Characterization of a Factor IX Variant With a Glycine to Glutamic Acid Mutation

By Shu-Wha Lin, Chia-Ni Lin, Nobuko Hamaguchi, Kenneth J. Smith, and Ming-Ching Shen

Factor IX<sub>Taipei</sub> is a factor IX variant from a hemophilia B patient with reduced levels of circulating protein molecules (cross-reacting material, reduced, CRM<sup>−</sup>). This variant contained a glycine (Gly) to glutamic acid (Glu) substitution at the 207th codon of mature factor IX. The functional consequences of the Gly→Glu mutation in factor IX<sub>Taipei</sub> (IXG207E) were characterized in this study. Plasma-derived IXG207E exhibited a mobility similar to that of normal factor IX on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its specific activity was estimated to be 3.5% that of the purified normal factor IX in a one-stage partial thromboplastin time assay (aPTT). Cleavage of factor IXG207E by factor XIa or factor Vlla-tissue factor complex appeared to be normal. When the calcium-dependent conformational change was examined by monitoring quenching of intrinsic fluorescence, both normal factor IX and IXG207E exhibited equivalent intrinsic fluorescence quenching. Activated factor IX<sub>Taipei</sub> (IXaG207E) also binds antithrombin III equally as well as normal factor IXa. However, aberrant binding of the active site probe p-aminobenzamidine was observed for factor XIa-activated factor IXG207E, indicating that the active site pocket of the heavy chain of factor IXG207E was abnormal. Moreover, the rate of activation of factor X by factor IXG207E, as measured in a purified system using chromogenic substrates, was estimated to be 1/40 of that of normal factor IXa. A computer-modeled heavy-chain structure of factor IXa predicts a hydrophobic environment surrounding Gly-207 and this Gly forms a hydrogen bond to the active site serine-365. The molecular mechanism of the Gly→Glu mutation in factor IX<sub>Taipei</sub> might result in the alteration of the microenvironment of the active site pocket which renders the active site serine-365 inaccessible to its substrate.

From the Graduate Institute of Medical Technology, National Taiwan University, School of Medicine, Taipei, Taiwan, Republic of China; Glaxo Research Institute, Research Triangle Park, NC; and the Department of Pathology and Medicine, University of New Mexico, Albuquerque, NM.

Submitted December 27, 1993; accepted May 18, 1994.

Supported by grants to S.W.L. from the National Science Council, Republic of China on Taiwan, Grants No. NSC81-0412-B002-44, NSC82-0412-B002-M02-154, and NSC83-0412-B002-136-M02.

Address reprint requests to Shu-Wha Lin, PhD, Graduate Institute of Medical Technology, National Taiwan University, School of Medicine, Taipei, Taiwan, Republic of China.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Patient. Factor IX<sub>Taipei</sub>, a Chinese man born and raised in Taiwan, is a hemophilia B patient with severe bleeding episodes. The
level of his circulating factor IX (IXG207E), as measured by immuno-electrophoresis (Stago, Paris, France), was estimated to be 30% to 40% of normal (a CRM phenotype). The clotting activity of factor IX was less than 1%, as measured by one-stage partial thromboplastin time assay (aPTT) (Behring, Marburg, Germany). The other clotting factors of factor IX (eg, factor X, factor VII, factor V, and prothrombin), were estimated to be 73%, 70%, 92%, and 92%, respectively (Behring). Patient's prothrombin time (PT) was normal when measured with human thromboplastin (Thromborel S, Behring, Germany). But his plasma prolonged the PT in a thrombotest assay or one stage PT using bovine brain thromboplastin (41 seconds, compared with 29 seconds from normal controls). This Bm phenotype might be caused by the lower than normal levels of factor VII (70%) in his plasma.

The molecular defect of factor IXG207E has been determined previously by the polymeerase chain reaction combined with direct sequencing. A single nucleotide transition (guanine to adenine) at residue 30,073 was identified for this factor IX variant, causing a glycine (G) to glutamic acid (E) substitution at the 207th codon (IXG207E).

**Purification of factor IX (or IXG207E) and enzyme-linked immunosorbent assay (ELISA).** Collection of patient's plasma was performed after appropriate informed consent at a time when the patient had not been transfused with factor IX for at least 4 weeks. Plasma factor IX was purified by binding to a diethyl aminoethyl-sepharose CL-6B and a metal ion-dependent antifactor IX monoclonal antibody (MoAb; designated A-7, which recognizes the Gla domain) columns as previously described. Fractions containing factor IX were determined by ELISA using monoclonal antifactor IX antibodies A-4 and peroxidase-conjugated A-1 or A-7. Peak fractions were pooled and concentrated with an Amicon microconcentrator (Centricon 30). A-4 recognizes the heavy chain of factor IXA. A-1 recognizes the activation peptide. The absorbance of ELISA was measured as an ELISA plate reader (Molecular Devices, Menlo Park, CA).

**Polyacrylamide gel electrophoresis and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described. After electrophoresis, proteins were visualized by staining with Coomassie brilliant blue, or silver-nitrate (Silver stain kit, Bio-Rad Laboratories, Richmond, VA), or subjected to immunoblotting using monoclonal antifactor IX antibodies.

**Activation of factor IX by factor Xa.** Human factor Xa was a gift from D. Monroe (Department of Medicine, University of North Carolina at Chapel Hill). The activation of normal factor IX or factor IXG207E by human factor Xa in the presence of 5 mmol/L CaCl2 was performed at enzyme to substrate ratios of 1:20, 1:40, 1:80, 1:120, and 1:160 (wt:wt). The final concentration of factor IX (or factor IXG207E) was 1.14 to 1.5 nmoVL. Aliquots from the reaction mixtures were also removed at timed intervals and analyzed by SDS-PAGE.

**Antithrombin III (ATIII) binding.** Approximately 0.1 to 0.3 ng of factor Xa-activated factor IX or IXG207E (designated IXG207E) was incubated with 0.1 ng of ATIII (Sigma Chemical Co, St Louis, MO) in the presence of heparin for 10 minutes before SDS-PAGE analysis under nonreducing conditions. The gels were silver stained or electrotransfered and immunoblotted with antifactor IX or antihuman ATIII antibodies.

**Factor X activation kinetics.** Activation of factor X by factor Xa (or IXaG207E) was analyzed by monitoring kinetically the sequential hydrolysis of a chromogenic substrate (Spectrozyme Xa; Sigma) at 37°C in a spectrophotometer equipped with a thermal controller (Hitachi U-3000; Hitachi, Tokyo, Japan). The concentration of the enzymes was 4 nmol/L or 250 nmol/L. In addition, the reaction mixtures contained 600 nmol/L of factor X (Enzyme Research Laboratory, South Bend, IN), 300 nmol/L Spectrozyme Xa, 10 mmol/L CaCl2, and 12 μL phospholipids (Behring). The reaction rate was calculated according to the following equation: ΔAbs = aV + Vo, where a is the acceleration rate, ΔAbs is the absorbance at 405 nm minus the initial value (Abs0), and V0 is the rate of Spectrozyme Xa hydrolysis in the absence of factor X. Calculation was performed using the SAS program (SAS Institute, Cary, NC).

**Molecular modeling.** To predict the structure of a zymogen form of factor IX, a factor IXa model was modified at the activation site (residues 181 and 182) and residues in contact with it based on an x-ray crystallographic structure of trypsinogen (PDB1TGC.ENT). This model was further modified to create a model for factor IXG207E, where its Gly at the 207th position was replaced by Glu. Both models were energy minimized using AMBER 3.1A to reduce unfavorable contacts introduced by changes. All calculations and molecular dynamics were performed as described.

**RESULTS**

**Biochemical characteristics of factor IXG207E.** Because the antigen level of the patient's circulating factor IX was 30% to 40% of normal, purification of enough quantities of factor IXG207E was aided by binding of the plasma zymogen to a metal ion-dependent conformational specific antifactor IX antibody column that has been used previously to isolate normal and abnormal factor IX. Isolated factor IXG207E, when analyzed by SDS-PAGE under reducing condition, ran as a single band with a mobility similar to that of normal factor IX purified by the same procedure (Fig 1A). Factor IXG207E was also recognized by several monoclonal antifactor IX antibodies A-1 (recognizing the activation peptide), A-4, and A-5 (both recognizing the heavy chain of factor IX), as shown by ELISA and by immunoblotting (Fig 1B). This indicates that the global structure of the protein molecules are macroscopically similar to plasma-derived normal factor IX. However, purified factor IXG207E molecules were not functionally active as measured by the one-stage aPTT assays using factor IX-deficient plasma. Its specific activity was to be 3.5% that of purified normal factor IX.

**Interaction of factor IXG207E with factor Xla and factor VII:TF complex.** The activation of normal and abnormal factor IX molecules by factor Xa was performed in the presence of CaCl2. Different ratios of enzyme to substrate concentrations (wt:wt) were tested and the time course was
Fig 1. SDS-PAGE and Western blotting analysis of purified factor IX and IXG207E. Purified human factor IX (lane 1) and factor IXG207E (lane 2) were subjected to SDS-PAGE followed by staining with silver nitrate (A) or immunoblotting (B). The antibody used was A-1 recognizing the activation peptide of factor IX. Lane M is the molecular-weight marker.

followed. Figure 2 depicts the activation by factor XIa at an enzyme to substrate ratio of 1:120. As shown on panel B, the signals for the intact factor IX and IXG207E decreased with a concomitant increase of signals for both the light and heavy chains. Activation by factor VIIa-TF complex yielded similar results. It appears that the mutation at the 207th codon in factor IXTa,iF,c does not affect its activation by factor XIa or factor VIIa-TF complex.

Fluorescence quenching. The ability of factor IXG207E to undergo a metal-induced conformational transition was evaluated by monitoring the effects of calcium on the quenching of intrinsic fluorescence. Titration of IXG207E with calcium resulted in a decrease in fluorescence that was similar to that observed with native factor IX (Fig 3). Half-maximal transitions were observed at about 0.6 mmol/L calcium. The result suggests that in the presence of increasing concentrations of calcium, factor IXG207E is able to undergo the conformational change monitored by the quenching of intrinsic tryptophan fluorescence.

Structural analysis of factor IXG207E-Interaction of p-aminobenzamidine. Binding of p-aminobenzamidine to the active sites of activated factor IX (IXa) or IXG207E (IXaG207E) were examined by monitoring the change in fluorescence of p-aminobenzamidine upon activation of both proteins by factor XIa.20 The extent of exposure of the active site is proportional to the relative increase in fluorescence. The experiments were performed in the presence of human serum albumin as a stabilizer that had been determined to have no effect on the results (unpublished results). As shown in Fig 4A, whereas normal factor IX, upon activation by factor XIa, binds p-aminobenzamidine normally and exhibits an increase in fluorescence intensity, there was no increase in the level of fluorescence over the time course shown, and therefore, no exposure of a “normal” active site of IXaG207E. Both proteins were cleaved by factor XIa normally (Fig 4B) throughout the fluorescent measurement. Failure to bind p-aminobenzamidine by IXaG207E suggests an abnormal conformation of the active site pocket.

Fig 2. Activation by factor XIa. The activation by factor XIa was performed in the presence of CaCl₂. Aliquots of 1.2 μg of each protein were activated by factor XIa in the presence of 5 mmol/L CaCl₂ at a substrate to enzyme ratio of 120:1. At each time point, samples were withdrawn and added to a solution of 1% SDS, 5 mmol/L EDTA, and 4% β-mercaptoethanol, boiled and then analyzed by SDS-PAGE. After electrophoresis, the proteins were visualized by silver staining.

Fig 3. Fluorescence quenching. The fluorescent changes of the intrinsic aromatic residues of normal factor IX or IXG207E in the presence of different concentrations of calcium were measured. The amount of the purified protein molecules was 14 μg/mL.
CHARACTERIZATION OF FACTOR IX

Fig 4. Activation of factor IX and IXG207E in the presence of p-aminobenzamidine. (A) The activations of both proteins by factor Xla in the presence of 5 mmol/L CaCl₂ were performed at 37°C as described in the legend to Fig 2. Excitation was at 336 nm and emission was monitored at 376 nm using a Jasco fluorescence spectrophotometer equipped with a thermo-controller. (B) Aliquots of the reaction mixtures were withdrawn at timed intervals and subjected to SDS-PAGE followed by staining with silver nitrate.

Structural analysis of factor IXG207E-Binding to antithrombin III. To examine the substrate and/or inhibitor binding pocket of IXaG207E, activated IXG207E was reacted with ATIII that forms a covalent bond to the heavy chain of factor IXa at 1:1 ratio in the presence of heparin. As shown in Fig 5, a complex of molecular weight 110 kDa was observed at three different concentrations of factor IXa (A) and IXaG207E (B), suggesting that IXaG207E was able to bind ATIII.

Kinetics of activation of factor X. The ability of IXaG207E to cleave its macromolecular substrate, factor X, was examined in an attempt to obtain the experimental kinetic values of IXG207E. Two different concentrations (4 nmol/L and 250 nmol/L) of factor IXa and IXaG207E were incubated with factor X at 37°C over 6 hours in the presence of phospholipids, calcium ions, and the chromogenic substrate for factor X, Spectrozyme Xa. As shown in Fig 6, over the time course studied, IXaG207E was unable to activate factor X. The calculated reaction rate for normal factor IXa at 250 nmol/L was $6.1 \times 10^{-5}$ min⁻¹ and for IXaG207E, $1.4 \times 10^{-4}$ min⁻².

DISCUSSION

The purpose of this study was to investigate the interactions between a naturally-occurring factor IX variant and the other components in the coagulation system, and to analyze the mechanisms by which the mutation results in dysfunction of the protein. Factor IXaG207E was investigated for several
reasons. First, although its level in circulation was 30% to 40% of normal, the abnormal protein is obtainable. Secondly, its mutation was at evolutionally conserved glycine residue that is found at the 207th codon of factor IX and comparable positions of several other serine proteases. These include a number of related coagulant proteins such as factors VII, X, prothrombin, and protein C, as well as trypsin and chymotrypsin. It is inferred that Gly-207 is structurally and/or functionally important. Moreover, whereas mutations at the amino- and carboxyl-termini of the catalytic domain of factor IX have been studied extensively, mutations at and around this region (encoded by the 6th exon) have not been analyzed at the level of the protein. Our biochemical findings show that mutant factor IXG207E had a normal electrophoretic mobility on the SDS-PAGE, and reacted normally with at least 4 different MoAbs (A-1, A-4, A-5, and A-7) for different regions of factor IX. The protein also bound calcium and was able to undergo subsequent conformational changes. Cleavage of factor IXG207E appeared to be normal by factor Xa or VIIa-TF complex, using either TF from human or bovine thromboplastin (data not shown). Therefore, one can assume that the overall structure of factor IXG207E was not affected by the substitution of glutamic acid at 207. However, the molecule was unable to bind p-aminobenzamidine. Functional analysis showed that factor IXG207E was a poor enzyme with a reaction rate of one fortieth of that of normal factor IXa with respect to its physiologic substrate, factor X. Based on what has been established for trypsin and chymotrypsin, the critical event during activation of the serine proteases is the formation of the ionic interaction between their positively-charged amino termini and a critical aspartic acid residue (Asp-364 corresponding to Asp-194 of chymotrypsin) to generate the substrate binding pocket (Ser-384 to Gly-386 and Asp-359 to Gln-362 in factor IX). In the case of factor IX, conformational rearrangement occurs upon activation by factor Xla or the factor VIIa-TF complex. Assuming similarity of the activation mechanism with the other serine proteases, the newly-generated amino terminal Val-181 would pass through the opening of the activation site pocket to form a salt bridge with the negatively-charged carboxyl group of Asp-364. This rearrangement makes the active site of factor IXa accessible to its physiologic substrate, factor X, the active site inhibitor, p-aminobenzamidine, and to the macromolecular inhibitor, ATIII. Based upon the structural conservation of the serine proteases, we have used the crystal structures of trypsin and other serine proteases to generate a three-dimensional homology model for the heavy chain of factor IXa (Fig 7A). We have used the model to predict the spatial relationships of the catalytic triad of both normal and mutant factor IXa molecules and to estimate the effect of the mutation at a molecular level. Three-dimensional models of factor IX have been useful to predict the effects of point mutations in several studies. As depicted in Fig 7, Gly-207 is in the hydrophobic core, and its amide proton interacts with the carbonyl oxygen of the active site Ser-365 (at the edge of the active site pocket) through hydrogen bonding (bond length of 2.8 Å; see Fig 7A). Contacts also occur between Asp-364 (194 in chymotrypsin) and Val-181 (residue 1 in chymotrypsin), Ser-319 (residue 139), Ala-320 (residue 140), and Gln-362 (residue 182).
Fig 7. Molecular modeling. A factor IXa model was modified at the activation site (residues 181 and 182) and the residues in contact with the activation site, based on the x-ray crystallographic structure of bovine trypsinogen (PDB1TGC.ENT). Both the wild-type and 207 mutation models were energy minimized using AMBER 3.1A to reduce unfavorable contacts introduced by changes. (A) The structure of the catalytic domain of factor IXa was predicted by this model. The backbone of factor IXa’s heavy chain is shown as a ribbon. The catalytic triad residues, Ser-365 (195), Asp-269 (102), and His-221 (57) are shown with side chains. Residue 207 was shown as glycine as in a normal factor IX molecule. A white dotted line indicates a hydrogen bond between the carboxyl group of Gly-207 and the amino group of Ser-365 with its distance (2.8 Å) between the heavy atoms. His-315 is also shown to indicate the position of surface loop whose deletion was shown to have a significant effect on ATIII binding in thrombin. (Note: the deleted residues in thrombin des-ETW were not conserved among serine proteases.) (B) A model depicting binding of p-aminobenzamidine. Yellow depicts disulfide bonds. The numbering system of factor IX is shown. Dotted area denotes p-aminobenzamidine. In both panels, oxygens are red, nitrogens are blue, and carbons are green.
The mechanism of the defect caused by the mutation at residue 207 is possibly caused by local conformational change. The retained affinity toward the MoAbs against the catalytic domain indicates no significant global change in the structure. The energy-minimized structure of the homology-built mutant molecule showed that the hydrogen bond between the amide proton of residue 207 and the carbonyl oxygen of active site residue Ser-365 (residue 195) is conserved in factor IXG207E. However, the hydroxyl group of serine was displaced 2Å away from the comparable position in our normal factor IX model. Further examination of this model indicated that introduction of a bulky and negatively charged amino acid at position 207 will also likely affect the conformation of an adjacent loop (residues 362 to 365), and this slight change is likely to be reflected in the catalytic activity. Another possibility is that the structural change caused by this mutation affects an electrostatic interaction between Asp-364 (residue 194) and Val-181 (residue 1). This interaction occurs upon the activation (the cleavage between residues 180 and 181), which was displaced 2Å away from the comparable position in our normal factor IX model. Further examination of this model indicated that introduction of a bulky and negatively charged amino acid at position 207 will also likely affect the conformation of an adjacent loop (residues 362 to 365), and this slight change is likely to be reflected in the catalytic activity. Another possibility is that the structural change caused by this mutation affects an electrostatic interaction between Asp-364 (residue 194) and Val-181 (residue 1). This interaction occurs upon the activation (the cleavage between residues 180 and 181), which is required for the correct rearrangement of the active site to obtain the catalytic activity. This electrostatic interaction was disrupted in the model of the mutated molecule during the energy minimization. It should be mentioned that the negative charge introduced at residue 207 was not stabilized in the mutant structure. Thus, p-aminobenzamidine failed to monitor the formation of the active site pocket, indicating the activation process did not result in the correct conformation of this pocket. The results from the ATIII binding experiments appear to indicate that a sufficient conformational change occurred in factor IXG207E upon activation that allowed ATIII to bind. However, the molecule failed to bind p-aminobenzamidine and was not able to activate factor X. Several other mutant factor IX molecules have been shown to behave like factor IXG207E in this regard, that they bind ATIII, but not factor X or p-aminobenzamidine. It is likely that the requirement for binding factor X is not exactly the same as that for binding ATIII. The interaction between factor IXa and factor X is very specific, whereas ATIII, which is less specific for it, is able to form a complex with factor X, factor IX, and thrombin. Whether the structures involved in the ATIII binding are tightly conserved among these three proteins is questionable. A study by Le Bonniec et al showed that a thrombin variant (des-ETW) deleting three amino acid residues (146 through 148) (thrombin des-ETW) failed to bind ATIII efficiently. However, the three amino acids are not conserved among the clotting factors, suggesting a distinct region for ATIII binding in thrombin. Therefore, it is reasonable to predict that factor IX’s binding of factor X and ATIII might not be totally identical.

The ability of factor IXG207E to prolong the ox brain PT, a Bm phenotype, was found to be caused by a lower (70%) factor VII level in the patient. Purified factor IXG207E did not inhibit the ox brain PT (data not shown). Therefore, it is not likely that factor IXG207E is a Bm variant. This is consistent with the notion that mutations associated with hemophilia Bm are usually found in the activation pocket region or at the second cleavage site of factor IX (residues 180 and 181). Moreover, the patient’s older brother, who is also a hemophilia B patient, had a normal level of factor VII and did not have a prolonged ox brain PT. Interestingly, the older brother is a moderate hemophilia B who does not require infusion therapy as often as his younger brother. It is not clear that the lower level of factor VII contributes to the clinical severity of the younger brother.

According to the annual database for hemophilia B, four independent mutations including factor IXa were found at the 207th codon. Arginine is found in two cases (UK43 and Luanda), a stop codon in one (unnamed), and glutamic acid in factor IXa. All four patients suffer from severe hemophilia B with clotting activities of 1% (Luanda) or less (the other three). The antigen levels of the two Arg mutants are not reported and that of the stop-codon mutant was less than 1%, which was predictable (CRM). However, the patient with factor IXa has an antigen level of 30% to 40% (CRM), as does his brother who also suffers from moderate hemophilia B. It is not clear if replacement of Gly-207 by Arg with a positively charged side chain would result in the same CRM phenotype as the Glu mutation in factor IXa. Although factor IXG207E tended to be degraded to a higher extent than normal factor IX during purification, how the Glu mutation contributes to the reduced antigen levels of factor IX in the patient is unclear. Using an in vitro expression system, one might be able to address the question of whether the reduced antigen level in factor IXG207E is caused by increased susceptibility of factor IXG207E to proteolysis or to increased intracellular turnover, and to determine whether differences in the substituted amino acids at this position will affect the CRM phenotypes.

ACKNOWLEDGMENT

The authors would like to thank Drs David Straight and Paul Charlison for reading the manuscript and Shu-Ying Tang at the Department of Biochemistry for free access to the Jasco fluorescent spectrophotometer. We also thank U-Lei Lao and Chia-Cheng Lee for excellent technical assistance during their summer-training program. We are grateful to Mei-Hua Hung and Tru-Hui Chen for providing clinical information and collection of samples.

REFERENCES

8. Skogen WF, Esmon CT, Cox AC: Comparison of coagulation
CHARACTERIZATION OF FACTOR IX takeaway

factor Xa and Des (1-44) factor Xa in the assembly of prothrombinase. J Biol Chem 259:2306, 1984
30. Nobuko H, Roberts H, Stafford DW: Mutations in the catalytic domain of factor IX that are related to the subclass hemophilia Bm. Biochemistry 32:6324, 1993
34. Freer ST, Kraut J, Robertus JD, Wright HT, Xuong NH: Chymotrypsinogen: 2.5 Å crystal structure, comparison with a-chymotrypsin, and implications for zymogen activation. Biochemistry 9:1977, 1970
39. Bertina RM, Linden IK, Mannucci PM, Reinalda-Poot HH, Cupers R, Poort SR, Reitsma PH: Mutations in hemophilia Bm occur at the Arg180-Val activation site or in the catalytic domain of factor IX. J Biol Chem 265:10876, 1990
Characterization of a factor IX variant with a glycine207 to glutamic acid mutation

SW Lin, CN Lin, N Hamaguchi, KJ Smith and MC Shen