

Structural Integrity of the γ -Carboxyglutamic Acid Domain of Human Blood Coagulation Factor IXa Is Required for Its Binding to Cofactor VIIIa*

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This report describes the analysis of a novel mutant human factor IX protein from a patient with hemophilia B (factor IX activity <1%; factor IX antigen 45%). Enzymatic amplification of all eight exons of the factor IX gene followed by direct sequence analysis reveals a single nucleotide change (a guanine → adenine transition) in exon 2 at nucleotide 6409 which results in a glycine → arginine substitution at amino acid 12 in the γ -carboxyglutamic acid rich (Gla) domain of the mature protein. Factor IX was isolated by immunoaffinity chromatography from plasma obtained from the proband. The purified protein is indistinguishable from normal factor IX by polyacrylamide gel electrophoresis. Characterization of the variant in purified component assays reveals that it is activated normally by its physiologic activator factor XIa, but its phospholipid-dependent activation by the factor VIIa-tissue factor complex is diminished. In the presence of phospholipid and 5 mM Ca²⁺, the activities of variant and normal plasma-derived factor IX are similar; however, in the presence of activated factor VIIIa (intrinsic tenase complex), the normal augmentation of the cleavage of the specific substrate of factor IX, factor X, is not observed. The determination of the association constants for normal and variant factor IXa with factor VIIIa shows that the affinity of the activated variant factor IX for the cofactor factor VIIIa is 172-fold lower than normal. Competition studies using active site-inactivated factor IXas in the intrinsic tenase complex confirm that the defect in the variant protein is in its binding to factor VIIIa. We conclude that the structural integrity of the Gla domain of human factor IX is critical for the normal binding of factor IXa to factor VIIIa in the intrinsic tenase complex. In addition, a glycine at amino acid 12 is necessary for normal activation of factor IX by the factor VIIa-tissue factor complex.

ine protease which functions in the middle phase of blood coagulation. F.IX is activated by either factor VIIa-tissue factor (F.VIIa-TF) or factor XIa (F.XIa) in Ca²⁺-dependent reactions. Once activated, factor IXa (F.IXa), with its cofactor factor VIIIa (F.VIIIa) in the presence of Ca²⁺ and phospholipid, forms the intrinsic tenase complex and is responsible for the cleavage of the Arg⁵²-Ile⁵³ bond in the heavy chain of its specific substrate, factor X (F.X), generating activated F.X (F.Xa) (F.X numbering according to Leytus *et al.* (1)). Inherited deficiency of F.IX results in a bleeding disorder, hemophilia B.

Factor IX circulates as a 415-amino acid single chain zymogen with a molecular mass of 55,000 daltons and is present in normal plasma at approximately 5 μ g/ml. Factor IX is synthesized in the hepatocyte as a precursor containing an amino-terminal signal sequence and a propeptide. The signal sequence and propeptide are cleaved off in separate reactions prior to secretion, and failure to remove these sequences results in a nonfunctional protein (2). The signal sequence directs the protein to the endoplasmic reticulum (ER). The propeptide contains elements which are important for recognition of F.IX by the vitamin K-dependent γ -glutamyl carboxylase which is associated with the inner surface of the ER (3). The amino-terminal 38 residues of the mature protein comprise a functional region known as the Gla domain, so named because its first 12 glutamic acid residues undergo a post-translational vitamin K-dependent carboxylation at the γ -carbon (Gla residues). The Gla domain functions in calcium-dependent lipid binding and in binding to endothelial cells (residues 3–11) (4).

The gene encoding F.IX is present on the X chromosome at Xq27.1 near the F.VIII locus; it spans 33.5 kilobases and is composed of eight exons which correspond to the functional domains of F.IX (5). Exon 1 encodes the signal peptide, exon 2 the propeptide and Gla domain, exon 3 a small aromatic amino acid-rich domain, exons 4 and 5 the 2 epidermal growth factor-like domains, exon 6 the activation peptide, and exons 7 and 8 the trypsin-like catalytic domain. Point mutations in the Gla domain of F.IX (and other vitamin K-dependent clotting factors) result in mild to severe bleeding disorders (6–8).

This report describes a point mutation in exon 2 of the factor IX gene which results in a glycine to arginine substitution at

Factor IX (F.IX)¹ is a vitamin K-dependent trypsin-like ser-

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¹ The abbreviations used are: F.IX, factor IX; F.IXa, activated F.IX; F.VIIa, activated factor VII; TF, tissue factor; F.XIa, activated factor XI;

F.X, factor X; F.Xa, activated factor X; F.Va, activated factor V; F.VIIIa, activated factor VIII; AT-III, antithrombin III; F.IIa, thrombin; PCR, polymerase chain reaction; PSPC, dioleoylphosphatidylserine dioleoylphosphatidylcholine; DEGR, dansyl-glutamyl-glycyl-arginyl; ER, endoplasmic reticulum.

amino acid 12 in the Gla domain of mature F.IX.² Analysis reveals that the major defect associated with the variant protein is a markedly diminished cleavage of the substrate F.X by F.IXa when the variant F.IXa is associated with its cofactor F.VIIIa in the membrane-bound intrinsic tenase complex. The data suggest that the binding of the variant F.IXa to F.VIIIa is diminished 172-fold, and that this defect accounts for the decreased activity of F.IXa in the intrinsic tenase complex. In addition, the functional characterization of this variant protein provides evidence that this substitution affects its normal activation by the membrane-bound F.VIIa-TF complex.

EXPERIMENTAL PROCEDURES

Materials

Taq polymerase was obtained from Perkin-Elmer, and Sequenase was obtained from U. S. Biochemical Corp. Nonidet P-40, Fast Flow Q-Sepharose, sodium heparin (porcine), and poly-L-lysine were obtained from Sigma. Rabbit brain thromboplastin (Simplastin Excel) and rabbit brain phospholipid (Platelin) were obtained from Organon Teknika, Research Triangle Park, NC. Kaolin was obtained from Fisher. Antiserum to human F.IX for crossed immunoelectrophoresis was obtained from Diagnostica Stago, Asnieres France, and polyclonal F.IX antibody coupled to horseradish peroxidase for immunoblotting was obtained from Dako-Patts, Carpinteria, CA. A-5 and A-7 monoclonal human F.IX antibodies were produced as described previously (9). Coomassie protein assay was obtained from Pierce. D-Phenyl-Pro-Arg-chloromethyl ketone, phenylmethylsulfonyl fluoride, *p*-nitrophenyl *p*-guanidinobenzoate, and dansyl-Glu-Gly-Arg-chloromethyl ketone (DEGR) were obtained from Calbiochem. Silver staining kit was obtained from Bio-Rad. Chemiluminescence detection system (ECL) was obtained from Amersham, Buckinghamshire, United Kingdom. Spectrozyme-F.Xa was obtained from American Diagnostica, Greenwich CT. Bovine brain phosphatidylserine and phosphatidylcholine were obtained from Avanti Polar Lipids, Birmingham, AL. A V_{max} microtiter plate reader (Molecular Devices Corp., Menlo Park, CA) was used for all spectrophotometric assays.

Proteins

Monoclonally purified F.VIII (AHF-Method M) was obtained from the American Red Cross, Washington, D. C. Thrombin (F.IIa) and antithrombin III (AT-III) were the generous gift of Dr. F. Church (University of North Carolina, Chapel Hill, NC). Hirudin was obtained from Accurate Chemical and Scientific Corp., Westbury, NY. Recombinant F.VIIa was obtained from NovoNordisk (Bagsvaerd, Denmark). Recombinant relipidated tissue factor was obtained from Baxter Dade (Miami, FL). Plasma-purified human F.X was obtained from Enzyme Research Laboratories, South Bend, IN. Normal F.IX was purified from frozen plasma obtained from the blood bank at Children's Hospital of Philadelphia.

Human Subject

The affected patient is a 35-year-old male with severe hemophilia B (<1% F.IX activity). There is no evidence of antibodies to F.IX, and his F.IX antigen level is 45%. The subject gave his informed consent for phlebotomy and underwent manual plasmapheresis.

Mutation Analysis

Genomic DNA was purified according to the method of Bell (10) and stored in Tris-EDTA at -20°C . Genomic DNA was amplified by the polymerase chain reaction (PCR) (11). Fifty-microliter reaction mixtures contained 0.1 μg of genomic DNA, 0.1 μM amplification primers, 200 mM dNTP, in a buffer of 10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM MgCl_2 , 0.01% gelatin. Amplification primers were derived from intron sequences such that all exons and intron-exon junctions were amplified. Exons 3 and 4 were amplified together, and the large exon 8 was amplified in two fragments as described previously (12). After the addition of 1.5 units of *Taq* polymerase, the reaction mixtures were overlaid with mineral oil and heated to 94°C for 10 min. Thirty amplification cycles were performed consisting of 2 min at 55°C (primer annealing), 3 min at 72°C (primer extension), and 2 min at 94°C (denaturation). PCR reaction mixtures were subjected to 1% agarose gel electrophoresis in Tris borate-EDTA buffer at 95 V. DNA bands were

visualized by ethidium bromide staining and excised. DNA was purified from the isolated agarose fragments and resuspended in 8 μl of distilled, deionized water for dideoxy sequence analysis. Sequencing reactions were carried out by adding 2 μl of $5 \times$ Sequenase reaction buffer and 0.4 μl of 10% Nonidet P-40 to resuspended PCR-amplified DNA fragments and heating to 100°C for 3 min. Two microliters of water were added to the reaction, and dideoxy sequencing reactions were carried out with Sequenase according to the manufacturer's instructions. Termination reactions were separated on a 6% denaturing polyacrylamide gel at 75 watts, and autoradiographs were produced by exposing dried gels to film for 24–72 h.

F.IX Coagulation and Antigen Assays

Prothrombin time and activated partial thromboplastin time were performed by standard tilt tube methods using rabbit brain thromboplastin as reagent for the prothrombin time assay and platelin and kaolin for the activated partial thromboplastin time. F.IX activity assays were performed on patient plasma diluted into F.IX-deficient plasma using a one-stage activated partial thromboplastin time-based assay. F.IX antigen level was determined by crossed immunoelectrophoresis (13) using commercially available antiserum to human F.IX. F.IX antigen level was also determined by an enzyme-linked immunosorbent assay in which the monoclonal anti-human F.IX antibody A-5 was utilized as the capture antibody and the A-7 monoclonal anti-human F.IX antibody as the detecting antibody.

Purification of Plasma-derived F.IX

Citrated patient plasma was stored at -18 or -80°C until use. Forty to sixty milliliters of plasma were thawed at 4°C and centrifuged at $5000 \times g$ for 10 min to remove cryoprecipitable proteins. The cryosupernatant was diluted 1:1 into 20 mM Tris, pH 7.4, and adjusted to a concentration of 10 mM benzamidine, 1 mM EDTA, and 1 unit/ml heparin. This solution was applied to 5 ml of Fast Flow Q-Sepharose equilibrated in 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM benzamidine at 4°C . Beads were washed with 500 ml of 20 mM Tris, pH 7.4, 150 mM NaCl, and 10 mM benzamidine, followed by a 30–50-ml wash with 20 mM Tris, pH 7.4, 150 mM NaCl. Protein was eluted with 20 mM Tris, pH 7.4, 750 mM NaCl at a flow rate of 0.5–1 ml/min. Four-milliliter fractions were screened for protein by Coomassie assay. Fractions containing protein were pooled, added to 4 volumes of 20 mM Tris, 10 mM benzamidine, 25 mM MgCl_2 , and applied, at a rapid rate, to a 2-ml column containing agarose coupled to the A-7 metal ion-dependent monoclonal anti-human F.IX antibody (14) (3 mg of antibody/1 ml of agarose) which had been equilibrated in 20 mM Tris, pH 7.4, 150 mM NaCl, 20 mM MgCl_2 . The column was washed with 200 ml of 20 mM Tris, 150 mM NaCl, and 20 mM MgCl_2 , then eluted at 22°C with 20 mM Tris, 150 mM NaCl, 20 mM EDTA at a flow rate of 6 ml/h. Eluted protein was detected by absorbance at 280 nm. Protein eluting in a sharp peak was pooled, and the protease inhibitors D-phenyl-Pro-Arg-chloromethyl ketone, dansyl-Glu-Gly-Arg-chloromethyl ketone, and phenylmethylsulfonyl fluoride were added to a concentration of 1 μM . Purified material was then dialyzed extensively against 20 mM HEPES, pH 7.4, 150 mM NaCl and frozen in aliquots at -70°C . Normal F.IX was purified in an identical fashion from human plasma collected from volunteer whole blood donors. Excluding thawing of plasma and dialysis, the purification was accomplished in less than 10 h. Yields of both variant and normal plasma protein were approximately 40–75%.

Analysis of Protein

Protein separation was performed using an 8–25% gradient SDS-polyacrylamide gel, and protein was visualized by a commercial silver staining method (15). Polyacrylamide gel separated protein was transferred to a nylon membrane by a semidry electrophoretic method. Immunoblotting was performed using a chemiluminescence detection system employing a peroxidase-conjugated polyclonal F.IX antibody. Gla analysis was performed according to the modified alkaline hydrolysis method of Price (16). Amino-terminal protein sequence analysis was performed by automated Edman degradation to confirm the mutation at position 12.

Activation of F.IX by F.XIa

F.XIa was prepared from human plasma as described previously (17). In 200 μl of HEPES-buffered saline with albumin, 250 nM F.IX was activated using 1 nM F.XIa. The reaction was started by the addition of Ca^{2+} to a concentration of 5 mM. At various time points, 10 μl were removed and added to 190 μl of 2 mM EDTA to quench activation. F.IX activity was determined by adding 40 μl of the quenched activation time

² F.IX G12R. In the hemophilia B data base (6), this variant is referred to as F.IX_{Los Angeles 4}.

point mixtures to 60 μl of 500 nM F.X, 1.7 mM Spectrozyme-F.Xa, 100 nM poly-L-lysine in a microtiter plate. F.IXa-mediated activation of F.X was detected by the release of pNA from Spectrozyme-F.Xa by generated F.Xa and monitored by sequential spectrophotometric readings taken over 30 min at 405 nm on a thermally controlled kinetic microtiter plate reader at 25 °C.

Activation of F.IX by F.VIIa-TF

Factor VIIa and recombinant relipidated tissue factor were incubated for 5 min at 37 °C. In a total volume of 200 μl of Hepes-buffered saline with albumin and 3 mM Ca^{2+} , 100 nM F.IX was activated at 37 °C with 1 nM F.VIIa and 1 nM TF (reaction was started by the addition of F.VIIa-TF). At various time points, 10 μl of this reaction mixture were removed and quenched with 190 μl of 2 mM EDTA. Determination of F.IXa activity was performed as described above.

Purification of F.IXa and Determination of F.IXa Concentration by AT-III Titration

Normal or variant F.IX was activated to completion by F.XIa as determined by the chromogenic substrate assay. EDTA was added to reaction mixtures to chelate Ca^{2+} . Reaction mixtures were then run over Fast Flow Q-Sepharose equilibrated in Hepes-buffered saline with 1 mg/ml albumin and eluted with 20 mM Hepes, pH 7.4, 250 mM NaCl, 20 mM CaCl_2 . Fractions containing protein were pooled and dialyzed into Hepes-buffered saline. Concentrations of these stock F.IXa solutions (normal and variant) were determined by active site titration using AT-III as described previously (12) (data not shown). Stock AT-III solutions were titrated against *p*-nitrophenyl-*p'*-guanidinobenzoate-titrated thrombin and diluted into Hepes-buffered saline containing 5 $\mu\text{g}/\text{ml}$ heparin. F.IXa solutions were incubated with varying concentrations of AT-III-heparin, and the concentration of AT-III required to neutralize the activity of F.IXa completely was determined. All subsequent experiments were performed using these stock F.IXa solutions.

Preparation of Phospholipid Vesicles

Phospholipid vesicles consisting of a molar ratio of 30% phosphatidylserine:70% phosphatidylcholine (PSPC vesicles) were prepared by a rapid extrusion procedure (18).

F.IXa Affinity for PSPC Vesicles

As a measure of F.IXa (normal and variant) affinity for phospholipid, varying concentrations of F.IXa between 0 and 7.5 nM were used to activate F.X. In a total reaction volume of 100 μl in Hepes-buffered saline with albumin and 10 mM Ca^{2+} , F.IXa was incubated for 5 min at 25 °C with 40 mM PSPC vesicles. Reactions were started by the addition of F.X for a final concentration of 100 nM and Spectrozyme-F.Xa for a final concentration of 0.5 mM. F.X activation was detected spectrophotometrically using the F.Xa-specific chromogenic substrate. In this and subsequent assays, experiments were performed in duplicate, and curves were fitted using all data points.

Activation of F.X by F.IXa in the Presence of Phospholipid

In a 50- μl volume of Hepes-buffered saline with albumin with 10 mM Ca^{2+} , 10 nM F.IXa (normal or variant) was incubated with 40 μM PSPC vesicles in a microtiter plate well. Factor X (varying concentrations between 0 and 1000 nM) and Spectrozyme-F.Xa (final concentration 1 mM) were added to this mixture, and the absorbance at 405 nm was measured for 30 min on the kinetic plate reader.

Activation of F.X by F.IXa-F.VIIIa in the Presence of Phospholipid (Intrinsic Tenase Complex)

Factor VIIIa was freshly prepared by incubating 16 nM monoclonal F.VIII with 20 nM thrombin (F.IIa) for 5 min at 25 °C. After 5 min, hirudin was added to a concentration of 50 nM to stop the reaction. PSPC vesicles, Ca^{2+} , and 1 mM Spectrozyme-F.Xa were added to this reaction, and 30 μl of the PSPC-F.VIIIa-Spectrozyme-F.Xa were then added to 20 μl of F.IXa (normal or variant) in a microtiter plate well and incubated for 5 min at 25 °C to form the intrinsic tenase complex. This method of F.VIII activation results in a concentration of F.VIIIa that is 25% of the starting concentration of F.VIII as determined by coagulation assays. The activated F.VIII retains 88% of its maximal activity at 5 min as determined by F.Xa generation in a purified component intrinsic tenase assay (data not shown). Intrinsic tenase activity was detected kinetically on the microtiter plate reader upon the addition of 50 μl of F.X (varying concentration). Final concentrations were 0.1 nM F.IXa, 0.25 nM F.VIIIa, 40 μM PSPC, 0–200 nM F.X, and 0.5 mM Spectrozyme-F.Xa.

Binding of F.IXa to F.VIIIa

Factor VIII was activated by incubating 100 μl of F.VIII (165 nM) with 20 nM thrombin for 5 min at 25 °C. Thrombin was then inhibited by incubation of the F.VIIIa reaction mixture with 1900 μl of a solution containing 50 nM hirudin, Ca^{2+} , and PSPC vesicles. Twenty-five microliters of this F.VIIIa-containing solution were mixed with 25 μl of F.IXa (varying concentration), and the reaction was incubated for 5 min at 25 °C. Activity of the intrinsic tenase formed by binding of F.VIIIa to F.IXa was then measured by the addition of 50 μl of a mixture of F.X and Spectrozyme-F.Xa. Reaction buffer was Hepes-buffered saline with 1 mg/ml albumin. Final concentrations were 0.5 nM F.VIIIa, F.IXa varied between 0 and 15 nM, 40 μM PSPC, 0.5 mM Spectrozyme-F.Xa, 5 mM Ca^{2+} , 100 nM F.X.

Inhibition of F.X Activation by the Intrinsic Tenase Complex with Active Site-inactivated F.IXa (Intrinsic Tenase Inhibition Assay)

Active site-inactivated F.IXa species (normal and variant) were prepared by incubating 5 nM F.IXa with 10 mM dansyl-Glu-Gly-Arg-chloromethyl ketone (DEGR) for 3 h at 37 °C. Following incubation, inactivated F.IXa was dialyzed extensively against 20 mM Hepes, pH 7.4, 150 mM NaCl to remove residual free DEGR. Varying amounts of normal or variant inactivated F.IXa (0 nM–50 nM final concentrations) were combined with 50 μM normal F.IXa. The tenase complex was formed by the addition of PSPC vesicles and F.VIIIa (freshly prepared as described above) to the F.IXa and incubated for 5 min at 25 °C. Intrinsic tenase activity was then measured as the rate of generation of F.Xa as detected by the addition of F.X and Spectrozyme-F.Xa. Final concentrations were 50 μM normal F.IXa, 2.75 nM F.VIIIa, 40 μM PSPC, 100 nM F.X, 0.5 mM Spectrozyme-F.Xa, and 5 mM Ca^{2+} .

Data Analysis

F.VIIa-TF Activation—Activation of F.IX by F.VIIa-TF fits the equation:

$$\text{relative [F.IXa]} = 1 - e^{-kt} \quad (\text{Eq. 1})$$

where k is the rate of F.IX activation (19). This equation assumes that the rate is normalized to 1 as the maximum, and that at t_0 there is no F.IXa. Raw data can be accommodated with the equation:

$$(1 - e^{-kt})([\text{F.IXa}]_{\text{max}} - [\text{F.IXa}]_0) + [\text{F.IXa}]_0 \quad (\text{Eq. 2})$$

thus, for normal F.IX, $k = 0.050$; for variant F.IX, $k = 0.013$.

F.IXa Activity—This is determined from a coupled assay in which both F.X and a chromogenic substrate for F.X are added to F.IXa and cofactors. The amount of cleavage of the chromogenic substrate is plotted against time and fitted to the equation:

$$\text{absorbance} = a_0 + a_1t + a_2t^2 \quad (\text{Eq. 3})$$

where a_0 gives the amount of cleaved substrate at time 0; a_1 gives the amount of F.Xa present in the zymogen F.X, and a_2 reflects the rate at which F.Xa cleaves the chromogenic substrate, the rate at which F.X is cleaved, and the amount of enzyme (F.IXa/PSPC) or (F.IXa-F.VIIIa/PSPC) present in the reaction (20).

F.IXa Binding to F.VIIIa—Data are analyzed using equations described by Krishnaswamy *et al.* (21) and by Duffy *et al.* (22). The rate of F.Xa generation is given by

$$v = k_{\text{cat}} [\text{F.IXa-F.VIIIa}] \quad (\text{Eq. 4})$$

The maximal rate is given by:

$$V_{\text{max}} = k_{\text{cat}} [\text{F.IXa-F.VIIIa}]_{\text{max}} \quad (\text{Eq. 5})$$

When F.VIIIa is limiting,

$$V_{\text{max}} = k_{\text{cat}} [\text{F.VIIIa}]_t \quad (\text{Eq. 6})$$

(subscript t indicates the total concentration) and

$$v = V_{\text{max}} \frac{[\text{F.IXa-F.VIIIa}]}{[\text{F.VIIIa}]_t} \quad (\text{Eq. 7})$$

By definition:

$$[\text{F.IXa-F.VIIIa}] = \frac{[\text{F.IXa}][\text{F.VIIIa}]}{K_d} \quad (\text{Eq. 8})$$

Since $[\text{F.IXa}] = [\text{F.IXa total}] - [\text{F.IXa in the intrinsic tenase complex}]$,

then:

$$[\text{F.IXa-F.VIIIa}] = \frac{([\text{F.IXa}]_t - [\text{F.IXa-F.VIIIa}]_t)([\text{F.VIIIa}]_t - [\text{F.IXa-F.VIIIa}]_t)}{K_d} \quad (\text{Eq. 9})$$

Solving for [F.IXa-F.VIIIa] gives:

$$[\text{F.IXa-F.VIIIa}] = \frac{([\text{F.IXa}]_t + [\text{F.VIIIa}]_t + K_d)}{2} - \frac{\sqrt{([\text{F.IXa}]_t + [\text{F.VIIIa}]_t + K_d)^2 - 4[\text{F.IXa}]_t[\text{F.VIIIa}]_t}}{2} \quad (\text{Eq. 10})$$

which can be substituted into Equation 7. Curves were derived from a least-squares fit of the data to substituted Equation 7.

DEGR-F.IXa Inhibition of F.IXa-F.VIIIa Activity—Data are analyzed by a variation of the above scheme. Since the concentration of the F.IXa-F.VIIIa complex is small compared to the total concentration of F.VIIIa, the equation for the concentration of the complex simplifies to:

$$[\text{F.IXa-F.VIIIa}] = \frac{[\text{F.IXa}]_t[\text{F.VIIIa}]_t}{K_d + [\text{F.VIIIa}]_t} \quad (\text{Eq. 11})$$

In the presence of DEGR-F.IXa:

$$[\text{F.IXa-F.VIIIa}] = \frac{1}{K_d} \cdot ([\text{F.IXa}]_t - [\text{F.IXa-F.VIIIa}]_t) \cdot ([\text{F.VIIIa}]_t - [\text{F.IXa-F.VIIIa}]_t - [\text{DEGR-F.IXa-F.VIIIa}]_t) \quad (\text{Eq. 12})$$

Since [F.IXa-F.VIIIa] is small compared to [F.VIIIa]_t, this equation simplifies to:

$$[\text{F.IXa-F.VIIIa}] = \frac{[\text{F.IXa}]_t([\text{F.VIIIa}]_t - [\text{DEGR-F.IXa-F.VIIIa}]_t)}{K_d + [\text{F.VIIIa}]_t - [\text{DEGR-F.IXa-F.VIIIa}]_t} \quad (\text{Eq. 13})$$

Since the rate of F.Xa generation is a direct function of the amount of F.IXa-F.VIIIa, the data can be expressed as:

$$\frac{\text{rate with DEGR-F.IXa}}{\text{rate without DEGR-F.IXa}} = \frac{[\text{F.IXa-F.VIIIa}] \text{ with DEGR-F.IXa}}{[\text{F.IXa-F.VIIIa}] \text{ without DEGR-F.IXa}} \quad (\text{Eq. 14})$$

which simplifies to:

$$\text{relative rate} = \left(\frac{K_d + [\text{F.VIIIa}]_t}{[\text{F.VIIIa}]_t} \right) \cdot \left(\frac{[\text{F.VIIIa}]_t - [\text{DEGR-F.IXa-F.VIIIa}]_t}{K_d + [\text{F.VIIIa}]_t - [\text{DEGR-F.IXa-F.VIIIa}]_t} \right) \quad (\text{Eq. 15})$$

The concentration of DEGR-F.IXa-F.VIIIa can be determined as described for the F.IXa-F.VIIIa complex in Equations 8–10 above assuming that the concentration of the F.IXa-F.VIIIa complex is small compared to the total amount of F.VIIIa and is given by the equation:

$$[\text{DEGR-F.IXa-F.VIIIa}] = \frac{([\text{DEGR-F.IXa}]_t + [\text{F.VIIIa}]_t + K_i)}{2} - \frac{\sqrt{([\text{DEGR-F.IXa}]_t + [\text{F.VIIIa}]_t + K_i)^2 - 4[\text{DEGR-F.IXa}]_t[\text{F.VIIIa}]_t}}{2} \quad (\text{Eq. 16})$$

which can be substituted into Equation 15. Curves were derived from a least-squares fit of the data to substituted Equation 15. Curve fits were performed using the SlideWrite program (Advanced Graphics Software Inc., Carlsbad, CA).

RESULTS

Direct sequence analysis of amplified genomic DNA from all eight exons and the exon-intron junctions of the variant F.IX gene demonstrated a single G → A transition at nucleotide 6409 in exon 2 (Fig. 1). The mutation was confirmed by sequence analysis of the opposite strand. This single nucleotide change results in the substitution of arginine for glycine at amino acid 12 in the Gla domain of the mature F.IX protein. The mutation neither creates nor destroys an endonuclease

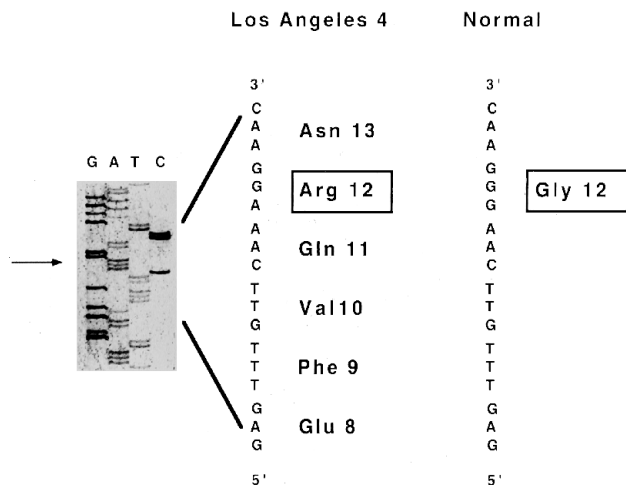


FIG. 1. **Sequence analysis of genomic DNA from the proband.** A G → A transition at nucleotide 6409 (denoted by arrow) results in the substitution of arginine for glycine at amino acid 12 of the mature protein.

cleavage site useful for confirming the mutation by restriction analysis.

Screening coagulation studies on patient plasma showed a prothrombin time of 10.4 s (control = 10.6) and a partial thromboplastin time of 64.9 s (control = 37). F.IX activity was <1%. F.IX antigen level was 41% by crossed immunoelectrophoresis and 45% by enzyme-linked immunosorbent assay (normal range 60–150%).

Immunoaffinity-purified F.IX from patient plasma comigrated with normal F.IX on electrophoresis in an 8–25% gradient SDS-polyacrylamide gel (Fig. 2). Immunoblotting confirmed this material to be human F.IX (data not shown). In order to confirm the mutation, purified variant F.IX was subjected to amino-terminal amino acid analysis. Edman degradation demonstrated a single amino acid species, arginine, at position 12. Gla analysis demonstrated 9.1 mol of Gla/mol of variant F.IX and 8.7 mol of Gla/mol of normal plasma-derived F.IX.

F.IXa activities in the following purified component assays were determined by the calculation of initial rates of F.IXa-mediated activation of F.X. The optimal concentration of PSPC vesicles (40 μM) was determined by varying the concentration of PSPC from 5 μM to 1 mM and determining the rate of F.Xa generation using normal F.IXa (10 nM) and normal F.X (300 nM) (data not shown). The rate of activation of purified variant F.IX by F.XIa was identical with that of normal F.IXa (Fig. 3). However, activation of the variant F.IX by the F.VIIa-TF complex was slower (Fig. 4), approximately 25% of normal based on kinetic analysis of time courses of activation (rate for normal F.IX is 0.050 ± 0.002 min⁻¹, for variant F.IX is 0.013 ± 0.001 min⁻¹; see data analysis).

Varying F.IXa between 0 and 10 nM showed the expected linear increase in the rate of F.Xa generation in the presence of 40 μM PSPC vesicles at 5 mM Ca²⁺ (Fig. 5). As shown in the inset to Fig. 5, there was no difference in F.Xa generation between normal F.IXa and variant F.IXa at subnanomolar concentrations (between 40 and 700 pM), indicating no defect in phospholipid binding by the variant F.IXa. As shown in Fig. 6A, the kinetics of F.X activation by purified variant F.IXa and normal F.IXa in the absence of F.VIIIa (in the presence of 40 μM PSPC vesicles at 5 mM Ca²⁺) were not significantly different ($K_m = 341 \pm 21.7$ nM, $k_{cat} = 9.0 \times 10^{-4}$ M F.Xa s⁻¹ M⁻¹ F.IXa for normal F.IXa; $K_m = 217 \pm 8.3$ nM, $k_{cat} = 9.2 \times 10^{-4}$ M F.Xa s⁻¹ M⁻¹ F.IXa for variant F.IXa). These data are summarized in Table I. When the cofactor F.VIIIa was added to the reaction to

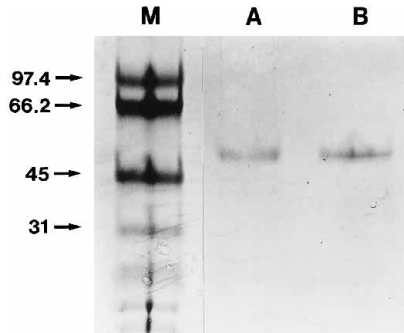


FIG. 2. 8–25% gradient SDS-polyacrylamide gel electrophoresis of purified F.IX. Protein is visualized by silver staining. Lane M, molecular weight markers. Lane A, immunopurified normal plasma-derived F.IX. Lane B, immunopurified variant F.IX.

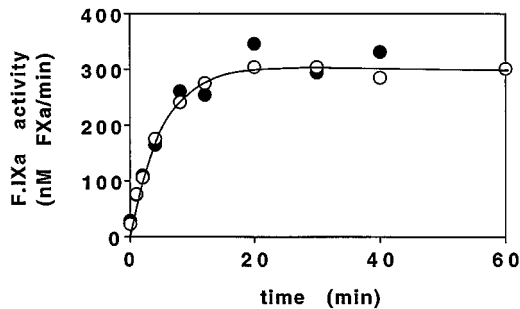


FIG. 3. Time course of F.IX activation by F.XIa. Variant (●) and normal (○) F.IX (250 nM) are activated by F.XIa (1 nM) at 5 mM Ca^{2+} . Activation is complete at 20 min. F.IXa activity is expressed as the concentration (nM) of F.Xa generated/min.

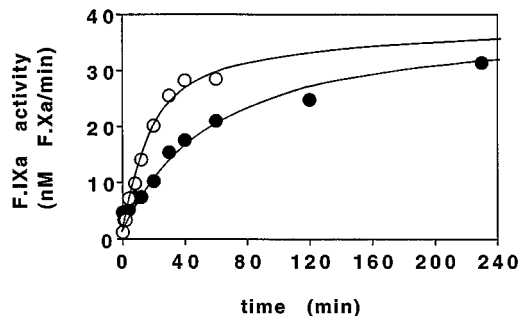


FIG. 4. Time course of F.IX activation by F.VIIa and tissue factor. Variant (●) and normal (○) F.IX (100 nM) are activated by F.VIIa (1 nM) and TF (1 nM) at 3 mM Ca^{2+} . F.IXa activity is expressed as the concentration (nM) of F.Xa generated/min. The rate of activation of variant F.IX by F.VIIa-TF is 25% that of normal ($0.050 \pm 0.002 \text{ min}^{-1}$ for normal F.IX and $0.013 \pm 0.001 \text{ min}^{-1}$ for the variant). Rates were determined by a least-squares fit of the data to Equation 2 (see "Experimental Procedures").

form the intrinsic tenase complex, however, the variant F.IXa did not show the normal augmentation in the rate of cleavage of F.X (Fig. 6B). This was due to a minor extent to a difference in K_m values (45 nM for normal F.IXa-F.VIIIa complex, 142 nM for the variant complex), but principally to a 26-fold difference in V_{\max} between normal and variant F.IXa (Fig. 6B). This defect is the likely explanation for the patient's severe bleeding diathesis.

Failure of the variant F.IXa to show enhanced cleavage of F.X in the intrinsic tenase complex may be due either to altered affinity of variant F.IXa for the cofactor, F.VIIIa, or failure of the variant F.IXa to undergo a required conformational change following its binding to F.VIIIa on the phospholipid surface (23). To determine whether diminished binding of F.IXa to F.VIIIa was responsible for the defect in the activated variant F.IX, we first determined K_d values for the variant and normal

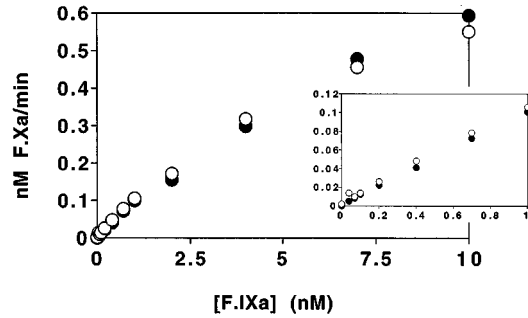


FIG. 5. F.IXa activation of F.X in the presence of phospholipid. Variant (●) and normal (○) F.IXa (between 0 and 7.5 nM) were used to activate 100 nM F.X in the presence of 40 μM PSPC vesicles. The inset shows rates of activation at subnanomolar concentrations of F.IXa (between 0 and 1 nM).

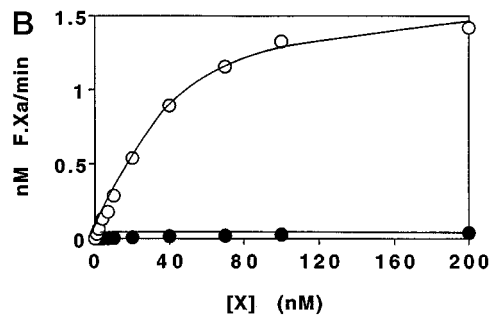
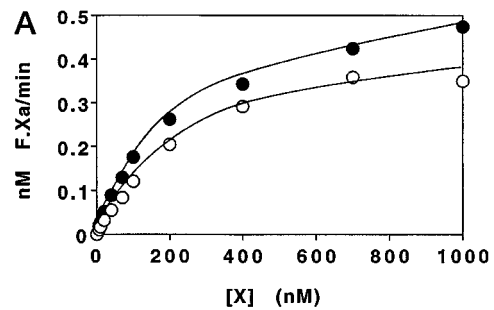


FIG. 6. F.IXa activation of F.X. A, F.IXa (10 nM normal (○) or variant (●)) are used to activate F.X in the presence of 40 μM PSPC vesicles and 5 mM Ca^{2+} and in the absence of F.VIIIa. The K_m and V_{\max} values of variant F.IXa are $217 \pm 8.3 \text{ nM}$ and $0.546 \pm 0.009 \text{ nM F.Xa/min}$, respectively. For normal F.IXa, the K_m is $341 \pm 21.7 \text{ nM}$ and the V_{\max} is $0.536 \pm 0.017 \text{ nM F.Xa/min}$. B, F.IXa (0.1 nM normal (○) or variant (●)) are used to activate F.X in the presence of 40 μM PSPC vesicles and 5 mM Ca^{2+} with 0.25 nM F.VIIIa (intrinsic tenase complex). The K_m for F.X of the intrinsic tenase complex containing variant F.IXa is $142 \pm 10.0 \text{ nM}$, and the V_{\max} is $0.067 \pm 0.003 \text{ nM F.Xa/min}$. The K_m for F.X of the normal intrinsic tenase complex is $45 \text{ nM} \pm 4.6$, and the V_{\max} is $1.834 \pm 0.071 \text{ nM F.Xa/min}$ (see Table I). Curves from which the constants were derived were determined based on a least-squares fit of the data to the Michaelis-Menten equation.

F.IXas (Fig. 7). The association of F.IXa with F.VIIIa was determined by the addition of F.VIIIa and PSPC vesicles to varying concentrations of F.IXa and measured by the increased activation of F.X by generated intrinsic tenase (F.IXa-F.VIIIa-PSPC vesicles- Ca^{2+}). Both the variant and normal F.IXa showed a similar V_{\max} ($V_{\max} = 25.7 \pm 0.5$ for normal; $V_{\max} = 27.5 \pm 4.9$ for variant); however, the K_d of the variant F.IXa for F.VIIIa-PSPC vesicles was 172-fold higher than that of normal F.IXa ($K_d = 30.9 \pm 7.27 \text{ nM}$; $K_d = 0.18 \text{ nM} \pm 0.02$, respectively). Since the kinetic parameters describing the interaction of variant F.IXa are extrapolated values, we sought to corroborate the estimated K_d using a second method. We determined K_i values for the intrinsic tenase using normal and variant F.IXas that had been blocked at the active site as competitive inhibi-

TABLE I
Kinetic analyses of normal and variant F.IXa in the presence and absence of F.VIIIa

	K_m	V_{max}	V_{max}	[Enzyme]	k_{cat}^a	k_{cat}/K_m^b
	nM	nM F.Xa/min	nM F.Xa/s	nM		
Normal F.IXa	341 ± 21.7	0.536 ± 0.017	0.0090	10	9.0 × 10 ⁻⁴	2640
Normal F.IXa-F.VIIIa	45 ± 4.6	1.834 ± 0.071	0.0305	0.053 ^c	0.575	12.7 × 10 ⁶
Variant F.IXa	217 ± 8.3	0.546 ± 0.009	0.0092	10	9.2 × 10 ⁻⁴	4240
Variant F.IXa-F.VIIIa	142 ± 10.0	0.067 ± 0.003	0.0012	0.001 ^c	1.200	8.45 × 10 ⁶

^a Units are M F.Xa, M⁻¹ F.IXa (or F.IXa-F.VIIIa), s⁻¹.

^b Units are M F.Xa, M⁻¹ F.IXa (or F.IXa-F.VIIIa), s⁻¹, M⁻¹ F.X.

^c Concentrations of F.IXa-F.VIIIa complex derived from experimental conditions and K_d values of normal and variant F.IXas for F.VIIIa (see Fig. 6).

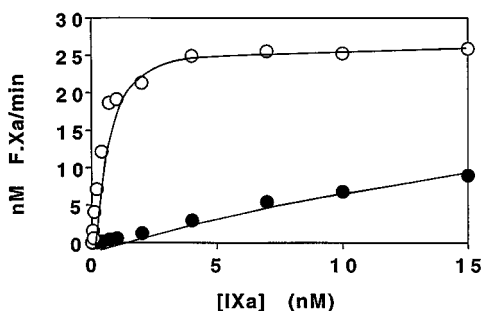


FIG. 7. Determination of K_d for normal or variant F.IXa and F.VIIIa. Thrombin-activated F.VIIIa (0.5 nM) is incubated with variant (●) or normal (○) F.IXa with 40 μM PSPC vesicles and 5 mM Ca²⁺. The binding of F.IXa to F.VIIIa was determined by measuring intrinsic tenase activity and detected by the generation of F.Xa. The V_{max} for normal F.IXa is 25.7 ± 0.5 nM F.Xa/min and for variant is 27.5 ± 4.9 nM F.Xa/min. The K_d describing the binding of normal F.IXa to PSPC-F.VIIIa is 0.18 ± 0.02 nM; for variant F.IXa, the K_d is 30.9 ± 7.3 nM as determined from curves derived from least-squares fit of the data to Equation 7 (see "Experimental Procedures").

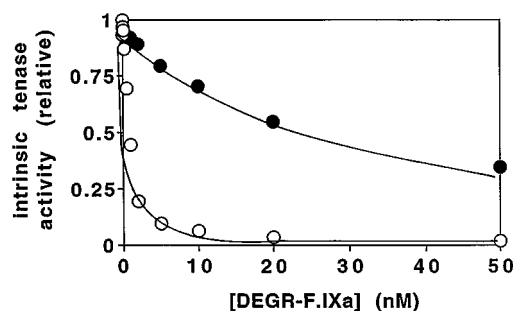


FIG. 8. Inhibition of the normal intrinsic tenase complex by dansyl-Glu-Gly-Arg (DEGR) inactivated F.IXas. Increasing concentrations of DEGR-inactivated F.IXa normal (○) and variant (●) are used as competitors for normal F.IXa in the intrinsic tenase complex (50 pM F.IXa, 0.55 nM F.VIIIa, 40 μM PSPC vesicles, 5 mM Ca²⁺). Binding of the inactivated species is measured by the inhibition of F.Xa generation by the normal intrinsic tenase complex. K_i for DEGR-inactivated F.IXa normal is 0.17 ± 0.01 nM and for DEGR-inactivated F.IXa variant is 7.71 ± 0.50 nM as determined from curves derived from least-squares fit of the data to Equation 15 (see "Experimental Procedures").

tors of normal F.IXa in the intrinsic tenase complex. In this inhibition assay, activated species of normal and variant F.IX were inhibited by the covalent linkage of the small peptide, dansyl-Glu-Gly-Arg (DEGR), at the active site triad. If the defect in the variant enzyme were due to abnormal binding to F.VIIIa, the variant would be expected to exhibit diminished or no displacement of normal F.IXa from intrinsic tenase. If, however, the defect were due to a failure of the variant to undergo a conformational change (upon binding of F.VIIIa) necessary for normal activity in the intrinsic tenase complex, inactivated variant protein would bind F.VIIIa normally and displace normal F.IXa from the intrinsic tenase complex in a fashion similar to inactivated normal F.IXa. Varying amounts of these active site-inactivated F.IXas were added to the intrinsic tenase complex consisting of F.IXa, F.VIIIa, and PSPC vesicles (Fig. 8). K_i values determined from this intrinsic tenase inhibition assay were consistent with results obtained in the binding experiment and showed a 45-fold difference between normal F.IXa ($K_i = 0.17 ± 0.011$ nM) and variant F.IXa ($K_i = 7.71 ± 0.50$ nM).

DISCUSSION

We describe a novel mutation in the gene for human blood coagulation factor IX which results in the substitution of the large basic amino acid, arginine, for the normal glycine at position 12 in the Gla domain of the mature protein. This protein is of special interest because it provides evidence that the structural integrity of the Gla domain is critical for binding of F.IXa to the cofactor F.VIIIa. The variant protein is secreted into the circulation, but has no activity in coagulation. We have characterized the effects of the substitution on the function of the variant protein in coagulation by using immunoaffinity-purified plasma-derived F.IX in a series of functional assays. Other F.IX mutants reported in the Gla domain have not been

studied in purified component assays (6).

On functional analysis, the major defect of the variant F.IXa is the absence of the normal augmentation of the F.IXa-mediated activation of F.X in the presence of phospholipid, Ca²⁺, and the cofactor F.VIIIa (intrinsic tenase) (Fig. 6B). Phospholipid binding by the variant is normal at concentrations of F.IXa between 40 pM and 10 nM as demonstrated by F.IXa-mediated generation of F.Xa (Fig. 5). Given the normal function of the variant in the presence of Ca²⁺ and phospholipid alone (Fig. 6A), the diminished activity observed in the intrinsic tenase complex is due to a F.VIIIa-dependent interaction of F.IXa within the intrinsic tenase complex. This reduced activity could be due either to a defect in variant F.IXa binding to F.VIIIa or a failure of the variant to undergo a conformational change upon binding F.VIIIa that is necessary for normal activity in the intrinsic tenase complex. Reduced binding of the variant F.IXa to F.VIIIa is demonstrated by both the binding and competitive inhibition assays. In the binding experiment, the K_d of variant F.IXa (30.9 nM) was 172-fold higher than that of normal F.IXa ($K_d = 0.18$ nM) (Fig. 7). Under the conditions used in this binding experiment, the K_d determined for F.VIIIa binding by variant F.IXa exceeded the highest concentration of variant F.IXa used in the experiment. To reasonably approach saturating conditions with variant F.IXa in this experiment would require concentrations of F.IXa in the range (>100 nM) where background F.X activation by F.IXa alone would affect the analysis of the results. In addition, under such conditions, the concentration of F.IXa would approach the concentration of F.X used in the assay resulting in competition between F.IXa and F.X for phospholipid binding. The use of higher concentrations of F.X to overcome this limitation would result in unmeasurable reaction rates. The intrinsic tenase inhibition assay was performed to overcome these limitations and to corroborate the estimated K_d using a second method. Normal F.IXa was 45

times more effective as an inhibitor in the intrinsic tenase inhibition assay ($K_i = 0.17$ nM for normal F.IXa and 7.71 nM for variant F.IXa) (Fig. 8). Several groups have published constants between 0.02 and 16 nM describing the interaction of F.VIIIa with F.IXa under conditions different from those used in the experiments described here (22, 24–28). Our values are at the lower limit of other reported ranges for binding constants but are not inconsistent with previous studies which evaluated human factors (25, 27). In the inhibition assay, similar K_i values (similar inhibition pattern) would have been expected for both the normal and variant if the defect observed in the intrinsic tenase (Fig. 6B) were due solely to the variant's failure to undergo the necessary conformational change. If one assumes that there is no effect of the small peptide blocking the active site of F.IXa on the binding of inactivated F.IXa (normal or variant) to F.VIIIa, then the intrinsic tenase inhibition assay, in which F.X activation is accomplished only by normal F.IXa, measures only the difference in binding of F.VIIIa between normal and variant F.IXa. Factor X activation in the binding assay, however, relies on the measurement of F.X activation by either normal F.IXa or variant F.IXa and, therefore, measures *both* binding and conformational effects. Thus, the inhibition assay confirms that the major effect is due to abnormal binding of variant F.IXa to F.VIIIa. The 4-fold discrepancy between the relative differences in the normal and variant K_i values (45-fold) and the normal and variant K_d values (172-fold) ascertained in these two experiments is likely to be within the range of experimental error. Alternatively, there may be a small contribution to the defect from differences in the conformational change undergone by wild-type *versus* variant F.IXa upon binding to F.VIIIa. (A conformational difference would be reflected in the binding assay shown in Fig. 7, but not in the competitive inhibition assay shown in Fig. 8.) In any case, it is clear that the major factor accounting for the reduced activity in the intrinsic tenase complex is altered binding of the variant F.IXa to F.VIIIa.

Catalytic efficiencies (k_{cat}/K_m) were calculated from experimentally determined K_m and V_{max} (Table I). In the experiments characterizing F.IXa activity in the intrinsic tenase complex, the true enzyme is the F.IXa-F.VIIIa complex. The concentration of the F.IXa-F.VIIIa complex was determined using observed K_d values (Fig. 7) and Equation 10 (see "Experimental Procedures," *Data Analysis*). Note that the 172-fold difference between the K_d values for normal and variant F.IXa results in a marked difference in the concentration of the true enzyme complex (normal or variant F.IXa-F.VIIIa) at identical concentrations of F.IXa and F.VIIIa. In contrast to the difference in rates of F.X activation at a given concentration of F.IXa between the normal and variant intrinsic tenase (Fig. 6B), catalytic efficiencies of the normal and variant complexes are similar, since they are determined using concentrations of the true enzyme complex (Table I).

In the solution phase activation of F.IX by F.XIa in which two bonds in the catalytic domain of F.IX (Arg¹⁴⁵-Ala¹⁴⁶ and Arg¹⁸⁰-Val¹⁸¹) are cleaved by F.XIa, variant F.IX is activated normally. However, in the phospholipid-dependent activation of F.IX by the F.VIIa-TF complex, in which the same two bonds are cleaved, the variant is activated at a rate which is 25% of normal. These data suggest that amino acid 12 of F.IX is either involved in the binding site for F.VIIa-TF or has distal effects on the conformation of the F.VIIa-TF binding site on phospholipid-bound F.IX.

The substitution of arginine, a bulky negatively charged residue, for the small neutral glycine at position 12 would be predicted to affect Ca²⁺ binding by perturbing the surrounding Gla residues. Such an effect might be expected to result in

altered phospholipid binding since Ca²⁺ binding in the Gla domain is responsible for a conformational change which is necessary for coagulant activity (29–32), and this activity is correlated to phospholipid binding (33). However, when the kinetic activation of F.X by F.IXa was studied with phospholipid and Ca²⁺, there was little difference between the variant and normal F.IXa, implying that binding of the variant to F.X and phospholipid is normal and that the effect of the amino acid substitution has minimal, if any, effect on calcium binding.

The data indicate that the variant F.IX undergoes γ -carboxylation to the same degree as the normal protein. Based on extensive work with recombinant proteins (3, 34–36) and synthetic peptides (37, 38), the γ -glutamyl carboxylase recognition site is known to include residues at the amino-terminal portion of the propeptide in vitamin K-dependent proteins. Observations from naturally occurring F.IX variants with mutations affecting residues -1 and -4 at the carboxyl terminus of the propeptide, are not consistent; some have reported normal γ -carboxylation (39, 40) and others, reduced γ -carboxylation (2, 41). Peptide substrates which contain these same changes, however, have no effect on carboxylation (38, 42). Although a contribution to the carboxylase binding site from the Gla domain has been suggested based on sequence homology between vitamin K-dependent coagulation factors and matrix Gla protein (43), peptide substrates which include the propeptide and this purported binding site in the Gla domain have not been shown to affect carboxylase binding when compared to the propeptide substrate alone (44). Zhang *et al.* (45) have observed that only 10–20% of recombinant protein C containing substitutions of aspartic acid for Gla residues 16, 20, and 26 is fully carboxylated. These data have led to the hypothesis that amino acid substitutions which perturb the structure of the Gla domain or the intervening portion of the propeptide may affect carboxylation. Normal carboxylation of the variant F.IX described here indicates that glycine 12 is not involved in carboxylase recognition, and that a nonconservative amino acid substitution at this position in F.IX does not affect γ -carboxylation of the appropriate glutamic acid residues by the carboxylase.

Alignment of the amino acid sequences of the human vitamin K-dependent factors (F.IX, prothrombin, F.VII, F.X, protein C, and protein S) reveals that the residue corresponding to glycine 12 of mature factor IX is conserved in these proteins, with the exception of protein C in which another small neutral polar amino acid (serine) is present. This suggests a critical role for glycine (or serine) at this position. Two other mutations have been reported in F.IX affecting amino acid 12 (6, 46). In F.IX_{Hong Kong1}, an alanine substitution results in a molecule with 3% normal activity. F.IX_{UK182}, in which the charged residue, glutamic acid, is substituted for glycine 12, has <1% normal activity. The mechanism for defective activity has not been characterized for either of these variants.

The data presented here demonstrate that a glycine to arginine substitution at position 12 in human F.IX prevents the normal augmentation of activity of activated F.IX that occurs in the presence of the cofactor F.VIIIa. Competition studies with active site-inactivated F.IXa provide strong evidence that variant F.IXa binding to F.VIIIa is diminished and accounts for the severe defect in the activity of the variant F.IXa in the intrinsic tenase complex. These data thus indicate a critical role for the structural integrity of the Gla domain in the binding of F.VIIIa by F.IXa. Although it is possible that the Gla domain of F.IXa contacts the F.VIIIa molecule directly, it is more likely that the alignment of proper intermolecular contact points is critically dependent on residue 12 of F.IXa, and that the Gly \rightarrow Arg mutation exerts its effect on F.VIIIa binding through this mechanism.

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