Molecular Defect in Factor IX Tokyo: Substitution of Valine-182 by Alanine at Position P2′ in the Second Cleavage Site by Factor XIa Resulting in Impaired Activation†

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ABSTRACT: Utilizing polymerase chain reaction and directly sequencing the amplified exon 6 of the factor IX gene derived from a mild hemophilia Bm patient, we have identified a T to C mutation at nucleotide 20 525. This point mutation predicted a Val^{182} to Ala substitution in the abnormal factor IX molecule, designated as factor IX Tokyo. The patient manifested a low factor IX activity and a moderately prolonged ox-brain prothrombin time but a normal factor IX antigen level in plasma. Immunopurified factor IX derived from the patient was found to have a normal molecular weight but a reduced specific activity (23% of normal). Limited proteolysis by activated factor XI or by a snake venom-derived factor X-activating enzyme was considerably delayed, indicating the presence of structural alteration(s) most probably at or near the second enzyme-cleavage site. Once activated, however, factor IXa Tokyo was able to activate factor X normally and was inactivated by antithrombin III also in a normal fashion. The structural model of factor IXa and a docking model of factor IX and activated factor VII (factor VIIa) suggested that the Val^{182} to Ala substitution would not affect the local conformation of the catalytic domain. This mutation would rather loosen the fitness of the molecule into the substrate-binding pocket of factor VIIa due to a shorter side chain of the Ala substitution at the P2′ position of the second cleavage site.

Factor IX is a single-chain vitamin K-dependent glycoprotein circulating in plasma as a zymogen of a serine protease and plays an important role in both the extrinsic and intrinsic coagulation pathways (Thompson, 1986). During activation by both factor XIa (intrinsic pathway) and the factor VIIa/tissue factor complex (extrinsic pathway), the two peptide bonds at Arg^{145}–Ala^{146} and Arg^{180}–Val^{181} are cleaved with the release of an activation peptide leaving a disulfided-linked two-chain molecule, factor IXaβ (DiScipio et al., 1978; Østerud et al., 1977). Factor IX is also activated to factor IXaα by a snake venom-derived factor X activator (RVV-X)[1] by the cleavage of the peptide bond at Arg^{180}–Val^{181} (DiScipio et al., 1978). A deficiency or functional defect of factor IX is related to an X-chromosome-linked bleeding disorder known as hemophilia B. To date, the DNA sequence of the factor IX gene has been determined (Yoshitake et al., 1985), and there has been substantial evidence that hemophilia B is due to different types of single point mutations or short deletions or additions in the factor IX gene (Giannelli et al., 1992). Hemophilia Bm characterized by abnormal factor IX with a prolonged plasma ox-brain prothrombin time (Hougie & Twomey, 1967) was found to possess a variety of mutations occurring either near the Arg^{180}–Val^{181} activation site (Suehiro et al., 1989; Huang et al., 1989; Sakai et al., 1989; Bertina et al., 1990; Taylor et al., 1990) or in the catalytic domain (Bertina et al., 1990; Spitzer et al., 1988; Sugimoto et al., 1988).

Here we report an abnormal factor IX, designated as factor IX Tokyo, due to a new type of point mutation adjacent to the activation site of factor IX (Val^{182} to Ala) which resulted in the delayed activation by both factor IXa and RVV-X.

MATERIALS AND METHODS

Patient. Informed consents were given by the patient and his parents for this study. The patient is a 15-year-old boy suffering from persistent hematemesis due to a gastric ulcer, diagnosed by gastrofiberscopy. He and his family had no other history of bleeding disorders, and the diagnosis of hemophilia B had not been made until the time of examination at the age of 15. His plasma contains a normal level of factor IX antigen with low procoagulant activity. The activated partial thromboplastin time was moderately prolonged to 55.1 s (control 34–38 s).

Materials. Factor X, activated factor X (Xa), α-thrombin, and antithrombin III (AT-III) were prepared as described previously (Tanabe et al., 1991), and AT-III was coupled to CNBr-activated Sepharose 4B. RVV-X was purified from a crude venom according to the method as described (Furie & Furie, 1977). Factor XI was purified from fresh frozen human plasma according to Naito and Fujikawa (1991) using a kinin-free high molecular weight kininogen–Sepharose column. Factor XIa was prepared by activating factor XI with β-factor XIIa and purified by FPLC using a Mono S column

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[1] Abbreviations: RVV-X, the factor X-activating enzyme from Russell's viper venom; HRP, horseradish peroxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; AT-III, antithrombin III; BPTI, bovine pancreatic trypsin inhibitor; rmsd, root mean square deviation.

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(Pharmacia, Uppsala, Sweden) as described (Meijers et al., 1988). Factor VIII was obtained from the Chemo-Sero-Therapeutic Institute, Kumamoto, Japan. Factor VIIIa was freshly prepared by activation with thrombin–Sepharose for 10 min at 37 °C. After removal of thrombin–Sepharose, the supernatant was used as factor VIIIa within 15 min. A monospecific rabbit anti-human factor IX antibody and a Ca^{2+}-specific anti-human factor IX monoclonal antibody, JK-IX-2, both coupled to horseradish peroxidase (HRP), were prepared essentially as described previously (Sugo et al., 1990). The absorption coefficients at 280 nm used for calculating the protein concentration were 13.3 for both normal and abnormal factor IX, 11.6 for factor X (DiScipio et al., 1977), and 10.0 for RVV-X (Furie & Furie, 1977). The protein concentrations of normal and abnormal factors IX and IXa and factor XIa were also determined by the method of Bradford (1976). Phospholipid vesicles composed of 25% Folch fraction III and 75% phosphatidylcholine were prepared as described previously (Sugo et al., 1990). Boc-Ile-Glu-Gly-Arg-4-methylcoumarin-7-amide (MCA) and 7-amino-4-methylcoumarin (AMC) were obtained from the Peptide Institute, Osaka, Japan.

Factor IX Assay. The factor IX clotting activity (IX:C) was measured by the partial thromboplastin time, using congenitally factor IX-deficient plasma as substrate. The ox-brain prothrombin time was measured using the Thrombotest reagent (Eisai Co., Tokyo, Japan) as a source of bovine tissue factor. Factor IX antigen (IX:Ag) was determined by a double-antibody sandwich ELISA using a Ca^{2+}-dependent or nondependent monoclonal antibody as the first antibody and a HRP-conjugated rabbit anti-factor IX antibody as the second antibody.

Purification of Abnormal Factor IX. Eighty micrograms of factor IX Tokyo was isolated from 45 mL of the patient's plasma by one-step purification using a Ca^{2+}-dependent JK-IX-2–Sepharose column as described (Sugo et al., 1990). Purified factor IX Tokyo was found to be more than 95% pure by SDS–PAGE stained with Coomassie Brilliant Blue R-250. The same procedure was used to isolate normal factor IX.

Activation of Factor IX by Factor XIa or RVV-X. Both normal factor IX and factor IX Tokyo were activated by incubation at 37 °C with an activation mixture consisting of factor IX (25 μg/mL), RVV-X (0.62 μg/mL) or factor XIa (0.5 μg/mL), and 2.5 mM $CaCl_2$ in 40 μL of 50 mM Tris-HCl and 0.1 M NaCl, pH 7.4. At 0, 0.5, 1, 2, and 16 h, an aliquot of the mixture was withdrawn for SDS–PAGE, followed by immunoblot analysis utilizing a HRP-conjugated rabbit anti-factor IX antibody. The bands were visualized by the peroxidase–carbazole staining. For the preparation of factor IXaβ, 70 μg of factor IX Tokyo was completely cleaved with factor XIa/Ca^{2+} and purified by immunoaffinity chromatography.

Activation of Factor X by Factor IXa. Factor X (0–56 μg/mL) was activated with factor IXa (10 ng/mL) at 37 °C in the presence of factor VIIIa (1.6 units/mL), phospholipid vesicles (10 μg/mL), and 2.5 mM $CaCl_2$ in HEPES buffer (10 mM HEPES, 0.137 M NaCl, 4 mM KCl, 11 mM glucose, and 4 mg/mL bovine serum albumin, pH 7.45). Factor Xa activity generated in the reaction mixture after different incubation times was measured by transferring a 50-μL aliquot to a cuvette containing 200 μL of 0.30 mM Boc-Ile-Glu-Gry-Arg-MCA and 10 mM EDTA in HEPES buffer, and the rate of AMC release was monitored as described (Morita et al., 1977). Factor Xa concentration was determined by using an active-site-titrated reference preparation of factor Xa.

Inhibition of Factor IXa Activity by AT-III. Inhibition experiments by AT-III were performed by using an AT-III–Sepharose gel in the absence or presence of heparin (1 μg/mL). One milliliter of AT-III–Sepharose gel could completely inhibit 0.8 NIH unit of α-thrombin after 30 min of incubation at 23 °C. On the rolling platform, 100 ng of factor IXa was incubated with the suspension of AT-III–Sepharose (5-μL gels) in a total volume of 150 μL of HEPES buffer at 23 °C. At timed intervals, the suspension mixture was centrifuged for 2 min at 3000 rpm, and residual factor IXa activity in 10 μL of the supernatant was assayed by measuring its factor X-activating activity as described above. The rate of factor Xa generation in the absence of AT-III–Sepharose gel was expressed as 100%.

Identification of Point Mutation. For DNA analysis, genomic DNAs were extracted from leukocytes derived from the patient and a normal volunteer using a standard procedure. One microgram of DNA was subjected to polymerase chain reaction by the method of Saiki et al. (1988). Two primers were used to amplify the segment of exon 6 as described previously (Nishimura et al., 1990). They were primer A, ATGTGGACTATGTAAATTCT (nucleotide 20 435–20 454, according to Yoshitake et al., 1985), and primer B, TGAC-CTGGTTTGGCATCTTC (the complementary sequence of nucleotide 20 533–20 552). The amplified DNA fragments were purified by electroelution and subjected to the direct DNA sequencing. For use with the automatic DNA sequencer, model 373A (Applied Biosystems, Foster City, CA), the Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) was used. The fluorecent dye-labeled dideoxynucleotides were identified by the DNA sequencer according to the method described by Kobayashi et al. (1991).

Structural Modeling. The molecular modeling was performed on an NEC EWS 4800/220 computer using the programs BIOCES [E]. The modeling procedures including the alignment of the sequence and modeling of the three-dimensional structure were essentially as described previously (Miyata et al., 1991). The structural refinement by molecular mechanics minimization was done on a Silicon Graphics Personal IRIS 4D/35 using the AMBER 3.0 Revision A united atom force field (Weiner et al., 1984) and programs of KOPT (Kamiya and Umeyama, unpublished). The cutoff distance for nonbonded interaction was 10 Å, and a distance-dependent dielectric constant was used. The effect of the amino acid substitution in factor IXa Tokyo on the conformational change(s) in the catalytic domain of normal factor IXa was analyzed by calculating the root mean square deviations (rmsd) in the positions of the main-chain atoms between normal and abnormal factor IXa. A factor VIIa model was constructed by Dr. Shigetaka Yoneda, Department of Pharmaceutical Sciences, Kitasato University, and the coordinates were kindly provided for our modeling study. The modeling of factor IX zymogen Thr[179]–Thr[415], lacking the NH_2-terminal Tyr[1]–Phe[178] peptide, was based on the high-resolution structures of trypsinogen defined by X-ray crystallography (Bolognesi et al., 1982; Walter et al., 1982). The structures of trypsinogen are entered as the names 1TGS and 1TGT in the Brookhaven Protein Data Bank (Bernstein et al., 1977; Abola et al., 1987). The docking model of factor IX zymogen and factor VIIa was based on the complex structure of bovine trypsin and bovine pancreatic trypsin inhibitor (BPTI) (PDB entry 2PTC; Marquart et al., 1983). The coordinates of the polypeptide backbone of the cleavage site of factor IX (Thr[179]–Val[182]) were initially positioned at the same coordinates as those of the reactive site of BPTI. Then the trypsin model and the BPTI model were replaced by those of factor VIIa and factor IX zymogen, respectively. The docking model was further accommodated to get the maximum congruence in the cavity

Table I: Factor IX Assay and Ox-Brain Prothrombin Time[a]

plasma	factor IX:C (%)	factor IX:Ag (%) total (a)	factor IX:Ag (%) Ca^{2+}-specific (b)	ox-brain prothrombin time (s)
patient	20	98	100	56
mother	60	80	89	42
normal	100	100	100	34

[a] Factor IX antigen was measured by a sandwich ELISA using either a Ca^{2+}-nondependent antibody (a) or a Ca^{2+}-dependent antibody, JK-IX-2 (b), as the first antibody and a HRP-conjugated rabbit anti-factor IX antibody as the second antibody. The binding activity of normal plasma was expressed as 100%.

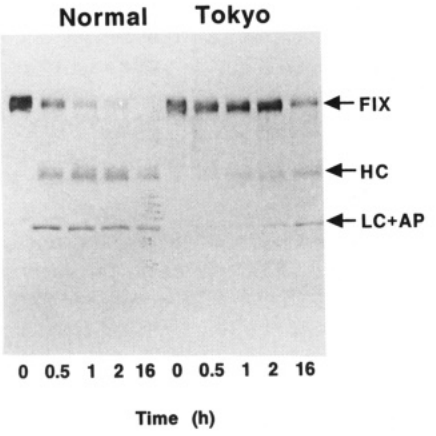

FIGURE 1: Activation of normal factor IX and factor IX Tokyo by RVV-X. Factor IX was incubated at 37 °C with RVV-X at an enzyme/substrate ratio of 1/50 (w/w). At indicated times, aliquots were withdrawn and examined by immunoblot analysis under the reducing conditions described under Materials and Methods. The arrows indicate the positions of the purified heavy-chain peptide (HC) and the light-chain-activation peptide (LC+AP).

of the catalytic site of factor VIIa by positioning the main chain. The three-dimensional structures of the side chains were not determined because of the rough docking process. Mutant factor IX's Tokyo, Cardiff II, and Milano were treated as the substitution of the side-chain coordinates.

RESULTS

Coagulation Studies. The factor IX clotting activities, antigen levels, and ox-brain prothrombin times of the patient's and his mother's plasmas are shown in Table I. Their plasmas contained normal levels of factor IX antigen but reduced coagulant activities. The ox-brain prothrombin time was moderately prolonged in the patient and slightly prolonged in his mother. Addition of 0.2 volume of a monoclonal antibody, Mc-IX (7.5 mg/mL), to the patient's plasma shortened the mild prolongation of the ox-brain prothrombin time (63 to 38 s) when compared to that of normal plasma (38 to 36 s). This indicated that the Bm effect of the patient's plasma is almost completely neutralized by an anti-factor IX antibody and that the factor IX Tokyo molecule was related to the mild prolongation of the ox-brain prothrombin time of the patient's plasma.

Abnormalities of Factor IX Tokyo. Since the patient's factor IX could bind to the Ca^{2+}-dependent antibody as normal factor IX, we applied immunoaffinity chromatography utilizing JK-IX-2–Sepharose to purify the abnormal protein. Purified factor IX Tokyo had a normal molecular weight (67 000) but a reduced specific clotting activity at only 23% of normal. Although factor IX Tokyo was resistant to RVV-X/Ca^{2+}, it was eventually converted by the enzyme to an active form of factor IXaα after longer incubation (Figure 1). In the activation of factor IX Tokyo by factor XIa/Ca^{2+} at an enzyme/substrate ratio of 1/50 (w/w), the cleavage of factor IX Tokyo was considerably delayed (Figure 2A). Immunoblot analysis of the reaction products under reducing conditions gave a faint blur band at the position around 35 kDa, indicating the generation of an intermediate peptide, the heavy-chain-activation peptide (Figure 2B). Although not shown here, the intensity of this band increased with time at a higher enzyme/substrate ratio, suggesting that the cleavage at Arg^{145}–Ala^{146} of factor IX Tokyo by factor XIa proceeded normally, while the second cleavage at Arg^{180}–Val^{181} was impaired.

Point Mutation in the Factor IX Tokyo Gene. Since the second cleavage site at Arg^{180}–Val^{181} in factor IX Tokyo was resistant to both enzymes, the abnormality of factor IX Tokyo was expected to reside at or adjacent to the second cleavage site. Therefore, we selected the primers to amplify the segment of exon 6 of the factor IX Tokyo gene that coded the region at issue. Part of the nucleotide sequence determination pattern of the normal DNA fragment (upper panel) and that of the patient's (lower panel) are shown in Figure 3. In the codon for Val^{182}, a point mutation of T to C at nucleotide 20 525 was detected. This transversion changes the codon for Val^{182} to that for an Ala.

Enzymatic Activity of Factor IXaβ Tokyo. Factor IX Tokyo was completely activated by factor XIa/Ca^{2+} at an enzyme/substrate ratio of 1/15 as evidenced by complete disappearance of the native molecule upon SDS–PAGE (24-h incubation), and the resultant factor IXa Tokyo was purified by immunoaffinity chromatography. Purified factor IXa Tokyo was found to have a gross structure similar to that of normal factor IXa (Figure 4, inset). Within the range of enzyme concentrations up to 100 ng/mL, the rate of factor Xa generation was proportional to the concentration of factor IXa, and there was no significant difference between the normal factor IXa and factor IXa Tokyo. The rate of factor X activation at various factor X concentrations was also determined, and the data are presented in the form of Lineweaver–Burk plots (Figure 4). The values of the apparent K_m for factor X and V_{max} in the factor X activation by factor IXa Tokyo fell in ranges similar to those obtained by normal factor IXa [K_m = 0.07 μM; V_{max} = 3.1 × 10^{-7} mol of factor Xa min^{-1} (mg of factor IXa)$^{-1}$]. Furthermore, as shown in Figure 5, factor IXa Tokyo reacted with the native inhibitor, AT-III, normally regardless of the presence or absence of heparin. From these results we concluded that factor IXa Tokyo possessed a normal reactivity to both the native substrate and inhibitor.

DISCUSSION

In this report, we have presented the molecular basis of a mild form of hemophilia Bm designated as factor IX Tokyo. Hemophilia Bm is one of the variant forms of hemophilia B and is associated with an abnormal factor IX molecule with prolongation of the prothrombin time performed with ox-brain thromboplastin. It is classified into two major groups, i.e., one with an amino acid substitution in the catalytic domain of factor IX and the other with a substitution at the second cleavage site for activation (Bertina, 1990). Our patient can be classified as hemophilia Bm, since he has an abnormal factor IX with a Val^{182} to Ala substitution manifesting a prolonged ox-brain prothrombin time. Purified factor IX Tokyo was found to have a reduced procoagulant activity at 23% of normal, and the cleavage of factor IX Tokyo by factor XIa was delayed at a low enzyme/substrate ratio. However, it could be activated by factor XIa at a high enzyme/substrate ratio, and activated factor IX Tokyo exhibited normal

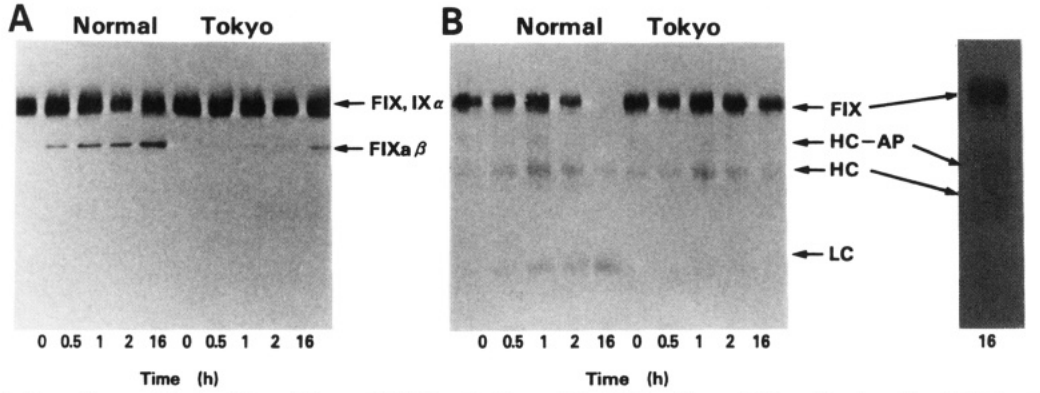

FIGURE 2: Activation of normal factor IX and factor IX Tokyo by factor XIa/Ca^{2+}. Factor IX was incubated at 37 °C with factor IXa at an enzyme/substrate ratio of 1/50 (w/w). At indicated times, aliquots were withdrawn and examined by immunoblot analysis under nonreducing (A) and reducing (B) conditions. The arrows indicate the positions of the purified heavy-chain-activation peptide (HC-AP) and the heavy-chain (HC) and light-chain (LC) peptides. A picture of 16-h incubation printed after a longer exposure was added at the right to show the HC-AP peptide intermediate.

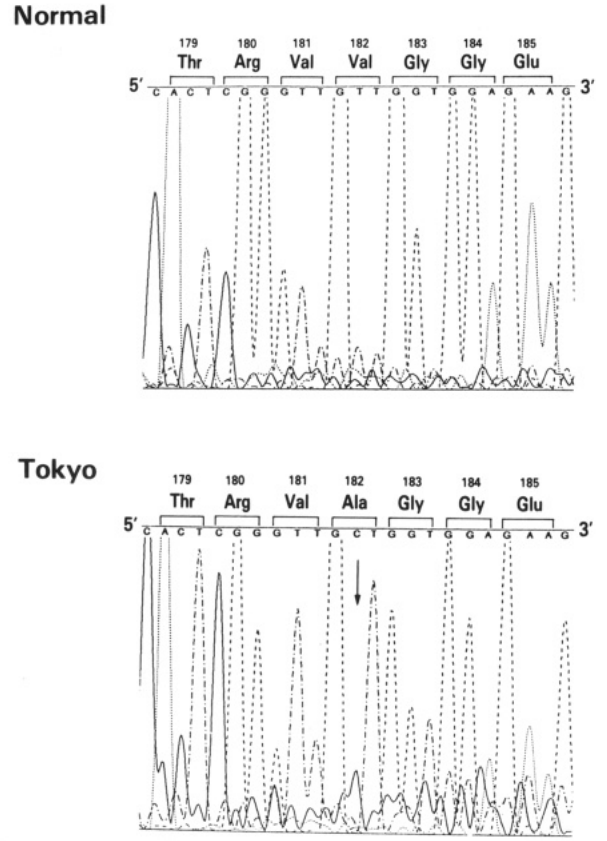

FIGURE 3: Partial nucleotide sequence of the coding strand of exon 6 of the gene for human factor IX. The sequences from the normal gene (top) and the factor IX Tokyo gene (bottom) are shown in the form of the outputs of an automatic DNA sequencer. The arrow indicates the point mutation at nucleotide 20 525. The signals of A, G, C, and T are shown by ···, – –, —, and – • –, respectively.

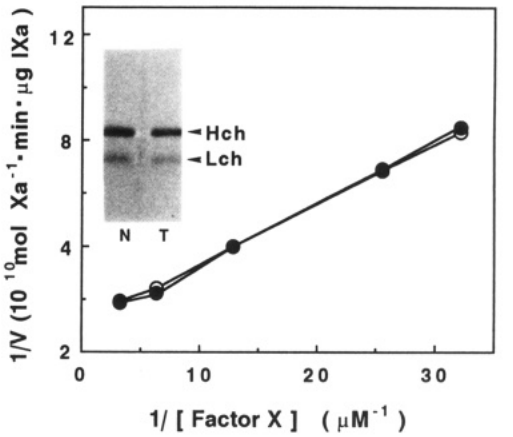

FIGURE 4: Activation of factor X by normal factor IXa and factor IXa Tokyo. Intrinsic factor X activation by factor IXa was measured in the presence of factor VIIIa and phospholipid vesicles as described under Materials and Methods. The rate of factor Xa generation was plotted according to a $1/V$ vs $1/S$ plot. Symbols: (○) normal factor IXa; (●) factor IXa Tokyo. Inset: SDS–PAGE (10–20% polyacrylamide gradient gel) of the purified factor IXa and factor IXa Tokyo under reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250.

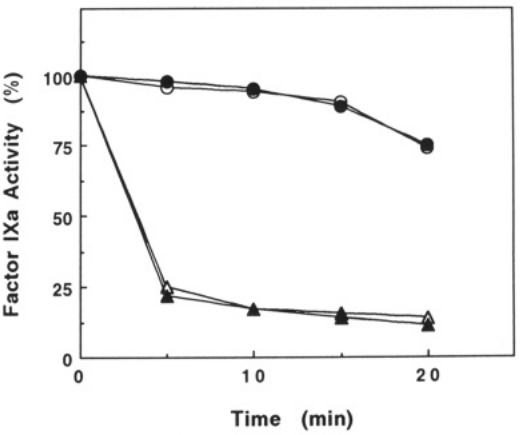

FIGURE 5: Inactivation of factor IXa activity by immobilized AT-III. Factor IXa or factor IXa Tokyo was incubated with a suspension of AT-III–Sepharose in the presence or absence of heparin at 23 °C. At timed intervals, the residual factor IXa activity was measured as described under Materials and Methods. Symbols: normal factor IXa in the presence (△) or absence (○) or heparin; factor IXa Tokyo in the presence (▲) or absence (●) of heparin.

enzymatic activity. Under the conditions where large amounts of factor XIa are generated, factor IX Tokyo would be activated without substantial delay and would function in a normal fashion. This may account for the absence of an apparent bleeding tendency in this patient.

To clarify how the amino acid substitution at position 182 affects the conformation of factor IXa, we have applied a model structure of the serine protease domain of factor IXa constructed for the analysis of factor IX Amagasaki (Miyata et al., 1991). Although this model has limitations with respect to the total structure because of the absence of the light chain of factor IXa, it appeared to be useful to evaluate the influence of the amino acid replacement. Table II shows rmsd values of the model structures of the catalytic domain of various aberrant factor IXa molecules with a mutation at either position 181, e.g., Milano, or position 182, e.g., Tokyo and Cardiff II, for normal factor IXa. The rmsd values in the

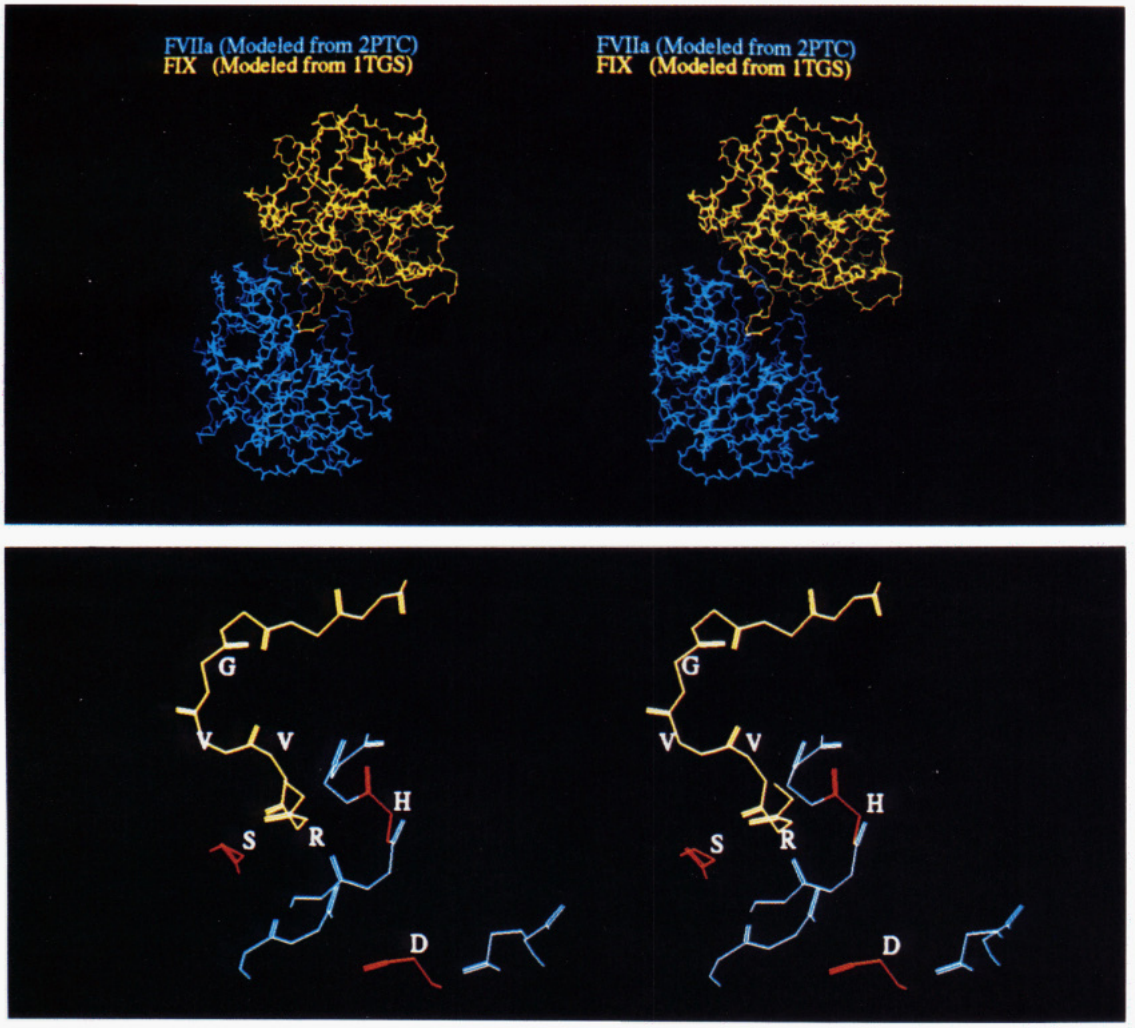

FIGURE 6: Stereoview of a docking model of factor IX and factor VIIa. (A, top) The main chain of the zymogen factor IX, Thr^{179}–Thr^{415} (yellow), and the catalytic domain of factor VIIa (blue) in the docking model are indicated. (B, bottom) A model view of the environment of the reactive site of factor IX, Arg^{180}–Gly^{183} (yellow), and the active site of factor VIIa (blue and red at catalytic residues His, Asp, and Ser) are indicated.

Table II: rmsd Values of the Model Structures of Variant Factor IXa with a Mutation at Position 181 or 182 for Normal Factor IXa

type of mutation	factor IX:C (%)	rmsd with variant IXa (Å) all main chain	active His-57	mutated C^{α}
V^{181}–V^{182} (normal)	100			
V^{181}–A^{182} (Tokyo)	23	0.009	0.012	0.021
V^{181}–L^{182} (Cardiff II)	15	0.174	0.180	0.193
F^{181}–V^{182} (Milano)	<1	0.154	0.138	0.195

positions of the main-chain atoms of factor IXa Tokyo were calcualted to be less than 0.02 Å for all main chains and in the range of 10 Å around the mutated Ala^{182} C^{α} as well as around the active site. In the mutant molecules in the type of factor IX's Milano and Cardiff II, the rmsd values are more than 10 times larger than those of factor IXa Tokyo. It is known that the movement of the active site amino acid residues more than 0.2 Å greatly affects the potential barrier of the proton transfer in relation to the enzymatic reaction (Nakagawa & Umeyama, 1982). Thus we concluded that the two models of factor IXa, normal and Tokyo, have similar conformations. This result was consistent with the finding that activated factor IX Tokyo had almost normal reactivities toward the native substrate and inhibitor.

Bertina et al. reported that factor IX Milano with a substitution of Val^{181} to Phe was activated by factor XIa but was unable to react with AT-III (1990). In this molecule, the aromatic side chain of the Phe at the NH_2 terminus of factor IXa was supposed to interfere with the formation of an ion pair with Asp^{364}. The substitution of Val^{181} to Phe changed the conformation of the enzyme part slightly as suggested by the rmsd values of less than 0.2 Å (Table II), whereas factor IX Tokyo has a normal residue of Val^{181}, which can form the ion pair with Asp^{364} at the distance of 2.60 Å (our calculation) although an Ala residue is present at the next position. From these results, we postulated that the Val^{182} to Ala substitution in Factor IX Tokyo affected only the rate of activation by factor XIa or by factor VIIa, resulting in reduced procoagulant activity.

Since direct comparisons between the kinetic analysis data utilizing factors VIIa and XIa have not so far been presented, we have attempted to construct an enzyme–substrate docking model and have selected factor VIIa rather than factor XIa as an activating enzyme. The reasons are that both enzymes cleave the same peptide bonds and probably yield the same cleavage products, as reported by a direct comparison study (Enfield & Thompson, 1984), and that the model of factor VIIa was available. The model of factor IX zymogen was based on the trypsinogen structure, especially at its cleavage segment. The peptide region comprising residues 16–26 in trypsinogen is mobile and hardly retains a stable conformation in the crystalline state (Fehhlhammer et al., 1977; Walter et al., 1982; Bologenesi et al., 1982). Residues 179–183 in factor IX (trypsinogen numbering 14–18) that span the P2 to P3′

region were not easily assigned. Among several models of the cleavage segment projecting outward of the factor IX molecule, we have selected a model that could accommodate the coordinates of residues 179–183 of factor IX to those of the reactive residues of BPTI in the trypsin–BPTI complex. Although this model has several limitations, we attempted to predict a putative factor IX zymogen–factor VIIa complex as shown in Figure 6. In this model, the protruding side chain of Val[181] should be oriented toward the factor IX molecule, while the side chain of Val[182] should be oriented into the substrate binding pocket of factor VIIa. Although both Val[181] and Val[182] in the reactive site of normal factor IX could be assigned close to the catalytic site region of the factor VIIa molecule, their individual isopropyl side chains were not exactly positioned because of the limited data available at this stage of investigation. Nevertheless, the introduction of a shorter side chain at position 182 in factor IX Tokyo, i.e., the methyl group of Ala instead of an isopropyl group of Val, would loosen the fitness of the relevant two molecules. This may partly account for the reason why IX Tokyo was activated slowly by factor XIa and RVV-X.

Recently, two other mutant molecules of hemophilia Bm were reported to have a substitution for Val[182] of Phe in factor IX Kashihira (Sakai et al., 1989) and Leu in factor IX Cardiff II (Taylor et al., 1990). Interestingly, these two abnormal factor IX molecules manifested different procoagulant activities, i.e., substantially no activity in factor IX Kashihara and 15% of normal in factor IX Cardiff II, although the mutation resided at the same position. Referring to our docking model, the complex formation between factor IX Kashihara and factor VIIa may not be easily accommodated since the aromatic ring of Phe[182] in factor IX Kashihira is more difficult to fit into the cavity of the binding pocket of factor VIIa due to steric hindrance. On the other hand, a less bulky side chain of Leu[182] in factor IX Cardiff II could be somehow, though not tightly, fitted into the enzyme pocket. These results suggest that the amino acid at position 182 corresponding to the P2′ site of the second cleavage site in factor IX is also responsible for the recognition by factor XIa and factor VIIa.

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SUPPLEMENTARY MATERIAL AVAILABLE

Atomic coordinates will be offered by mail order.

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