioleoyl phospholipids [Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS)] were purchased from Avanti Polar Lipids (Alabaster, AL). The reagents α -thrombin, FVIIa, FIXa β , FX, and FXa (Enzyme Research Laboratories, South Bend, IN), hirudin (DiaPharma, West Chester, OH), the chromogenic FXa substrate, Pefachrome Xa (Pefa-5523, CH₃OCO-D-CHA-Gly-Arg-pNA·AcOH; Centerchem Inc. Norwalk CT), Enhanced Chemifluorescence reagent (GE Healthcare Bioscience, Piscataway, NJ), recombinant human tissue factor (rTF: Innovin, Dade Behring, Deerfield, IL), fluorogenic substrate (Z-Gly-Gly-Arg-AMC: Calbiochem, San Diego, CA), and thrombin calibrator (Diagnostics Stago, Parsippany, NJ) were purchased from the indicated vendors.

Construction, expression and purification of WT and variant FVIII

WT FVIII and FVIII variants with mutation of 336(P4-P3')562 [where residues 333–339 (PQLRMKN) that flank the fast FXa-cleavage site at Arg336 are replaced with residues flanking the slow cleavage site at Arg562 (residues 559–565, VDQRGNQ)] combined with mutation of Ala108Ile, Asp519Val/Glu665Val, or Asp519Val/Glu665Val /Ala108Ile were constructed as B-domainless FVIII, lacking residues Gln744-Ser1637 in the B-domain as described before (8, 9, 19, 21). Recombinant WT and variant FVIII forms were stably expressed in BHK cells and purified as described previously (22). Protein yields for the variants ranged from >10 to ~100 μ g from two 750 cm² culture roller bottles, with purity from ~85% to >95% as judged by SDS-PAGE. The primary contaminant in the FVIII preparations was albumin. FVIII concentration was measured using an Enzyme-Linked Immunoabsorbant Assay (ELISA) and FVIII activity was determined by one-stage clotting and two-stage chromogenic FXa generation assays described below.

ELISA

A sandwich ELISA was performed as previously described (23) using purified recombinant FVIII (Kogenate, Bayer Corporation) as a standard. FVIII capture used the anti-C2 monoclonal antibody (GMA8003, Green Mountain Antibody) and the anti-A2 monoclonal antibody (R8B12, Green Mountain Antibody) was employed for FVIII detection following its biotinylation.

One-stage clotting assay

One-stage clotting assays were performed using substrate plasma chemically depleted of FVIII according a method as previously described using pooled normal plasma (George King Bio-Medical, Overland Park, KS) as a FVIII standard (24) and assayed using a Diagnostica Stago clotting instrument.

FXa generation assay

The rate of conversion of FX to FXa was monitored in a purified system (25) according to methods previously described (26, 27). FVIII (1 nM) in 20 mM HEPES, 0.1 M NaCl, 5 mM CaCl₂, 0.01% Tween 20, 0.01% BSA, pH 7.2 (HEPES buffer), containing 20 μ M PSPCPE vesicles (PS:PC:PE = 3:2:5) was activated with 30 nM α -thrombin for 1 min. Reactions were stopped by adding hirudin (10 U/ml) and the resulting FVIIIa was reacted with FIXa (40 nM) for 1 min. FX (300 nM) was added to initiate reactions that were quenched after 1 min by the addition of 50 mM EDTA. FXa generated was determined following reaction with the chromogenic substrate Pefachrome Xa (0.46 mM final concentration). All reactions were run at 23°C.

FVIII thermal decay

FVIII variants (4 nM) in HEPES buffer were incubated at 57°C, aliquots were removed at indicated time points, and activity was determined using the FXa generation assay.

FVIIIa activity decay

FVIII variants (1.5 nM) in HEPES buffer were activated using 20 nM thrombin for 1 min at 23°C. Reactions were immediately quenched by hirudin (10 U/ml) to inactivate thrombin, aliquots removed at the indicated times, and activity was determined using the FXa generation assay.

Thrombin Generation Assay

The amount of thrombin generated in plasma was measured by Calibrated Automated Thrombography (28) using methods previously described (7). Briefly, FVIII deficient plasma (<1% residual activity, platelet-poor) from a severe hemophilia A patient lacking FVIII inhibitor (George King Bio-Medical) was mixed at 37°C with a final concentration of 0.13 nM FVIII, 0.5 pM rTF, 4 μ M PSPCPE vesicles, 433 μ M fluorogenic substrate, 13.3 mM CaCl₂, and 105 nM thrombin calibrator. The development of a fluorescence signal was monitored at 8 second intervals using a Microplate Spectrofluorometer (Spectramax Gemini, Molecular Devices, Sunnyvale, CA) with a 355 nm (excitation)/460 nm (emission) filter set. Fluorescence signals were corrected by the reference signal from the thrombin calibrator samples (28) and actual thrombin generation in nM was calculated as previously described (7).

Inactivation of FVIIIa by FXa

FVIII (150 nM) was activated by thrombin (30 nM) in HEPES buffer for 10 minutes at 37°C. After thrombin was inhibited by addition of hirudin (20 U/mL), the FVIIIa product was reacted with FXa (5 nM) in the presence of phospholipid vesicles (100 μ g/mL).

Reactions were run at 37°C. Aliquots were removed at the indicated time and assayed by one-stage clotting assay.

Von Willebrand factor (VWF) binding assay

VWF was purified from FVIII-VWF complex (Koate) as described previously (29, 30). FVIII binding affinity to VWF was measured in an ELISA-based assay performed on a micro-titer plate. VWF (18 µg/ml) was coated onto the micro-titer plate (Immulon 4 HBX, Thermo, Milford, MA) in 50 mM Tris, pH 7.5, 0.1 M NaCl at 4°C overnight. The surface was treated with 10% skim milk for 2 hours followed by washing with 20 mM HEPES, 0.1 M NaCl, 5 mM CaCl₂, 0.01% Tween 20, pH 7.2. Indicated concentrations of FVIII were added and incubated for 1 hour and optical density (O.D.) at 455 nm, which corresponds to bound FVIII was obtained as described above in the ELISA section after correction for binding in the control lacking VWF coating.

Data analysis

Values for FVIII/ FVIIIa activity decay as a function of time were fitted to a single exponential decay curve non-linear least squares regression using the equation,

$$A=A_0 \cdot e^{-k \cdot t}$$

where A is residual FVIIIa activity (nM/min/nM FVIII), A_0 is the initial activity, k is the apparent rate constant, and t is the time after FVIII activation when thrombin was quenched.

We utilized a second order polynomial equation as previously employed for an unbiased estimation of the initial reaction rate of FVIIIa inactivation by

$$[FVIIIa]=A+Bt+Ct^2$$

Where $[FVIIIa]$ is the FVIIIa concentration in nM, t is the time in minutes, A is the initial concentration in nM of FVIIIa and B is the slope at time zero. Rates of FVIIIa inactivation were calculated by dividing the absolute value of B by the concentration of FXa.

Data for FVIII binding to VWF were fitted to simple binding model using the equation,

$$B=B_{\max} \cdot [FVIII]/(K_d+[FVIII])$$

Where $[FVIII]$ is FVIII concentration applied, B is O.D. at 455 nm which corresponds to bound FVIII quantity, $B_{\max}$ is maximum O.D. at saturation, and K_d is a dissociation constant.

Computation for nonlinear least-squares regression analysis was performed using a standard curve-fitting algorithm (Gauss-Newton algorithm using the method of Levenberg-Marquardt).

Results

Activity of FVIII variants after combination mutagenesis

FVIII specific activity was measured by both one-stage clotting and two-stage FXa generation assays. Activity of 336(P4-P3')562 by one-stage assay was similar to the WT value (~111% relative to a 100% value for WT). The activity value of combination mutant (336(P4-P3')562/Ala108Ile) was slightly reduced to ~85% of WT, and this value likely reflects the Ala108Ile mutation which was previously determined to be ~82% of WT (9). The Asp519Val/Glu665Val variant showed somewhat increased activity (133%) as compared with WT, while this mutation in the combination with either 336(P4-P3')562 or 336(P4-P3')562/Ala108Ile showed significantly improved activity (148% and 169%, respectively relative to WT) in one stage assays. However, in two-stage assays there were no appreciable differences among all FVIII variants tested. These results suggest that the above combination of mutations did not yield any undesirable changes in cofactor function.

Stability of FVIII variants

The rate of loss of FVIII activity at 57°C as measured by FXa generation assays was used to monitor the thermal stability of the FVIII procofactor (6). Figure 1A shows results of the loss of activity over time at the elevated temperature for WT FVIII, each of the combined mutations variants and relevant controls. The activity of WT FVIII and the 336(P4-P3')562 variant showed similar, rapid losses in activity retaining ~20% of the initial activity value after 10 min, indicating that changing sequences surrounding the Arg336 cleavage site do not contribute to this physical parameter. The 336(P4-P3')562/Asp519Val/Glu665Val combination mutant and control Asp519Val/Glu665Val mutation decayed somewhat slower, retaining ~40% activity after 10 min and indicating that the increased stability in the combination mutant resulted from the Asp519Val/Glu665Val mutation. Similarly, 336(P4-P3')562/Ala108Ile mutation showed ~60% activity after 10 min and this enhanced stability relative to control could be attributed to the Ala108Ile mutation. Finally 336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val showed the greatest thermal stability retaining ~75% activity after 10 min. Table 1 lists the decay rate values following curve fitting. The 336(P4-P3')562/Asp519Val/Glu665Val and control Asp519Val/Glu665Val variants showed ~2 fold increases in stability relative to WT, while the 336(P4-P3')562/Ala108Ile and control Ala108Ile showed ~3 fold increase in stability. These results suggest a somewhat greater contribution of Ala108Ile compared with Asp519Val/Glu665Val mutations to FVIII stability. Furthermore, the stability of 336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val combination mutation was ~5 fold greater than WT, and suggested that this increase in stability resulted from the simple additive effects of the Ala108Ile mutation and Asp519Val/Glu665Val mutations with no contribution of the 336(P4-P3')562 to FVIII thermal stability.

Figure 1B shows results of FVIIIa activity decay for FVIII variants. WT and all FVIII variants lacking the Asp519Val/Glu665Val mutation showed similar decay profiles after

thrombin activation that were significantly faster than the Asp519Val/Glu665Val mutation-containing variants. The activities of WT and Ala108Ile were reduced to ~30% in 10 min while 336(P4-P3')562 and 336(P4-P3')562/Ala108Ile decayed somewhat faster, showing ~20% activity after 10 min with estimated decay rate values that were ~30% larger than the WT FVIIIa value (Table 2). However, the 336(P4-P3')562/Asp519Val/Glu665Val and 336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val variants were the most stable FVIIIa forms, showing ~90% activity after a 40 min incubation and yielding decay rate values that were reduced ~25-fold compared with WT FVIII value. This effect resulted from the Asp519Val/Glu665Val mutation which showed ~80% activity after 40 min representing an ~14-fold reduced rate of FVIIIa decay, a value similar to that observed previously and attributed to increased retention of the A2 subunit in FVIIIa (8).

Thrombin generation potential of FVIII variants

FVIII variant activity was assessed by thrombin generation assays performed at low rTF concentration (0.13 pM) using FVIII deficient plasma. Figure 2 shows a representative thrombogram of FVIII WT and selected variants with parameter values of all variants are listed in Table 3. Control experiments without FVIII yielded essentially no thrombin generation. Thrombin generation of WT FVIII initiated at ~10 min and peak thrombin generation (~47 nM) was reached at ~22 min. A similar profile was seen for the 336(P4-P3')562, Ala108Ile, and 336(P4-P3')562/Ala108Ile variants (data not shown in Figure 2). Both 336(P4-P3')562 and 336(P4-P3')562/Ala108Ile variants showed modest increases (~17%) improved endogenous thrombin potential (ETP) compared with the WT value (Table 3). On the other hand, all FVIII variants containing the Asp519Val/Glu665Val mutation (Asp519Val/Glu665Val, 336(P4-P3')562/Asp519Val/Glu665Val, and 336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val) showed markedly improved thrombin generation parameter values. These variants reached ~3.8 fold greater peak thrombin generation (~180 nM) in ~17 min, which was ~5 min earlier than WT FVIII. In addition, ETP values for these variants ranged from ~1310 to ~1380 nM·min, reflecting >2-fold increases compared with the WT value (Table 3). The observation that there were little if any differences with these variants indicates the primary reason for parameter value increases relative to WT resulted from stabilizing the A2 subunit interaction in FVIIIa by the Asp519Val/Glu665Val mutation.

FVIIIa inactivation by FXa for FVIII variants

The FVIII variant, 336(P4-P3')562 where sequences flanking the relatively fast-reacting Arg336 cleavage site are replaced with those flanking the slower-reacting Arg562 cleavage site, has been shown to possess a reduced rate of inactivation by FXa resulting from a slower rate of cleavage at Arg336 (19). We examined the effect on this property after combining the protease-resistant mutation with the high FVIII/FVIIIa stability mutations. FXa inactivation experiments were conducted by adding FXa after a high concentration FVIII (150 nM) was completely activated to FVIIIa by thrombin (30 nM for 10 min at 37°C). This high FVIIIa level was used to minimize the loss of activity due to spontaneous dissociation of A2 subunit. Figure 4 shows the rate of loss of FVIIIa activity following addition of 5 nM FXa and incubation at 37°C. Control experiments in the absence of FXa yielded essentially no activity reduction (<5%) after 30 min incubation and these values were used to correct

for the non-proteolytic decay in the FXa-containing reactions. Using these conditions, WT FVIIIa activity was rapidly inactivated by FXa showing activity attributed to ~50 nM FVIIIa remaining in 10 min. A similar result was observed for the Asp519Val/Glu665Val variant while 336(P4-P3')562 FVIII showed increased resistance to FXa inactivation, with ~90 nM FVIIIa remaining in 10 min consistent with earlier results (19). Interestingly the Ala108Ile variant showed somewhat reduced FXa inactivation profile, with ~70 nM FVIIIa remaining at 10 min. In addition, when 336(P4-P3')562 was combined with Ala108Ile, the inactivation was slower than the 336(P4-P3')562 mutation alone, showing ~110 nM FVIIIa remaining at 10 min. Together, these results suggest a contribution of Ala108Ile to resistance to proteolytic inactivation. However, the combining of the 336(P4-P3')562 mutation with either Asp519Val/Glu665Val or Ala108Ile/Asp519Val/Glu665Val mutations yielded highly resistant FVIIIa against FXa inactivation, showing ~130 nM FVIIIa remaining even after 30 min incubation. The estimated inactivation rates of these latter combined variants were reduced ~11-fold relative to WT value (Table 4). These results indicate a synergy of combining reduced rates of cleavage and increased A2 subunit retention in stabilizing FVIIIa to proteolytic inactivation.

VWF binding of FVIII variants

FVIII binding affinity to VWF was performed using an ELISA-based assay and, since this is a non-equilibrium assay, estimated binding affinity was determined and compared among FVIII variants. As shown in Figure 4, the amount of bound FVIII increased with saturation in all FVIII variants tested. Estimated K_d values after curve-fitting of WT FVIII was 0.95 ± 0.19 nM and values for other FVIII variants were only marginally different (<15%, Table 5). Data showed slightly higher K_d values compared with previously reported (0.2 – 0.5 nM) (32). This result may be due to the nature of assay we used which entailed several washings leading to uncertainty of free FVIII concentration on each point for curve-fitting. However, overall these results indicate there are no appreciable differences in affinity of WT or variant FVIII for VWF.

Discussion

Alternating FVIII function by enhancing properties such as activity, stability, and resistance to proteolytic degradation by selective mutagenesis represents potential benefits for improved therapeutics for the treatment of hemophilia A. We previously reported that the Asp519Val/Glu665Val mutation yields a FVIII with prolonged activity following activation by strengthening the interaction of the A2 subunit in FVIIIa (8). Furthermore, the Ala108Ile mutation was shown to yield a FVIII variant with improved FVIII thermal and chemical stability by optimizing hydrophobic interactions in a pocket at the interface between the A1 and C2 domains (9). On the other hand, the mutation 336(P4-P3')562 was shown to reduce rates of FXa as well as APC inactivation by introducing a slower-reacting sequence flanking the P1 Arg336 residue (19). In the current report, we show that multiple mutations may be combined into single molecules that possess the attributes of the individual variants without detrimental effects. In fact, in some of the combined mutations we actually observed additive and/or synergistic effects.

In a previous study we characterized the stability of each single mutation at 37°C in plasma to mimic a more physiological condition. In this assay the higher thermal stability variants also showed higher stability in this plasma assay (7). Further, the thrombin generation assays represent a good indicator of FVIIIa stability following thrombin activation and are monitored in a plasma-based assay.

Surprisingly, the 336(P4-P3')562 mutation potentially makes a small contribution to FVIIIa stability when combined with Asp519Val/Glu665Val or Ala108Ile/Asp519Val/Glu665Val mutation. This result was unexpected as this variant contains mutations at the beginning of a1 acidic region (residues 336–372) localized to the C-terminal of the A1 subunit. This region in FVIII is disordered so no structural information is available from the intermediated resolution X-ray structures (33, 34). However, zero-length cross-linking studies have indicated that residue(s) within the a1 region interact with the A2 subunit in FVIIIa (35). For this reason we speculate that the modest reduction in the rate of FVIIIa decay by replacing the Arg336 flanking sequence with that from the sequence flanking Arg562 may result from a direct contribution to A2 subunit retention and/or an indirect one through altering a1 conformation.

Our earlier results have demonstrated that the thrombin generation potential of Asp519Val/Glu665Val mutation is much greater than WT, with for example, ~2-fold increase in ETP (8). We also showed that 336(P4-P3')562 flanking sequence swap mutation yields a slight improvement thrombin generation potential (~17% this study; ~30% in a prior report (19)) by reducing the rate at which the generated FXa feed-back inactivates FVIIIa. Results from the current study did not distinguish the magnitude of thrombin generation potential between Asp519Val/Glu665Val or Ala108Ile/Asp519Val/Glu665Val with or without the 336(P4-P3')562 mutation. This suggests that the relatively small contribution of the flanking sequence to improved thrombin generation potential was masked by the Asp519Val/Glu665Val mutation.

Examination of FVIIIa inactivation rates revealed that each individual type of mutation yielded rate reductions relative to WT with an ~10% reduction for the Asp519Val/Glu665Val variant, an ~35% reduction for the Ala108Ile variant, and an ~45% reduction for the 336(P4-P3')562 flanking sequence variant (Table 4). While the effect of the latter was expected, the results obtained with the two high FVIII/FVIIIa stability variants were not. Regarding the Ala108Ile, we speculate that the increased stabilization of the A1-C2 domains achieved with this mutation may potentially have long-range interactions in further stabilizing the A1 domain interaction with A2 subunit. We also note that combining this high stability variant with the flanking sequence variant yielded a further ~15% reduction in inactivation rate relative to the flanking sequence alone suggesting a synergy of the mutations.

Interestingly, combining the flanking sequence mutation with the high FVIIIa stability variant, Asp519Val/Glu665Val with or with Ala108Ile resulted in a marked decrease in the rate of FVIIIa inactivation by FXa (~9% the WT value) compared with the flanking sequence mutation alone (57% the WT value). This result yields mechanistic insights and suggests that A2 subunit destabilization is a component of proteolytic inactivation following

cleavage at Arg336. This destabilization may result from Arg336 cleavage facilitating A2 subunit dissociation. In this case the Asp519Val/Glu665Val mutation would directly reduce this effect, consistent with earlier studies showing the mutation markedly reducing rates of FVIIIa decay (8) by increasing inter-A2 subunit affinity (36). Alternatively, we previously showed that cleavage of FVIIIa at Arg336 may alter the orientation of A2 subunit with FXase (37). In this case, the increase in affinity of the Asp519Val/Glu665Val variant may reduce the rate at which the change in orientation occurs.

The FVIII variants described in this report possess functional attributes that are clearly desirable for replacement FVIII therapeutics. Inasmuch as all FVIII variants show no appreciable difference in VWF binding affinity compared with WT, they should bind to VWF in the circulation with similar efficiency as WT FVIII, acquiring similar protection from clearance (38). In addition, the residues modified (Ala108, Asp519, and Glu665) are buried at interfaces between FVIII domains as visualized in the FVIII X-ray structure (33, 34). Thus, we do not predict that mutations at these sites would result in additional inhibitor development should these reagents be used for FVIII replacement therapy. Furthermore, these FVIII reagents could be used at a lower dose compared with current standard dose of WT FVIII to achieve the same pro-coagulant effect, and this reduction in antigen load could potentially decrease the chance of overall FVIII inhibitor antibody development. However, the 336(P4-P3')562 variant contains mutations in 6 residues and this region is surface-exposed rather than buried within the FVIII structure. As such the potential exists for this region in contributing to inhibitor development. We are currently working on reducing the number of residues that need to be mutated at this site in order to achieve a slower rate of FXa-catalyzed cleavage at Arg336.

In conclusion, we demonstrated several examples of combining of different gain-of-function mutation to increase FVIII/FVIIIa stability and resistance against proteolytic degradation. Importantly, the generated FVIII proteins show no detrimental effects on FVIII function.

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What is known about this topic?

- Replacing charged residues with hydrophobic ones in hydrophobic pockets at FVIII A domain interfaces yields FVIII with improved FVIII/FVIIIa stability.
- Altering the sequence flanking the fast-reacting APC/FXa-cleavage site at Arg336 yields FVIII with reduced rates of proteolytic inactivation.

What does this paper add?

- Combining the above gain-of function FVIII mutations results in FVIII proteins harboring multiple mutations possessing increased stability and resistance to proteolytic inactivation without any detrimental effects on FVIII function.
- Combining the mutation 336(P4-P3')562, which reduces the rate of FXa-catalyzed cleavage at Arg336, with the Asp519Val/Glu665Val, which reduces the rate of A2 subunit dissociation in FVIIIa, results in a markedly reduced rate of FXa-catalyzed inactivation of FVIIIa suggesting that A2 subunit destabilization is a component of proteolytic inactivation following cleavage at Arg336.

Fig. 1A

Fig. 1B

Figure 1. FVIII thermal stability at 57°C (A) and FVIIIa spontaneous decay (B)

(A) FVIII variants (4 nM) were incubated at 57°C, aliquots were taken at indicated time points and activity was measured by FXa generation assay as described in Methods. (B) Thrombin-activated FVIIIa (1.5 nM) was incubated at 23°C, aliquots were taken at indicated time points, and activity was measured by FXa generation assay as described in Methods. Each point represents a value averaged from three separate determinations. Data were fitted to a single exponential decay equation by non-linear least squares regression and dashed (WT) and solid (FVIII mutants) lines and were drawn. Symbols denote for WT (open circles), 336(P4-P3')562 (open triangles), Ala108Ile (open squares), Asp519Val/Glu665Val (open diamonds), 336(P4-P3')562/Ala108Ile (closed circles), 336(P4-P3')562/Asp519Val/Glu665Val (closed triangles), and 336(P4-P3')562/Asp519Val/Glu665Val/Ala108Ile (closed squares).

Figure 2. Thrombin generation of select variants

Thrombin generation assays were performed in the presence of 0.13 nM FVIII variant, 0.5 pM rTF, and 4 μ M PSCPE vesicles. Data represent the average values of triplicate samples. Representative thrombograms of WT (circles), Asp519Val/Glu665Val (triangles), 336(P4-P3')562/Asp519Val/Glu665Val (squares), and 336(P4-P3')562/Asp519Val/Glu665Val/Ala108Ile (diamonds) are shown. A negative control (0 nM FVIII) did not generate any detectable thrombin (not shown).

Figure 3. FXa inactivation of the P4-P3' FVIIIa variants

FVIII (150 nM) was activated by thrombin (30 nM) for 10 minutes. Thrombin activity was quenched with hirudin (20 U/mL) and FXa was added (5 nM) in the presence of phospholipid (100 μ g/mL), aliquots were removed at the indicated times, and activity was determined by a one-stage clotting assay as described in Methods. Active FVIIIa concentration is plotted as a function of time for WT (open circles), 336(P4-P3')562 (open triangles), Ala108Ile (open squares), Asp519Val/Glu665Val (open diamonds), 336(P4-P3')562/Ala108Ile (closed circles), 336(P4-P3')562/Asp519Val/Glu665Val (closed triangles), and 336(P4-P3')562/Asp519Val/Glu665Val/Ala108Ile (closed squares) proteins. Activity values were corrected for the spontaneous decay of FVIIIa in the absence of added FXa, which accounted for less than 5% loss over the 30 min time course. Each point represents a value averaged from three separate determinations. Data were fitted to a second order polynomial equation by non-linear least squares regression and dashed (WT) and solid (FVIII mutants) lines and were drawn.

Figure 4. VWF binding of FVIII variants

FVIII binding to VWF was measured using a micro-titer plate as described. OD₄₅₅ values corresponds to the amount of FVIII bound to VWF were plotted as a function of FVIII concentration added. Symbols denote for WT (open circles), 336(P4-P3')562 (open triangles), Ala108Ile (open squares), Asp519Val/Glu665Val (open diamonds), 336(P4-P3')562/Ala108Ile (closed circles), 336(P4-P3')562/Asp519Val/Glu665Val (closed triangles), and 336(P4-P3')562/Asp519Val/Glu665Val/Ala108Ile (closed squares) proteins. Each point represents a value averaged from three separate determinations. Data were fitted to a simple binding equation by non-linear least squares regression and dashed (WT) and solid (FVIII mutants) lines and were drawn.

Table 1

Specific activity values.

	One-stage assay (Unit/ μ g)	Two-stage assay nM $\cdot$ min ⁻¹ per nM FVIII
WT	4.5 $\pm$ 0.2 (1)	44.5 $\pm$ 4.1 (1)
336(P4-P3')562	5.0 $\pm$ 0.3 (1.11)	43.5 $\pm$ 0.5 (0.98)
Ala108Ile	3.7 $\pm$ 0.3 (0.82)	40.3 $\pm$ 4.5 (0.91)
Asp519Val/Glu665Val	6.0 $\pm$ 0.4 (1.33)	45.4 $\pm$ 1.5 (1.02)
336(P4-P3')562/Ala108Ile	3.8 $\pm$ 0.4 (0.85)	44.7 $\pm$ 0.8 (1.01)
336(P4-P3')562/Asp519Val/Glu665Val	6.7 $\pm$ 0.7 (1.48)	42.7 $\pm$ 2.5 (0.96)
336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val	7.6 $\pm$ 1.2 (1.69)	49.2 $\pm$ 2.9 (1.11)

Specific activity values were measured by one-stage clotting assay and FXa generation assay as described in Methods. Data represents average values $\pm$ standard deviations (SD) from three separate determinations. Values in parentheses are relative to the WT value.

Table 2

Factor VIII/VIIIa activity decay summary.

	Rate constant (min ⁻¹)	
	FVIII Decay	FVIIIa Decay
WT	0.161 ± 0.004 (1)	0.122 ± 0.009 (1.00)
336(P4-P3')562	0.166 ± 0.004 (1.03)	0.156 ± 0.010 (1.28)
Ala108Ile	0.051 ± 0.005 (0.32)	0.119 ± 0.008 (0.98)
Asp519Val/Glu665Val	0.091 ± 0.003 (0.57)	0.009 ± 0.001 (0.07)
336(P4-P3')562/Ala108Ile	0.058 ± 0.005 (0.36)	0.160 ± 0.008 (1.31)
336(P4-P3')562/Asp519Val/Glu665Val	0.097 ± 0.007 (0.6)	0.004 ± 0.001 (0.04)
336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val	0.032 ± 0.002 (0.2)	0.005 ± 0.001 (0.04)

FVIII thermal decay at 57°C and FVIIIa spontaneous decay were performed as described in Methods and plotted in Figure 1. Data were fitted to a single exponential decay equation and rate constant values ± SD were obtained. Values in parentheses are relative to the WT value.

Table 3

Thrombin generation assay parameter values.

	Latent time (min)	Peak time (min)	Peak value (nM)	ETP (nM·min)
WT	10.7 ± 0.9 (1)	22.1 ± 1.3 (1)	47 ± 1.7 (1)	611 ± 17 (1)
336(P4-P3')562	9.5 ± 0.5 (0.89)	23.7 ± 0.9 (1.07)	51.5 ± 4.9 (1.1)	714 ± 56 (1.17)
Ala108Ile	9.8 ± 1.2 (0.92)	24.0 ± 2.3 (1.09)	43.7 ± 4.3 (0.93)	570 ± 35 (0.93)
Asp519Val/Glu665Val	11.2 ± 0.1 (1.05)	16.2 ± 0.2 (0.73)	180.1 ± 7.4 (3.83)	1312 ± 119 (2.15)
336(P4-P3')562/Ala108Ile	17.6 ± 3.5 (1.64)	25.8 ± 2.5 (1.17)	48.7 ± 1.3 (1.04)	713 ± 17 (1.17)
336(P4-P3')562/Asp519Val/Glu665Val	11.7 ± 1.5 (1.09)	16.3 ± 1.3 (0.74)	172.5 ± 3.0 (3.67)	1378 ± 14 (2.26)
336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val	12.2 ± 0.2 (1.14)	17.4 ± 0.5 (0.79)	179.0 ± 1.1 (3.81)	1346 ± 22 (2.2)

Thrombin generation assays in the presence of 0.13 nM FVIII proteins, 0.5 pM rTF, and 4 μM PSPCPE vesicles were performed and parameter values were calculated as described in Methods. Data represent the average values ± SD of triplicate samples. Values in parentheses are relative to the WT value.

Table 4

Factor FVIIIa inactivation rate by factor Xa.

	nM·min⁻¹ per nM FXa
WT	3.45 ± 0.02 (1.00)
336(P4-P3')562	1.96 ± 0.06 (0.57)
Ala108Ile	2.24 ± 0.02 (0.65)
Asp519Val/Glu665Val	3.06 ± 0.02 (0.89)
336(P4-P3')562/Ala108Ile	1.46 ± 0.06 (0.42)
336(P4-P3')562/Asp519Val/Glu665Val	0.30 ± 0.07 (0.09)
336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val	0.28 ± 0.11 (0.08)

Factor VIIIa inactivation by 5 nM FXa was performed as described in Methods. The inactivation rates were determined by non-linear least squares regression analysis of data in Figure 3. Data were fitted to second order polynomial equation and inactivation rates (slope values at time 0) ± SDs were obtained. Values in parentheses are relative to the WT value.

Table 5

VWF binding affinity.

	Estimated K_d (nM)
WT	0.95 ± 0.19 (1.00)
336(P4-P3')562	0.82 ± 0.15 (0.86)
Ala108Ile	0.97 ± 0.28 (1.02)
Asp519Val/Glu665Val	0.90 ± 0.19 (0.95)
336(P4-P3')562/Ala108Ile	0.91 ± 0.19 (0.95)
336(P4-P3')562/Asp519Val/Glu665Val	0.87 ± 0.23 (0.92)
336(P4-P3')562/Ala108Ile/Asp519Val/Glu665Val	1.00 ± 0.24 (1.05)

FVIII binding to VWF was measured as described in Methods. VWF binding affinity (estimated K_d values) was determined by non-linear least squares regression analysis of data in Figure 4. Data were fitted to a simple binding equation and K_d s $\pm$ SDs were obtained. Values in parentheses are relative to the WT value.