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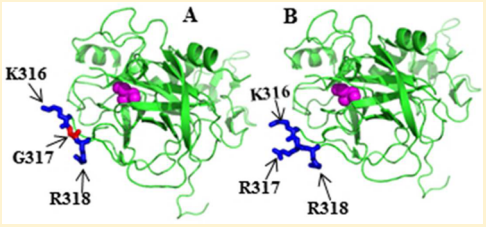
Expression and Characterization of Gly-317 Variants of Factor IX Causing Variable Bleeding in Hemophilia B Patients

Qiuya Lu,[†] Likui Yang,[‡] Chandrashekhara Manithody,[‡] Xuefeng Wang,*[,†] and Alireza R. Rezaie*[,‡]

[†]Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China

[‡]Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri 63104, United States

ABSTRACT: We recently identified two hemophilia B patients who carried Gly-317 to Arg (FIX-G317R) or Gly-317 to Glu (FIX-G317E) substitutions in their FIX gene. The former mutation caused severe and the latter moderate bleeding in afflicted patients. To understand the molecular basis for the variable clinical manifestation of Gly-317 mutations, we prepared recombinant G317R and G317E derivatives of FIX and compared their kinetic properties to those of recombinant wild-type FIX in appropriate assay systems. Both physiological activators, factor XIa and extrinsic Tenase (factor VIIa−tissue factor), activated both zymogen variants with an ∼1.5-fold elevated K_m; however, extrinsic Tenase activated FIX-G317E with an ∼2-fold improved k_{cat}. By contrast to zymogen activation, the catalytic activities of both FIXa-G317R and FIXa-G317E enzymes toward the natural substrate, factor X, were dramatically (>4 orders of magnitude) impaired, but their apparent affinity for interaction with factor VIIIa was only slightly (<2-fold) decreased. Further studies revealed that the reactivity of FIXa-G317R and FIXa-G317E with antithrombin has been impaired 10- and 13-fold, respectively, in the absence and 166- and 500-fold, respectively, in the presence of pentasaccharide. As expected, the clotting activities of FIX variants could not be measured by the aPTT assay. These results implicate a critical role for Gly-317 in maintaining normal catalytic function for FIX/FIXa in the clotting cascade. The results further suggest that improved k_{cat} of FIX-G317E activation in the extrinsic pathway together with dramatically impaired reactivity of FIXa-G317E with antithrombin may account for the less severe bleeding phenotype of a hemophilia B patient carrying the FIX-G317E mutation.



Factor IX (FIX) is a vitamin K-dependent trypsin-like serine protease zymogen in plasma, which upon activation to its active form (FIXa) binds to cofactor VIIIa (FVIIIa) on negatively charged membrane surfaces in the presence of Ca^{2+} to activate factor X (FX) in the intrinsic pathway of the blood clotting cascade.[1−5] FIX circulates in plasma as a single-chain inactive zymogen with 415 amino acids.[6] The structure of FIX protein is comprised of four functionally distinct domains that are encoded by different exons. These include an N-terminal γ-carboxyglutamic acid (Gla) rich domain followed by two epidermal growth factor (EGF)-like domains and a C-terminal catalytic domain.[7] The single-chain zymogen is converted to a two-chain enzyme upon activation by either of the physiological activators, the factor VIIa (FVIIa)−tissue factor (TF) complex or factor XIa (FXIa) during the initiation of the clotting cascade.[8−10] Proteolytic cleavage after two basic residues (Arg-145 and Arg-180) by either of these physiological activators releases the activation peptide, thereby converting the zymogen to an active enzyme consisting of a light chain (Gla and EGF domains) and a heavy chain (catalytic domain) linked together by a single disulfide bond.[7−10] Both the Gla and the N-terminal EGF domains as well as the 162-helix of the catalytic domain of FIXa facilitate the Ca^{2+}-dependent assembly of the protease with FVIIIa on the negatively charged membrane surfaces.[5−7] The complex assembly is essential for the catalytic function of FIXa in the intrinsic pathway of the coagulation cascade.

FIX plays a key role in maintaining normal hemostasis, and its deficiency is associated with mild to severe bleeding in hemophilia B patients.[3,11] FIX deficiency is inherited as an X-linked recessive disorder with a prevalence of 1 in 25000 in newborn males worldwide.[12] It is caused by a wide range of mutations that can include point mutations (nonsense and missense), insertions, deletions, and other complex rearrangements of the FIX gene. The mutations are distributed through the entire FIX gene, encoding different domains of the protein. More than 1000 FIX gene mutations causing FIX deficiency, most of which are located on the catalytic domain, have thus far been reported in the Human Gene Mutation Database. FIX deficiency causes a defect in the intrinsic pathway, and on the basis of the clotting activity, it can be classified as severe (<1%), moderate (1−5%), and mild (5−30%). In a recent study aimed at elucidating the spectrum of FIX gene mutations in Chinese hemophilia B patients,[13] we identified two missense mutations resulting in substitution of Gly-317 (Gly-149 in chymotrypsin numbering[14]) with an Arg (G317R) in one patient and with a Glu (G317E) in the other.[13] The Arg mutation in the first patient caused severe bleeding; however, the Glu substitution resulted in moderate bleeding in the second patient.[13] To

Received: March 11, 2015
Revised: May 26, 2015
Published: May 29, 2015

DOI: 10.1021/acs.biochem.5b00270
Biochemistry 2015, 54, 3814−3821

understand the molecular basis for different clinical manifestations of Gly-317 mutations in these patients, we expressed wild-type FIX and the two different mutants in mammalian cells, purified them to homogeneity, and characterized their properties in established coagulation assays. Analysis of data suggests that although both FIXa variants exhibit equally dramatically impaired activity toward factor X in the intrinsic Tenase pathway, the G317E mutant zymogen is, nevertheless, activated with an ~2-fold improved k_{cat} in the extrinsic pathway. Furthermore, while both mutants are poorly inhibited by antithrombin (AT), the G317E mutant loses its reactivity with the serpin at a significantly higher rate particularly in the presence of pentasaccharide. These results suggest that the improved activation of FIX-G317E by the FVIIa−TF complex together with its dramatically poor reactivity with AT may be responsible for the less severe bleeding phenotype of the hemophilia B patient carrying this mutation. These results predict a key role for Gly-317 in the catalytic function of FIXa in the intrinsic pathway.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of Recombinant Proteins. Both wild-type (FIX-WT) and Gly-317 to Arg (FIX-G317R) and Gly-317 to Glu (FIX-G317E) mutants of FIX (Gly-149 in chymotrypsinogen numbering[14]) were generated by standard polymerase chain reaction mutagenesis methods and expressed in the RSV-PL4 expression/purification vector system as described previously.[15] After confirmation of the accuracy of the mutagenesis by DNA sequencing, the constructs were introduced into HEK-293 cells and the mutant proteins were isolated from 20 L cell culture supernatants by a combination of immunoaffinity chromatography using the HPC4 monoclonal antibody followed by ion exchange chromatography using a Mono Q column as described previously.[15]

Human plasma factors FVIIa, FX, FXa, FXIa, and AT were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described previously.[16] Human recombinant FVIIIa was a generous gift from P. Fay (University of Rochester, Rochester, NY). Tissue factor lacking the cytoplasmic domain was incorporated into PC/PS phospholipids as described previously.[16] Normal pooled plasma and FIX-deficient plasma were purchased from George King Bio-Medical, Inc. (Overland Park, KS), and the activated partial prothrombin time reagent, TriniCLOT aPTT S, was purchased from Trinity Biotech (St. Louis, MO). The active AT-binding pentasaccharide fragment of high-affinity heparin (fondaparinux sodium) and unfractionated heparin was purchased from Quintiles Clinical Supplies (Mt. Laurel, NJ). The chromogenic substrates specific for FXa, MeO-CO-D-Chg-Gly-Arg-*p*-nitroanilide (SpFXa), and FIXa, CH_3SO_2-D-Leu-Gly-Arg-*p*-nitroanilide (CBS 31.39), were purchased from American Diagnostica (Greenwich, CT) and Midwest Bio-Tech Inc. (Fishers, IN), respectively.

FIX Activation by Physiological Activators. The concentration dependence of activation of FIX zymogens by the FVIIa−TF complex was monitored as described previously.[17] Briefly, FIX zymogens (0.06−4.0 μM) were incubated with FVIIa (0.5 nM) in complex with relipidated TF (5 nM) in TBS containing 1 mg/mL bovine serum albumin (BSA), 0.1% polyethylene glycol (PEG) 8000, and 5 mM Ca^{2+} (TBS/Ca^{2+}). Following activation for 10 min at room temperature, the activation reactions were stopped with 20 mM EDTA and the rate of FIXa generation was measured by an amidolytic activity assay using a standard curve prepared by total activation of each zymogen at the time of experiments. A similar assay was employed to monitor the concentration dependence of FIX activation (0.03−2.0 μM) by FXIa (0.5 nM) at room temperature for 10 min in TBS/Ca^{2+}. Apparent K_m and k_{cat} values for activation of FIX derivatives by each activator were obtained by nonlinear regression fits of kinetic data to the Michaelis−Menten equation.

Amidolytic Activity. The steady-state kinetics of hydrolysis of CBS 31.39 (0.06−8 mM) by FIXa and its mutant derivatives (10−30 nM) were studied in TBS containing 1 mg/mL BSA, 33% ethylene glycol, and 5 mM Ca^{2+}. The rate of hydrolysis was measured at 405 nm at room temperature in 96-well plates by a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) as described previously.[15,17] Apparent K_m and k_{cat} values for substrate hydrolysis were obtained by nonlinear regression fits of data to the Michaelis−Menten equation, and the catalytic efficiencies were expressed as the k_{cat}/K_m ratio.

FX Activation by FIXa and Its Mutant Derivatives. The initial rate of FX activation by FIXa and its mutants was measured on PC/PS vesicles in the presence of FVIIIa in TBS/Ca^{2+} at room temperature as described previously.[15] First, the ability of wild-type FIXa and its mutants to interact with FVIIIa was evaluated by incubating each protease (0.1 nM wild-type and 25 nM mutants) with varying concentrations of FVIIIa (1−80 nM) on PC/PS vesicles (50 μM), and the reaction was initiated by addition of FX (200 nM) in TBS/Ca^{2+}. Following a 2−30 min activation in 30 μL reaction volumes in a 96-well assay plate, the reactions were stopped by addition of EDTA to a final concentration of 20 mM, and the rate of FXa generation was measured by an amidolytic activity using SpFXa as described above. The concentration of FXa generated in the activation reactions was determined from a standard curve prepared from the cleavage rate of SpFXa by known concentrations of plasma FXa under exactly the same conditions. The apparent K_d for the interaction of FIXa and its mutants with FVIIIa was determined by nonlinear regression fits of data by a hyperbolic binding equation as described previously.[15] This assay was also used to analyze the concentration dependence of FX activation by each protease. In this case, FIXa-WT (0.1 nM) or mutants (25 nM) were incubated with a fixed and saturating concentration of FVIIIa (50 nM) on PC/PS vesicles (50 μM) and the reaction was initiated by addition of varying concentrations of FX (6−800 nM) in TBS/Ca^{2+}. Following a 2−30 min activation, EDTA was added to a final concentration of 20 mM, and the rate of FXa generation was measured as described above. Apparent K_m and k_{cat} values were calculated by nonlinear regression fits of data to the Michaelis−Menten equation.

Reaction with Antithrombin. The rate of inactivation of FIXa and its mutants by AT in the absence and presence of the active pentasaccharide fragment of heparin was measured under pseudo-first-order conditions by a discontinuous assay as described previously.[18] In the absence of a cofactor, FIXa and its mutant derivatives (5−20 nM) were incubated with AT (0.5−2.0 μM) in TBS/Ca^{2+} for 2−8 h in 50 μL volumes in 96-well polystyrene assay plates at room temperature. In the presence of pentasaccharide, the reaction conditions were the same except that proteases were incubated with AT (0.025−1.6 μM) in TBS/Ca^{2+} in complex with a saturating concentration of pentasaccharide (2 μM) for 5 min to 8 h. The reactions were

DOI: 10.1021/acs.biochem.5b00270
Biochemistry 2015, 54, 3814−3821

stopped by addition of 50 μL of CBS 31.39 (final concentration of 1.0 mM), and the remaining enzyme activity was measured with a V_{max} Kinetics Microplate Reader at 405 nm as described above. The observed pseudo-first-order rate constants (k_{obs}) were determined by fitting the time-dependent change in protease activities to a first-order rate equation, and second-order rate constants (k_2) were obtained from the slopes of linear plots of k_{obs} versus the concentration of the AT-pentasaccharide complex as described previously.[18]

Analysis of Thrombin Generation in Plasma. The thrombin generation (TG) assay was performed using previously described standard methods.[19,20] Each assay contained 80 μL of citrated pooled plasma, obtained from two different hemophilia B patients with severe bleeding, incubated with 20 μL of PPP reagent containing either 1 or 5 pM TF, 4 μM phospholipids, and 100 mM $CaCl_2$. In some experiments, the TG assay was performed in the presence of 0.1 unit/mL unfractionated heparin. TG was assessed by measuring the hydrolysis of a fluorogenic thrombin substrate as described previously.[19,20] Three parameters, including lag time (minutes), peak height (Peak, nanomolar), and area under the curve, referred to the endogenous thrombin potential (ETP, nM*minute), were used to assess TG dynamics and to evaluate the clotting activity of FIX and its mutant derivatives.

RESULTS

Clinical Data. In the process of investigating the spectrum of FIX mutations in hemophilia B patients, we identified two patients who carried two different missense mutations involving the Gly-317 codon of the catalytic domain, which resulted in the mutation of this residue to Glu (G317E) in one patient and to Arg (G317R) in the other. The FIX clotting activity (FIX:C) and antigen levels (FIX:Ag) were 0.9 and 63% for the G317R mutation, respectively. No FIX:Ag level was determined for the G317E patient (he can no longer be located to obtain blood samples); however, the FIX:C activity of the patient's plasma was determined to be 1.3%. While both patients had joint bleeding and the presence of arthropathy was diagnosed for both cases, the G317R patient also had bleeding on the skin. The first bleeding in the skin and joints occurred when the G317R patient (age 7) was 7 months old, but the G317E patient who was 23 years old first had joint bleeding when he was 3 years old. Both patients had normal FVIII clotting activity and were tested negative for the FIX inhibitors. The G317E patient experienced moderate but the G317R patient severe bleeding. The occurrence of bleeding in more than one site, the lower FIX:C activity, and the earlier onset of bleeding for the G317R mutation were consistent with the clinically severe bleeding phenotype for this patient. In light of this different clinical manifestation for mutations involving the same residue and our interest in the structure–function correlates in the coagulation factors, we decided to express the two different FIX mutants for further analysis.

Expression, Purification, and Activation of Recombinant FIX Derivatives. Both FIX-WT and its G317R and G317E substitution mutants were expressed in HEK-293 cells as described previously.[15] The recombinant zymogens from cell culture supernatants were isolated by a combination of immunoaffinity and ion exchange chromatography using the HPC4 monoclonal antibody and a Mono Q column as described previously.[15] Zymogen purification by the Mono Q column separated fully γ-carboxylated proteins from the partially modified species as described previously.[15] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis under nonreducing conditions indicated that the isolated proteins have been purified to homogeneity and that all three FIX zymogens migrate with similar and expected molecular masses of ~60 kDa (Figure 1A).

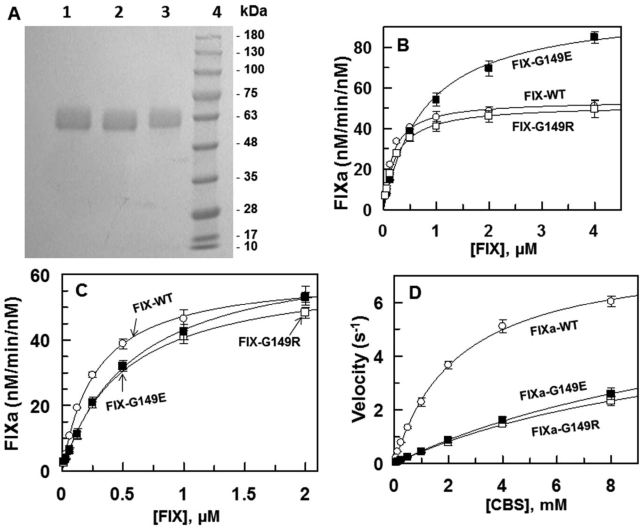


Figure 1. Characterization of FIX derivatives. (A) SDS–PAGE analysis of the recombinant FIX derivatives expressed in HEK-293 cells under nonreducing conditions: lane 1, FIX-WT; lane 2, FIX-G317R; lane 3, FIX-G317E; lane 4, molecular mass standards in kilodaltons. (B) Concentration dependence of activation of FIX derivatives by the extrinsic Tenase complex. Increasing concentrations of FIX-WT (○), FIX-G317R (□), and FIX-G317E (■) (*x*-axis) were incubated with FVIIa (0.5 nM) in complex with relipidated TF (5 nM) in TBS/Ca^{2+} for 10 min at room temperature. The rate of FIXa generation was monitored using CBS 31.39 as described in Experimental Procedures. Solid lines are computer fits of kinetic data to the Michaelis–Menten equation, yielding apparent K_m and k_{cat} values of 0.25 μM and 51.2 nM min^{-1} nM^{-1} for FIX-WT, 0.35 μM and 53.6 nM min^{-1} nM^{-1} for FIX-G317R, and 0.38 μM and 98.2 nM min^{-1} nM^{-1} for FIX-G317E, respectively. (C) Concentration dependence of activation of FIX derivatives by FXIa. Increasing concentrations of FIX-WT (○), FIX-G317R (□), and FIX-G317E (■) (*x*-axis) were incubated with FXIa (0.5 nM) in TBS/Ca^{2+}. Following activation for 10 min at room temperature, the rate of FIXa generation was measured using CBS 31.39 as described in Experimental Procedures. Solid lines are computer fits of kinetic data to the Michaelis–Menten equation, yielding apparent K_m and k_{cat} values of 0.28 μM and 60.4 nM min^{-1} nM^{-1} for FIX-WT, 0.50 μM and 61.1 nM min^{-1} nM^{-1} for FIX-G317R, and 0.58 μM and 68.1 nM min^{-1} nM^{-1} for FIX-G317E, respectively. (D) The amidolytic activity of FIXa and its mutant derivatives toward CBS 31.39 was monitored by incubating increasing concentrations of the chromogenic substrate (*x*-axis) with 10 nM FIXa-WT (○), 30 nM FIXa-G317R (□), and 30 nM FIXa-G317E (■) in TBS/Ca^{2+} containing 33% ethylene glycol at room temperature as described in Experimental Procedures. The kinetic values are listed in Table 1. Chymotrypsin numbering is used within panels B–D.

The initial rate of zymogen activation by the two physiological activators of FIX was evaluated. The concentration dependence of FIX activation by the FVIIa–TF complex, presented in Figure 1B, suggests that the apparent K_m for the activation of mutant zymogens by the extrinsic Tenase has been elevated ~1.5-fold. Interestingly, however, the k_{cat} of zymogen activation by the FVIIa–TF complex for the G317E mutant was improved ~2-fold, without a noticeable

DOI: 10.1021/acs.biochem.5b00270
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difference in the rate of activation between FIX-WT and FIX-G317R (Figure 1B). Analysis of zymogen activation rates by FXIa suggests that FXIa activates both zymogen mutants with similar catalytic rates; however, as seen for activation by the FVIIa–TF complex, the apparent K_m for the activation of the zymogen mutants by FXIa was also elevated ~2-fold for both FIX-G317R and FIX-G317E derivatives (Figure 1C). The complete activation of the mutant FIX zymogens was confirmed by SDS–PAGE (data not shown).

Amidolytic Activity. Following activation, the active-site concentrations of FIXa and its mutant derivatives were determined by stoichiometric titration with a calibrated concentration of AT in the presence of unfractionated heparin as described previously.[15,18] The amidolytic activities toward the chromogenic substrate CBS 31.39 (LGR-pNA) for all proteases are presented in Figure 1D, and the kinetic parameters for the cleavage of the substrate are listed in Table 1. FIXa-WT cleaved CBS 31.39 with relatively good

Table 1. Kinetic Constants for the Cleavage of the Chromogenic Substrate, CBS 31.39 (LGR-pNA), by FIXa and Its Mutant Derivatives[a]

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
FIXa-WT	2.3 ± 0.1	7.9 ± 0.2	3.4 ± 0.2
FIXa-G317R	12.5 ± 0.7	6.0 ± 0.3	0.48 ± 0.05
FIXa-G317E	14.6 ± 1.1	7.4 ± 0.4	0.51 ± 0.06

[a]The kinetic constants were calculated from the initial cleavage rates measured at increasing concentrations of CBS 31.39 (0.08–10 mM) by each FIXa (10–30 nM) in TBS buffer containing 5 mM Ca^{2+}, 1 mg/mL BSA, and 33% ethylene glycol. The kinetic values are derived from the data in Figure 1D. Values are the average of at least three measurements ± standard deviations.

catalytic efficiency in TBS/Ca^{2+} containing 33% ethylene glycol, thus yielding kinetic constants of 2.3 μM and 7.9 s^{-1} for K_m and k_{cat}, respectively (Table 1). While the FIXa and its mutants exhibited similar k_{cat} values of 6.0 s^{-1} (G317R) and 7.4 s^{-1} (G317E) toward the chromogenic substrate, nevertheless, the K_m values for both mutant proteases were impaired ~6-fold (12.5 and 14.6 μM for G317R and G317E, respectively), suggesting that the mutagenesis impedes the docking of the chromogenic substrate into the active-site pockets of mutant proteases. Normal k_{cat} values suggest that the mutagenesis may not have caused any adverse effect on the folding or reactivity of the catalytic triad but that the effect is limited to the secondary substrate-binding site(s) of the protease.

Factor X Activation. The catalytic property of FIXa and its mutant derivatives in the intrinsic Tenase complex (FVIIIa, PC/PS vesicles, and Ca^{2+}) toward activation of the natural substrate FX was studied. First, the ability of these proteases to interact with FVIIIa in the intrinsic Tenase complex was evaluated by measuring the initial rate of FXa generation as a function of increasing concentrations of the cofactor. The results presented in Figure 2A suggest that FIXa-WT interacts with FVIIIa with an apparent dissociation constant [$K_{d(app)}$] of ~13 nM. The $K_{d(app)}$ for the interaction of both FIXa-G317R (22 nM) and FIXa-G317E (20 nM) with FVIIIa was only slightly elevated; nevertheless, the initial rate of FX activation by both FIXa mutants (in particular by G317R) was dramatically impaired (Figure 2B). Comparison of the concentration dependence of FX activation by FIXa-WT (Figure 2C) and mutants (Figure 2D) in complex with a

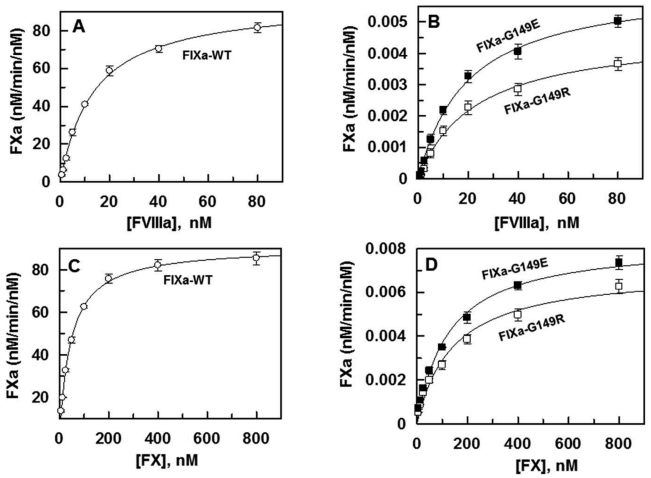

Figure 2. Cofactor and substrate concentration dependence of FX activation by FIXa and its mutant derivatives on PC/PS vesicles. (A) FIXa-WT (0.1 nM) was incubated with FX (0.2 μM) in the presence of increasing concentrations of FVIIIa (1–80 nM) on PC/PS vesicles (50 μM) in TBS/Ca^{2+}. Following activation for 2 min at room temperature, the reaction was terminated by addition of 20 mM EDTA and the rate of FXa generation was measured by an amidolytic activity assay using SpFXa as described in Experimental Procedures. (B) Same as panel A except that FIXa mutants (25 nM each) were incubated with FX in the presence of increasing concentrations of FVIIIa (*x*-axis). (C) Same as panel A, except that the concentration dependence of FX activation by FIXa-WT (0.1 nM) in complex with FVIIIa (50 nM) was monitored. (D) Same as panel B, except that the concentration dependence of FX activation by FIXa mutants (25 nM) in complex with FVIIIa (50 nM) was monitored. The symbols in all panels are as follows: (○) FIXa-WT, (□) FIXa-G317R, and (■) FIXa-G317E. The kinetic values are listed in Table 2. Chymotrypsin numbering is used within panels B and D.

fixed concentration of FVIIIa (50 nM) indicated that the mutants activate FX with a >4 order of magnitude decreased k_{cat} while exhibiting an ~3-fold elevated apparent K_m for the substrate (Table 2). These results suggest that Gly-317 plays a critical role in the catalytic function of FIXa and that its mutation effectively abrogates the physiological function of the mutant protease in the intrinsic Tenase complex. This interpretation is consistent with a complete loss of clotting function for both mutant zymogens in the FIX-deficient plasma-based aPTT assay (data not presented).

Reaction with AT. The reactivity of FIXa and its mutant derivatives with AT was evaluated in the absence and presence of the high-affinity AT-binding pentasaccharide (Figure 3 and Table 3). The G317R and G317E mutants reacted with AT with ~10- and ~13-fold slower second-order association rate constants (k_2), respectively. The cofactor function of pentasaccharide in accelerating the AT inhibition of the mutants was also dramatically impaired (Table 3). Thus, analysis of the inhibition rates as a function of increasing concentrations of the AT–pentasaccharide complex (Figure 3) suggested that the inhibition of G317R and G317E mutants by the cofactor–serpin complex has been impaired 166- and 500-fold, respectively (Table 3). These results suggest that the mutagenesis of Gly-317 (in particular to Glu) nearly eliminates the ability of FIXa mutants to recognize the heparin-activated conformation of AT.

Analysis of Thrombin Generation in Plasma. The clotting activities of recombinant FIX and its mutant derivatives

DOI: 10.1021/acs.biochem.5b00270
Biochemistry 2015, 54, 3814–3821

Table 2. Kinetic Constants for the Activation of the Natural Substrate, FX, and Interaction with FVIIIa by FIXa and Its Mutant Derivatives[a]

	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (nM^{-1} min^{-1})	$K_{d(app)}$ for FVIIIa (nM)
FIXa-WT	44 ± 2.1	90 ± 3.8	2.0 ± 0.18	13 ± 1.5
FIXa-G317R	142 ± 12.5	0.007 ± 0.0004	(4.9 ± 0.7) × 10^{-5}	22 ± 2.3
FIXa-G317E	125 ± 7.8	0.008 ± 0.0003	(6.4 ± 0.6) × 10^{-5}	20 ± 0.9

[a]The apparent K_m and k_{cat} values for activation of FX were determined from the saturable concentration dependence of the rate of FXa generation by each FIXa (0.1−25 nM) in complex with FVIIIa (50 nM) on PC/PS vesicles (50 μM) in TBS/Ca^{2+} for 2−30 min at room temperature as described in Experimental Procedures. The kinetic values are derived from panels C and D of Figure 2. The apparent dissociation constants for the interaction of FVIIIa with FIXa and its mutants were determined by the same FXa generation assay from the saturable concentration dependence of FVIIIa (1−80 nM) using a fixed concentration of FX (200 nM). The values are derived from panels A and B of Figure 2. Values are the average of at least three measurements ± standard deviations.

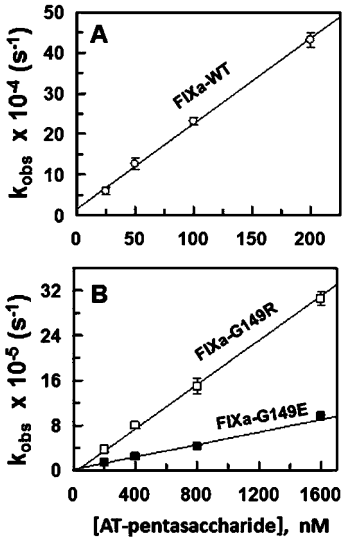

Figure 3. Dependence of the pseudo-first-order rate constant (k_{obs}) for the AT−pentasaccharide inhibition of FIXa and its mutant derivatives. (A) k_{obs} values for the inactivation of 5 nM FIXa-WT (○) at different concentrations of the AT−pentasaccharide complex (*x*-axis) were determined as described in Experimental Procedures. (B) Same as panel A except that k_{obs} values for the inactivation of 20 nM FIXa-G317R (□) or FIXa-G317E (■) by increasing concentrations of the AT-pentasaccharide complex were determined. The k_2 values were determined from slopes of the fitted straight lines and are listed in Table 3. Chymotrypsin numbering is used within panel B.

Table 3. Kinetic Constants for the AT Inhibition of FIXa and Its Mutant Derivatives in the Absence and Presence of Pentasaccharide[a]

	$k_{2,uncat}$ (×10 M^{-1} s^{-1})	$k_{2,penta}$ (×10^2 M^{-1} s^{-1})	acceleration $k_{2,penta}/k_{2,uncat}$ (*x*-fold)
FIXa-WT	5.2 ± 0.1	250.0 ± 30	480 ± 67
FIXa-G317R	0.5 ± 0.04	1.5 ± 0.2	30 ± 6
FIXa-G317E	0.4 ± 0.02	0.5 ± 0.05	12 ± 2

[a]The protease inactivation rates in the absence of a cofactor ($k_{2,uncat}$) were determined from the residual activities of FIXa and its mutants (20 nM) after incubation at room temperature with AT (0.5−2.0 μM) for 2−8 h in TBS buffer, containing 5 mM $CaCl_2$, 1 mg/mL BSA, and 0.1% PEG 8000, as described in Experimental Procedures. The $k_{2,penta}$ values were determined by the same procedures except that 5 nM enzyme was incubated with 25−1600 nM AT for 5 min to 8 h in the presence of 2 μM pentasaccharide. The k_2 values were determined from the slopes of linear plots of k_{obs} vs the concentration of AT or the AT−pentasaccharide complex as shown in Figure 3. Values are the average of at least three measurements ± standard deviations.

were evaluated by thrombin generation (TG) in pooled plasma prepared from two different severe hemophilia B patients as we have determined by clinical and laboratory tests in our hospital. We used a commercial kit and utilized TF concentrations of either 1 or 5 pM to initiate the clotting cascade. In the absence of exogenously added FIX, no detectable thrombin generation was observed if TG was initiated with a low (1 pM) concentration of TF (data not shown). However, when the FIX-deficient plasma was supplemented with a physiological level of FIX or its mutants, while the wild-type zymogen yielded normal TG at 1 pM TF, neither mutant showed any activity at this low TF concentration. Nevertheless, when plasma clotting was initiated with 5 pM TF, both FIX and its mutants exhibited normal TG (Figure 4A), suggesting that the assay is not sensitive to discrimination of the clotting defect caused by FIX mutations under these conditions. Therefore, the TG assay with 5 pM TF was conducted in the presence of 0.1 unit/mL heparin. Interestingly, the TG assay was sensitive in discriminating the thrombin generation levels between different forms of FIX under these conditions (Figure 4B). Thus, while nearly similar clotting parameters (ETP and Peak) were obtained for both wild-type FIX and FIX-G317E in this test in the presence of heparin, the clotting parameters for the FIX-G317R mutant were significantly diminished (Figure 4B).

■ DISCUSSION

Mutation database analysis of the FIX gene suggests that mutations involving Gly-317 of the catalytic domain are associated with different degrees of bleeding severity in hemophilia B patients depending on the nature of the amino acid substitution.[11,13] Among three types of substitutions reported for Gly-317 (Trp, Arg, and Glu), patients carrying the Trp or Arg substitution exhibit severe bleeding, whereas the Gly-317 to Glu substitution causes moderate bleeding.[13,21] To investigate the molecular basis for the variable clinical manifestation of bleeding in hemophilia B patients carrying Gly-317 mutations, we expressed two mutants of FIX in which Gly-317 was substituted with either Arg or Glu. Following purification to homogeneity, we characterized the zymogenic and enzymatic properties of the FIX mutants in established coagulation assay systems. The results suggest that both Arg and Glu substitutions dramatically impair the catalytic function of FIXa mutants toward the natural substrate FX in the intrinsic Tenase complex, accounting for the bleeding observed in patients carrying these mutations. However, the mutagenesis also modestly improved the k_{cat} of G317E activation by the FVIIIa−TF complex. This was evidenced by the observation that FIX-G317E was activated by the extrinsic Tenase complex with a catalytic rate ~2-fold higher than those of both FIX-WT

DOI: 10.1021/acs.biochem.5b00270
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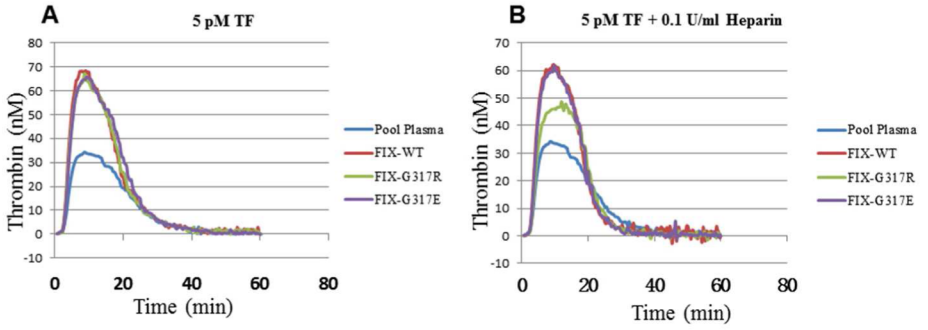

	Pooled plasma	FIX-WT	FIX-G317R	FIX-G317E
Lagtime (min)	3.0	2.9	3.0	3.0
ETP (nM*min)	628	1077	957	1020
Peak (nM)	34.3	68.4	67.2	65.7

	Pooled Plasma	FIX-WT	FIX-G317R	FIX-G317E
Lagtime (min)	3.0	2.9	3.0	3.0
ETP (nM*min)	628	902	765	878
Peak (nM)	34.3	62.3	48.8	61.9

Figure 4. Assessment of thrombin generation (TG) in the absence and presence of heparin in FIX-deficient plasma supplemented with recombinant FIX and its mutant derivatives. Citrated plasma (80 μL), supplemented with different forms of FIX (5 μg/mL) was incubated with 20 μL of PPP reagent in the absence (A) and presence (B) of 0.1 unit/mL heparin, and TG was analyzed as described in Experimental Procedures.

and FIX-G317R. Moreover, relative to the effect of the G317R mutation, the G317E mutation had a more dramatic effect on abolishing the susceptibility of the mutant protease to inhibition by the activated conformer of AT as evidenced by a 500-fold slower reactivity of FIXa-G317E with AT in the presence of pentasaccharide. Assuming that the binding of AT to anticoagulantly active glycosaminoglycans found in vivo is critical for the serpin inhibition of the vitamin K-dependent procoagulant proteases as has been hypothesized,[22,23] Gly-317 mutagenesis can have a dramatic effect in increasing the half-life of the mutant protease in circulation. These results suggest that the improved activation of the G317E zymogen variant by the extrinsic Tenase complex, together with a dramatically impaired reactivity of the FIXa-G317E mutant enzyme with AT, may account for the less severe bleeding phenotype of hemophilia B patients carrying this mutation. This hypothesis is consistent with the TG test presented in Figure 4 demonstrating that the clotting activity of exogenously added FIX-G317R in the FIX-deficient plasma is significantly lower than that of FIX-G317E if the TG assay is initiated with TF in the presence of a low concentration of heparin. It is worth noting that among seven reported hemophilia B patients carrying Gly-317 substitutions, five of them carry the less severe Glu[11,13,21,24] and the other two carry the severe Trp or Arg mutations.[13,21]

Structural data suggest that Gly-317 is located on an exposed surface loop termed variable loop 4 or the autolysis loop on the catalytic domain of FIX/FIXa.[25,26] It has become apparent in recent years that residues of this loop play key roles in determining the macromolecular substrate and inhibitor specificity of FIXa and other coagulation proteases.[18,27] The autolysis loop of FIX, consisting of residues 312−323 (143−154 in chymotrypsinogen numbering[14]), has three basic residues, Arg-312, Lys-316, and Arg-318, which play essential roles in the catalytic function of FIXa in its reaction with the physiological substrate, FX, and with the only serpin inhibitor, AT.[18] Previous results have demonstrated that both Arg-312 and Lys-316 of the autolysis loop of FIXa may directly interact with FX in the intrinsic Tenase complex in a manner independent of FVIIIa.[18,27] In a previous study, we also demonstrated that the Ala substitution mutant of Arg-318 does not significantly alter the interaction of the FIXa mutant with its natural substrate FX; however, it was discovered that this residue makes a key contribution to the interaction of protease with AT, in particular with the heparin-activated conformation of the serpin.[18] Thus, the basic residues of the autolysis loop of FIXa are directly involved in interaction of the protease with both the physiological substrate and the inhibitor. In light of such a critical role for the autolysis loop in FIXa, Gly-317 appears to play an important structural role in rendering high flexibility for this loop, thereby facilitating the dynamic interaction of the basic residues of the FIXa loop with its target macromolecules. Because of the small size and the fact that only one H atom is its side chain, the Gly residue can have a conformation with a greater degree of freedom, a structural feature that is suitable for the active-site pockets of allosteric enzymes because it can provide more flexibility for adjacent residues involved in the catalysis.[28] Gly-317 is strategically located between the two functionally critical basic residues, Lys-316 and Arg-318, in the autolysis of FIXa (Figure 5). This

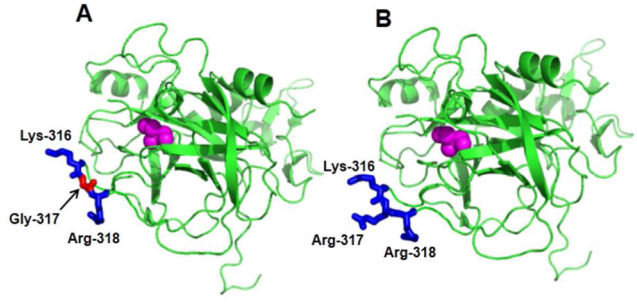

Figure 5. Crystal structure of the catalytic domain of FIXa in complex with *p*-aminobenzamidine. (A) Three autolysis loop residues of FIXa, Lys-316 (blue), Gly-317 (red), and Arg-318 (blue), are shown. The catalytic residue Ser-195 is colored magenta. (B) Same as panel A except that the wild-type Gly-317 residue is substituted with Arg (colored blue). The coordinates (Protein Data Bank entry 1RFN) of the C-terminal catalytic domain of FIXa were used to prepare this figure.[26]

DOI: 10.1021/acs.biochem.5b00270
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configuration allows these residues to adopt optimal conformations to effectively recognize and interact with FX and AT. Thus, substitution of this residue with bulky hydrophobic and/or charged residues in the natural variants of FIXa appears to impose unfavorable steric effects and/or restrict the flexibility of the loop, thereby locking it in a nonproductive conformation incapable of interacting with complementary sites of the target macromolecules.

Unlike its important role in the catalytic function of FIXa, the observation that the activation of the mutant FIX zymogens was not significantly altered suggests that the residues of the autolysis loop may not play a significant role in zymogen recognition by either of the physiological activators, FXIa or the FVIIa−TF complex. However, it was interesting to note that the k_{cat} of FIX-G317E activation by the FVIIa−TF complex specifically was improved ∼2-fold, suggesting that the negative charge of this residue may facilitate more efficient recognition of the mutant zymogen by the extrinsic Tenase complex, possibly accounting for the less severe bleeding phenotype of hemophilia B patients carrying this mutation. Furthermore, the loss of AT reactivity with the G317E substitution mutant of FIXa was also more dramatic than that of the G317R mutation. Thus, in addition to improved zymogen activation, a longer half-life for the FIXa-G317E mutant in plasma may also contribute to the less severe bleeding phenotype of hemophilia B patients carrying the G317E mutation. Results of the TG test in the presence of a low concentration of heparin (Figure 4B) support this hypothesis.

AUTHOR INFORMATION

Corresponding Authors

*Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, No. 197 Ruijin Second Road, Shanghai 200025, China. Telephone: 86 21 54667770. Fax: 86 21 64333548. E-mail: wangxuefeng6336@hotmail.com.

*Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1100 S. Grand Blvd., St. Louis, MO 63104. Telephone: 314 977-9240. Fax: 314 977-9205. E-mail: rezaiear@slu.edu.

Funding

This work was supported by grants awarded by the National Heart, Lung, and Blood Institute of the National Institutes of Health (HL 101917 and HL 62526 to A.R.R.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Audrey Rezaie for proofreading the manuscript.

ABBREVIATIONS

FIX, factor IX; FIXa, activated FIX; FIX-G317R and FIX-G317E, FIX mutants in which Gly-317 has been substituted with an Arg and a Glu, respectively; FVIIa, activated factor VII; FX, factor X; FXa, activated FX; FXIa, activated factor XI; FVIIIa, activated factor VIII; TF, tissue factor; AT, antithrombin; PC, phosphatidylcholine; PS, phosphatidylserine; SpFXa, Spectrozyme FXa; CBS 31.39, chromogenic substrate for FIXa; PPP, platelet poor plasma; PEG, polyethylene glycol; BSA, bovine serum albumin.

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