Hemophilia A mutations associated with 1-stage/2-stage activity discrepancy disrupt protein-protein interactions within the triplicated A domains of thrombin-activated factor VIIIa

Steven W. Pipe, Evgueni L. Saenko, Angela N. Eickhorst, Geoffrey Kemball-Cook, and Randal J. Kaufman

Thrombin-activated factor VIII (FVIII) is a heterotrimer with the A2 subunit (amino acid residues 373-740) in a weak ionic interaction with the A1 and A3-C1-C2 subunits. Dissociation of the A2 subunit correlates with inactivation of FVIIIa. Patients with hemophilia A have been described whose plasmas display a discrepancy between their FVIII activities, where the 1-stage activity assay displays greater activity than the 2-stage activity assay. The molecular basis for one of these mutations, ARG531His, is an increased rate of A2 subunit dissociation. Examination of a homology model of the A domains of FVIII predicted ARG531 to lie at the interface of the A1 and A2 subunits and stabilize their interaction. Indeed, patients with mutations either directly contacting ARG531 (ALA284Glu, ALA284Pro) or closely adjacent to the A1-A2 interface in the tightly packed hydrophobic core (SER289Leu) have the same phenotype of 1-stage/2-stage discrepancy. The ALA284Glu and SER289Leu mutations in FVIII were produced by transfection of COS-1 monkey cells. Compared to FVIII wild-type both mutants had reduced specific activity by 1-stage clotting activity and at least a 2-fold lower activity by 2-stage analysis (COAMATIC), similar to the reported clinical data. Analysis of immunofinity purified ALA284Glu and SER289Leu proteins in an optical biosensor demonstrated that A2 dissociation was 3-fold faster for both FVIIIa mutants compared to FVIIIa wild-type. Therefore, these mutations within the A2 subunit of FVIIIa introduce a similar destabilization of the FVIIIa heterotrimer compared to the ARG531His mutation within the A2 subunit and support that these residues stabilize the A domain interface of FVIIIa. (Blood. 2001;97:685-691)

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Introduction

Effective hemostasis is mediated by the regulated and sequential activation of serine proteases in the coagulation cascade. Factor VIII (VIII) functions in the intrinsic pathway of blood coagulation as a cofactor to accelerate by 100,000-fold the activation of factor X by factor IXa that occurs on a phospholipid surface in the presence of calcium ions. A quantitative or qualitative deficiency of FVIII leads to the phenotype of the bleeding disorder hemophilia A. The FVIII amino acid sequence, deduced from the cloned complementary DNA (cDNA), identified that the molecule is synthesized as a single-chain polypeptide having the domain structure A1-A2-B-A3-C1-C2. On secretion from the cell, FVIII is processed to a heterodimer consisting of a carboxy terminal-derived light chain (LC) of 80 kd in a metal ion-dependent association with a variably proteolyzed (90-200 kd) amino terminal-derived heavy chain (HC) fragment. The FVIII A domains share almost 40% amino acid identity with each other and to the factor V (FV) A domains, as well as with the A domains in the copper-binding protein ceruloplasmin, suggesting the A domains may be involved in copper ion binding. Indeed, studies have detected 1 mole copper ion per mole of both FV and FVIII protein.

In vivo, FVIII activity is regulated by proteolytic activation. On thrombin cleavage of FVIII there is a rapid 50-fold increase and subsequent first-order decay of procoagulant activity. The activation coincides with proteolysis of both the HC and LC of FVIII. Thrombin activates FVIII through proteolytic cleavage after ARG740, ARG1689, and ARG372 generating an FVIIIa heterotrimer consisting of the A1 subunit (50 kd) in a copper ion-dependent association with the thrombin-cleaved LC (73 kd) and a free A2 subunit associated with the A1 subunit through a weak ionic interaction. The FVIIIa heterotrimer exhibits a pH-dependent dissociation of the A2 subunit from the A1/A3-C1-C2 heterodimer that correlates with loss of procoagulant activity. Porcine FVIIIa exhibits an increased affinity for its A2 subunit compared to human FVIIIa and accordingly demonstrates an increased specific activity. In addition, a novel genetically engineered FVIII molecule, in which the A2 subunit remains covalently linked to the LC even after thrombin activation, has increased specific activity in vitro and abrogates the first-order decay of procoagulant activity as observed for native FVIII. These observations highlight the role of A2 dissociation in limiting FVIIIa activity in vitro. However, it is not yet known whether spontaneous A2 subunit dissociation or further proteolysis limits FVIIIa procoagulant activity in vivo. Insight into this question has been gained by the recent characterization of an unusual clinical phenotype of hemophilia A.

Patients with hemophilia A have been described whose plasmas display a discrepancy between their FVIII activities, where the...
1-stage (1-st) activity assay displays greater activity than the 2-stage (2-st) activity assay.\textsuperscript{12-18} Initially, the molecular mechanism for one of these mutations, a missense mutation, ARG\textsuperscript{531}HIS, was characterized to result from increased rate of inactivation of FVII\textalpha{} by increased rate of A2 subunit dissociation.\textsuperscript{19} On thrombin activation, the ARG\textsuperscript{531}HIS A2 subunit exhibited a 4-fold increased rate of dissociation from the A1/A3-C1-C2 heterodimer. The increased instability of the ARG\textsuperscript{531}HIS heterotrimer would reduce its specific activity in both 1-st and 2-st assays. However, the specific activity of ARG\textsuperscript{531}HIS is disproportionately reduced in the 2-step procedure, possibly due to the incubation phase in the first step. This hemophilia A phenotype therefore supports previous in vitro studies that have suggested that nonproteolytic regulation of FVII\textalpha{} activity, via spontaneous A2 subunit dissociation, is important in vivo.

Examination of a homology model of the A domains of FVIII\textsuperscript{20} identified ARG\textsuperscript{531} to lie at the interface of the A1 and A2 subunits. We hypothesize that if this region is important for FVII\textalpha{} stability, other mutations within this region would have similar phenotypes. Interestingly, there are 3 described hemophilia A mutations either directly contacting ARG\textsuperscript{531} (ALA\textsuperscript{284}GLU, ALA\textsuperscript{284}GLU and ALA\textsuperscript{284}GLU) or closely adjacent to ARG\textsuperscript{531}HIS at the A domain interface in the tightly packed hydrophobic core (SER\textsuperscript{289}LEU). All patients with these mutations have the phenotype of 1-st/2-st discrepancy. FVII\textalpha{} cDNA constructs were prepared containing either the ALA\textsuperscript{284}GLU or SER\textsuperscript{289}LEU mutations and the proteins were expressed and purified from transfected COS-1 monkey cells. FVIII harboring these missense mutations were secreted at similar levels to FVIII wild-type (WT), had reduced specific activity by 1-st clotting activity, and at least a 2-fold lower activity by 2-st assay.

**Materials and methods**

**Materials**

Anti-HC FVIII monoclonal antibody (F-8) conjugated to CL-4B Sepharose was a gift from Debra Pittman (Genetics Institute, Cambridge, MA). FVIII-deficient and normal pooled human plasma samples were obtained from George King Biomedical (Overland Park, KS). Activated partial thromboplastin (Activated aPTT reagent) and CaCl\textsubscript{2} were purchased from General Diagnostics Organon Teknika (Durham, NC). Anti-LEC FVIII monoclonal antibody ESH-8 was purchased from American Diagnostica (Greenwich, CT). Dulbecco modified Eagle medium (DMEM) and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD). COAMATIC was purchased from DiaPharma (West Chester, OH).
mutant proteins from immobilized ESH8 and those for dissociation of the A2 subunits on thrombin activation ofimmobilized FVIII proteins were calculated using the dissociation model with offset described by the following equation: \( R = R_i \times \exp(-k_{	ext{off}}A2) + R_f \). In the equation R is the observed surface plasmon resonance signal during A2 dissociation; \( k_{	ext{off}} \), the kinetic rate constant for the A2 dissociation on thrombin activation of FVIII WT or mutant proteins; \( R_i \), the initial resonance signal when thrombin was injected; \( R_f \), the final signal observed after all A2 dissociated (the final plateau achieved). \( R_i \) and \( k_{	ext{off}} \) were used as the independent parameters to fit observed resonance signal R versus time (t) by nonlinear regression analysis using SigmaPlot (Jandel Scientific, San Rafael, CA).

**Results**

**Recombinant-derived ARG531HIS, ALA284GLU, and SER289LEU proteins demonstrate a similar functional phenotype to patient plasma FVIII**

The synthesis and secretion of FVIII WT and ARG531HIS, ALA284GLU, and SER289LEU mutants were compared after transient DNA transfection into COS-1 monkey cells. FVIII antigen determinations by ELISA were performed on conditioned media collected from 24 to 64 hours after transfection and were similar for all mutants compared to FVIII WT suggesting no significant defect in synthesis or secretion.

FVIII activity (Figure 1) was measured by a 1-st aPTT clotting assay or by a modified 2-st method using the COAMATIC chromogenic assay. Activities were normalized to their expressed activities for recombinant-derived proteins are presented conditioned medium at 64 hours following transfection. One-stage activity (from recombinant-derived protein was obtained from assaying the activity in the Full-length cDNAs containing missense mutations generated by oligonucleotide mismatch (M) determined by aPTT-based assay. Two-stage activity (from thrombin-activated FVIII MUTATIONS INCREASE A2 SUBUNIT DISSOCIATION 687 BLOOD, 1 FEBRUARY 2001 pdFVIII 4.9 (± 0.3) × 10^−3 141 ± 6 s
FVIII WT 5.9 (± 0.6) × 10^−3 117 ± 9 s
ARG531HIS 2.9 (± 0.2) × 10^−2 24 ± 2 s
ALA284GLU 1.9 (± 0.1) × 10^−2 36 ± 1.4 s
SER289LEU 2.0 (± 0.1) × 10^−2 35 ± 1.6 s
BDN-FVIII 4.2 (± 0.02) × 10^−3 164 ± 2.6 s
BD-DD2S289-EU 2.3 (± 0.03) × 10^−2 30 ± 1.2 s

**Table 1. Analysis of FVIIIa A2-subunit dissociation by optical biosensor**

The relative rates of A2 dissociation for plasma-derived (pd) FVIII, FVIII WT, and mutant proteins were determined using an optical biosensor (Figure 2). An anti-LC antibody, ESH8, was covalently immobilized on a carboxymethylxyllecoated biosensor chip. Similar amounts (1.12 ng/mm^2) of immunoaffinity purified FVIII WT or mutant proteins were bound to ESH8 antibody. Unbound material was removed by washing with buffer and free (nonprotein-lytic) dissociation from antibody was measured. The values of dissociation rate constants, \( k_{	ext{off}} = 8.9 ± 0.23 \times 10^{-5}\text{s}^{-1}\), were similar for FVIII WT and mutant proteins. Subsequently, thrombin was added to a final concentration of 1 U/mL. Because the FVIII preparations are bound to an ESH8-coated chip via the C2 domain of their LCs, this interaction is not disturbed by thrombin cleavage and the A1 subunit remains associated with the LC through the copper ion–dependent linkage between the A1 and A3 domains. Thus, the thrombin-induced release of the A2 subunit from the heterotrimer can be measured as a dissociation curve registered by the optical biosensor. Control experiments with thrombin concentrations ranging from 0.05 to 2.25 U/mL demonstrated that maximal thrombin cleavage under these experimental conditions occurs at a thrombin concentration of 0.75 U/mL demonstrated that maximal thrombin cleavage under these experimental conditions occurs at a thrombin concentration of 0.75 U/mL (data not shown).

<table>
<thead>
<tr>
<th>Protein</th>
<th>koff (s⁻¹)</th>
<th>t½ (ln2/koff)</th>
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<tr>
<td>FVIII WT</td>
<td>5.9 (± 0.6) × 10^−3</td>
<td>117 ± 9 s</td>
</tr>
<tr>
<td>ARG531HIS</td>
<td>2.9 (± 0.2) × 10^−2</td>
<td>24 ± 2 s</td>
</tr>
<tr>
<td>ALA284GLU</td>
<td>1.9 (± 0.1) × 10^−2</td>
<td>36 ± 1.4 s</td>
</tr>
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<td>SER289LEU</td>
<td>2.0 (± 0.1) × 10^−2</td>
<td>35 ± 1.6 s</td>
</tr>
<tr>
<td>BDN-FVIII</td>
<td>4.2 (± 0.02) × 10^−3</td>
<td>164 ± 2.6 s</td>
</tr>
<tr>
<td>BD-DD2S289-EU</td>
<td>2.3 (± 0.03) × 10^−2</td>
<td>30 ± 1.2 s</td>
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**Figure 1. Recombinant-derived missense mutations in FVIII demonstrate a similar phenotype to described patient plasmas.**

(A) One-stage and 2-stage activity assays from plasmas obtained from hemophilia A patients with these missense mutations as reported in the hemophilia A mutation database. (B) Full-length cDNAs containing missense mutations generated by oligonucleotide site-directed mutagenesis were expressed in COS-1 monkey kidney cells. The data from recombinant-derived protein was obtained from assaying the activity in the conditioned medium at 64 hours following transfection. One-stage activity was determined by aPTT-based assay. Two-stage activity was determined by COAMATIC assay. Specific activities for recombinant-derived proteins are presented as a percent of recombinant FVIII WT specific activity.

2-st activity results obtained with the recombinant-derived mutant proteins are consistent with those obtained from the few reported patient plasmas where a chromogenic assay rather than the classical 2-st assay was used. Following immunoaffinity purification of FVIII WT and mutant proteins from the conditioned medium, similar discrepancy in 1-st/2-st activities were obtained (data not shown).

ARG531HIS, ALA284GLU, and SER289LEU proteins exhibit increased rate of A2 subunit dissociation from the thrombin-activated heterotrimer

The relative rates of A2 dissociation for plasma-derived (pd) FVIII, FVIII WT, and mutant proteins were determined using an optical biosensor (Figure 2). An anti-LC antibody, ESH8, was covalently immobilized on a carboxymethylxyllecoated biosensor chip. Similar amounts (1.12 ng/mm^2) of immunoaffinity purified FVIII WT or mutant proteins were bound to ESH8 antibody. Unbound material was removed by washing with buffer and free (nonprotein-lytic) dissociation from antibody was measured. The values of dissociation rate constants, \( k_{	ext{off}} = 8.9 ± 0.23 \times 10^{-5}\text{s}^{-1}\), were similar for FVIII WT and mutant proteins. Subsequently, thrombin was added to a final concentration of 1 U/mL. Because the FVIII preparations are bound to an ESH8-coated chip via the C2 domain of their LCs, this interaction is not disturbed by thrombin cleavage and the A1 subunit remains associated with the LC through the copper ion–dependent linkage between the A1 and A3 domains. Thus, the thrombin-induced release of the A2 subunit from the heterotrimer can be measured as a dissociation curve registered by the optical biosensor. Control experiments with thrombin concentrations ranging from 0.05 to 2.25 U/mL demonstrated that maximal thrombin cleavage under these experimental conditions occurs at a thrombin concentration of 0.75 U/mL demonstrated that maximal thrombin cleavage under these experimental conditions occurs at a thrombin concentration of 0.75 U/mL (data not shown).
prepared BDD forms of FVIII and the SER289LEU mutant so that only A2 subunit dissociation would be measured by the optical biosensor. Both BDD-FVIII and BDD-SER289LEU were expressed from COS cells by transient transfection and the purified proteins subjected to the same analysis as the full-length proteins. BDD-SER289LEU exhibited similar 1-st/2-st discrepancy as SER289LEU in activity assays (data not shown). Purified BDD-SER289LEU was then subjected to optical biosensor analysis and displayed 5-fold greater rate of A2 dissociation compared to the BDD-FVIII control. Because the amount of each immobilized FVIII was approximately 310 RU when thrombin was added, the reduction of the resonance signal on dissociation of A2 is 28% and 27.6% for BDD-FVIII and BDD-SER289LEU, respectively. This is identical to the expected reduction of the resonance signal (27.6%) on complete dissociation of the A2 subunit calculated using molecular weights of 170 kd and 123 kd for BDD-FVIII and its A1/A3-C1-C2 dimer. In contrast, B domain–containing samples in panels A and B of Figure 2 demonstrated an initial rapid loss of resonance signal immediately following addition of thrombin (consistent with loss of B-domain fragments) that was not observed for the BDD samples in panel C. However, all samples subsequently exhibited a relatively slower loss of resonance signal (consistent with A2 subunit dissociation) that was used to calculate the koff for dissociation.

Figure 2. Determination of the kinetic parameters for nonproteolytic and thrombin-mediated dissociation of FVIII and variants from monoclonal antibody ESH8. The monoclonal antibody ESH8 was covalently immobilized to a biosensor chip at 20 ng/mm². FVIII WT, SER289LEU, and pdFVIII (A); FVIII WT and ALA284GLU (B); or BDD-FVIII and BDD-SER289LEU (C) (2.5 nM) were bound to ESH8 at biosensor chip at 20 ng/mm². A resonance response of 200 Arc seconds corresponds to 1 ng protein.

Figure 3. ARG531HIS, ALA284GLU, and SER289LEU proteins exhibit unstable activity during the first phase of the 2-st chromogenic assay. Conditioned medium from COS cells expressing FVIII WT or mutant protein was collected 64 hours after transfection and analyzed in a modified 2-stage activity assay (COAMATIC). The first stage of the assay was modified such that each protein was subjected to increasing duration of the first stage. FVIII, ARG531HIS, SER289LEU, ALA284GLU.

To address the effect of the increased rate of A2 dissociation on the discrepant activity of the mutants in the 2-st assay, FVIII WT, ARG531HIS, ALA284GLU, and SER289LEU proteins were assayed in the chromogenic assay under conditions of increased duration of the first stage (Figure 3). Conditioned media samples collected at 64 hours following transfection were used for the assay. Antigen concentrations were similar for FVIII WT and the mutants and ranged between 10 and 20 ng/mL. The manufacturer’s protocol was modified whereby duplicated samples were incubated in the first stage for 2, 4, 8, and 16 minutes in parallel with FVIII WT. The first stage was initiated for the 16-minute samples first, followed by the 8-, 4-, and 2-minute incubation samples. The second stage was then initiated for all samples simultaneously and then measured together. During the first-stage incubation, the FVIII samples are activated due to the presence of thrombin in the reagent mix. The resultant FVIIIa is then available to exert cofactor activity with factor IXa to generate factor Xa from unactivated factor X. The total amount of factor Xa generated in the first stage is then quantified at the second stage by the amount of chromophore released from the chromogenic reagent by factor Xa. A standard curve was performed in parallel with dilutions of pooled normal plasma and incubated for 2 minutes during the first stage. The y-axis of Figure 3 is thus the relative FVIII activity of the samples following the various one-stage incubations as compared to this standard curve. We hypothesized that both FVIII WT and the mutants with increased rate of A2 dissociation would become fully activated in the first-stage incubation and exhibit FVIII activity as determined by the total amount of factor Xa generated. However, with increased rate of inactivation due to A2 dissociation, the mutants would be unable to sustain FVIII activity and the total amount of factor Xa generated would not increase at the same rate as FVIII WT. Indeed, FVIII WT was observed to have continued cofactor activity (as demonstrated by increasing factor Xa generation) throughout even the longest first-stage incubation of 16 minutes. In contrast, each of the mutants, though showing continued cofactor activity for up to 4 minutes of first-stage incubation fail to contribute to any further factor Xa generation beyond 4
minutes. This is consistent with complete spontaneous inactivation of the mutants after 4 minutes. Thus the relative amount of factor Xa generation decreases with increasing duration of first-stage incubation compared to FVIII WT.

Discussion

Despite the 4 decades of availability of both the 1-st and 2-st assays for measuring FVIII activity, there is still no consensus on which method most accurately represents FVIII cofactor function in vivo. Although there is little assay discrepancy between the methods when comparing human plasmas or various FVIII-containing concentrates, considerable variability has been described when not comparing FVIII molecules with similar molecular composition and structure. For instance, recombinant FVIII shows a reduced potency in the 1-st assay compared to the 2-st assay (chromogenic) when plasma FVIII is used as the standard.\(^{28}\) Similar discrepancy is observed following infusion of recombinant FVIII into patients with hemophilia, prompting recommendations that recombinant FVIII be used to prepare the standards for this type of analysis.

In addition, a naturally occurring patient mutation was described whereby the 1-st assay reports reduced activity compared to the 2-st assay.\(^{29}\) The mutation GLU\(^{720}\)LYS within FVIII results in a mild hemophilia A phenotype with detectable circulating FVIII antigen. Patients with this mutation exhibit normal levels of FVIII cofactor activity as measured by the chromogenic assay method but have reduced levels of procoagulant activity when measured by 1-st assay. Recombinant GLU\(^{720}\)LYS was purified and found to require higher concentrations of FIXa for full cofactor activity as measured by FXa generation, whereas lower concentrations of FIXa (as would be expected in a 1-st assay) resulted in 2- to 3-fold reduced activity. It was proposed that this mutation at residue 720, adjacent to one of 3 putative FIXa binding sites within the A2 domain (residues 698-712), could result in reduced affinity for FIXa and therefore contribute to the higher FXa concentration required for optimal cofactor activity.

The 1-st/2-st discrepancy described above relates to reduced FVIII activity in the 1-st compared to the 2-st assay. However, the hemophilia A phenotype analyzed in this report relates to FVIII activity that is reduced in the 2-st assay compared to the 1-st assay. Early reports characterizing this clinical phenotype were attributed to type 2N von Willebrand disease (vWD) where a mutation in the FVIII binding site within von Willebrand factor leads to reduced type 2N von Willebrand disease (vWD) where a mutation in the FVIII binding site within von Willebrand factor leads to reduced activity that is reduced in the 2-st assay compared to the 1-st assay. Recombinant GLU\(^{720}\)LYS was purified and found to require higher concentrations of FIXa for full cofactor activity as measured by FXa generation, whereas lower concentrations of FIXa (as would be expected in a 1-st assay) resulted in 2- to 3-fold reduced activity. It was proposed that this mutation at residue 720, adjacent to one of 3 putative FIXa binding sites within the A2 domain (residues 698-712), could result in reduced affinity for FIXa and therefore contribute to the higher FXa concentration required for optimal cofactor activity.

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Figure 4. Predicted destabilization of interdomain interaction in the ALA\(^{284}\)GLU\(^{720}\)LYS and ALA\(^{284}\)PRO region. (A) Close-up view of a portion of the A1-A2 interdomain boundary in the FVIII A domain model, showing the putative interaction of the ALA\(^{284}\)PRO with the main chain carboxyl of ALA\(^{284}\)PRO, together with the close packing of the ALA\(^{284}\)PRO side chain against that of ALA\(^{284}\)PRO. Replacement of the Ala side chain by the bulky polar Glu side chain (as in the hemophilia A variant ALA\(^{284}\)GLU) is predicted to disturb the ALA\(^{284}\)GLU\(^{720}\)LYS interaction. Side chains are colored by atom (C, green; O, red; N, blue; H, white). (B) Predicted destabilization of interdomain interaction in the SER\(^{289}\)GLU\(^{720}\)LYS and ALA\(^{284}\)PRO region. Close-up view of a portion of the A1-A3 interdomain boundary, showing the putative interaction of the SER\(^{289}\)GLU\(^{720}\)LYS side chain with the side chain of ALA\(^{284}\)PRO. Replacement of the Ser side chain by the bulky hydrophobic Leu side chain (as in the hemophilia A variant SER\(^{289}\)GLU\(^{720}\)LYS) is predicted to disturb the SER\(^{289}\)GLU\(^{720}\)LYS interaction and packing of surrounding residues. Side chains are colored by atom (C, green; O, red; N, blue; H, white).
case, inspection of the FVIII A domain model predicts an interdomain H-bond between the A1 and A3 domains, between the side chains of ARG 284 and TYR 1979 (Figure 4B). Replacement by the bulky leucine side chain is predicted to abolish this interdomain H-bond but also to disrupt packing very close to the interface between all 3 A domains at the pseudo-threefold axis of the model.

Our in vitro characterization of both of these FVIII missense mutations located within the A1 subunit (at residues 284 and 289) confirms the hemophilia A clinical phenotype of 1-st/2-st discrepancy and demonstrates the same molecular phenotype of increased rate of A2 subunit dissociation on thrombin cleavage. These observations provide evidence that the packing of the triplicated A domains is critical to FVIII function and that interdomain protein-protein interactions are required for stabilizing both the unactivated and activated forms of the molecule. These observations also provide first experimental evidence that validates the molecular model of the triplicated A domains based on their homology to ceruloplasmin.

The insights gained from studying this patient phenotype and the molecular characterization of these particular mutations has extended the observations made from analyzing the ARG 531 HIS mutation. Two additional reports have added to our observations. A family pedigree has been described in which all members have 2-st FVIII activities that are one half of that measured by 1-st assay.66 This pedigree included affected males as well as homozygous females. Molecular analysis identified a missense mutation at residue 694 within the A2 subunit where ILE is substituted for a HIS.37 This mutation predicts loss of intradomain hydrogen bonds as well as introduces severe side-chain packing constraints within an area adjacent to the A2-A3 interface. The second report describes a hemophilia A patient with the same 1-st/2-st discrepancy phenotype attributed to a missense mutation at residue 1954 within the A3 subunit where LEU is substituted for a HIS.37 This patient’s 1-st activity assay was within normal limits though the 2-st activity assay and chromogenic assay were only 18% and 35%, respectively. This residue is in close proximity to TYR 1979, and to residues ARG 698 and MET 1947, both previously indicated to lie at the A2-A3 interface and associated with the same 1-st/2-st discrepant mild hemophilia phenotype under discussion.18,35 This report is particularly interesting in that the hemophilia phenotype was more accurately reflected by the 2-st assay rather than the more widely used 1-st assay. Thus, mild hemophilia A variants can be missed when screened in laboratories that exclusively use the 1-st aPTT-based assay. These observations, as well as those reported here, show that the 2-st assay more closely reflects in vivo FVIII clotting activity, support that

or close to the A1-A2, A1-A3, and A2-A3 domain interfaces. Figure 5 shows a global view of the FVIII A domain model viewed down the pseudo-threefold axis, showing the predicted positions of the amino acids mentioned above, which are associated with the 1-st/2-st discrepancy in hemophilic subjects. There is a clear clustering of all these residues at or very close to the interface of the A2 domain with the A1 and A3 domains.

The proposed pseudo-threefold axis at the hydrophobic core of the A1-A2-A3 interface is tightly packed and predicts several interdomain interactions. These lie at the interface of A1-A2, A2-A3, and A1-A3. Sufficient disruption of these protein-protein interactions along the pseudo-threefold axis would lead to destabilization and increased propensity for dissociation particularly after thrombin cleavage whereby covalent bonds flanking the A2 subunit are lost.

The validation of the molecular modeling of the A domains and the characterization of these putative interdomain protein-protein interactions now allows the hypothesis that increased stabilization of this interface would lead to greater stability of FVIII and increased cofactor activity. There is now opportunity to explore complementary mutations that might allow better packing and stabilization of the A domains to test this hypothesis. Novel bioengineering strategies may ultimately enable strategic interdomain cross-linking to provide further stabilization of FVIIIa. Further characterization of FVIIIa-FIXa sites of interaction combined with both molecular modeling and crystallography observations may ultimately allow the design of a peptidomimetic for FVIIIa.

References

3. Toole JJ, Knopf JL, Wozney JM, et al. Molecular cloning of a cDNA encoding human antihempho-

4. Koschinsky ML, Funk WD, van OstBA, MacGil-

5. Bihoreau N, Pin S, de Keserbie AM, Vidot F, Fontaine-Aupar MP. Copper-atom identification in the active and inactive forms of plasma-derived FVIII and recombinant FVIII-delta II. Eur J Bio-

8. Eaton D, Rodriguez H, Vehar GA. Proteolytic pro-

cessing of human factor VIII. Correlation of spe-
cific cleavages by thrombin, factor Xa, and acti-

vated protein C with activation and inactivation of factor VIII coagulant activity. Biochemistry. 1986;

25:505-512.
10. Lollar P, Parker ET. Structural basis for the de-

11. Fay PJ, Smuzdin TM. Characterization of the in-


17. Duncan EM, Duncan BM, Tunbridge LJ, Lloyd JV. Familial discrepancy between the one-stage and two-stage factor VIII methods in a subgroup of patients with haemophilia A. Br J Haematol. 1996;94:400-406.


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