Characterization of γ-Carboxyglutamic Acid Residue 21 of Human Factor IX†

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Received February 29, 1996; Revised Manuscript Received May 6, 1996⊗

ABSTRACT: We investigated the functional role of γ-carboxyglutamic acid (Gla) residue 21 of human factor IX, using site-directed mutagenesis to change the glutamic acid residue to aspartic acid (FIX21D). FIX21D had reduced activity in an activated partial thromboplastin time (aPTT) assay and was activated by factor XIa more slowly than wild-type factor IX (FIXwt). FIX21D underwent normal, two-stage calcium-dependent intrinsic fluorescence quenching, indicating that a folding event similar to that seen in FIXwt occurred upon the addition of calcium ions. Antibody A-7, which recognizes factor IX-specific residues at positions 33−40, bound FIX21D as well as FIXwt; however, the calcium-specific monoclonal antibody, JK-IX-2, whose epitope includes residues 1 and 22, did not recognize FIX21D. FIX21D bound phosphatidylserine/phosphatidylcholine (PS/PC) vesicles with K_d approximately 10-fold greater than FIXwt, as measured by a fluorescence light scattering assay. Finally, although FIXwt binds endothelial cells with a K_d of 2.8 nM, FIX21D did not bind endothelial cells. Molecular modeling simulations of FIXwt and FIX21D indicate that mutating Gla 21 to Asp causes structural changes in residues 3−5 and 8−10, as well as in two exposed calcium ions, consistent with the reduced function of FIX21D. Immunological and intrinsic fluorescence quenching assays and the molecular dynamics simulations suggest normal folding in the C-terminal region of the Gla domain. Thus we hypothesize that FIX21D has reduced JK-IX-2 and phospholipid and endothelial cell binding due to localized structural changes in residues 3−10 and the exposed calcium ions. Our study suggests that the Gla 21 to Asp mutation disrupts function in the N-terminal region of the Gla domain without affecting structure in the C-terminal Gla domain region.

Factor IX is a 57 kDa serine protease zymogen which has sequence homology with other blood proteins including factor X, factor VII, protein C, protein S, and protein Z (Nelsestuen *et al*., 1974; Stenflo *et al*., 1974). These proteins require a vitamin K-dependent, posttranslational modification of glutamic acid residues to γ-carboxyglutamic acid (Gla)[1] for physiological activity. The modification occurs at a limited number of residues in the N-terminal region known as the Gla domain (Stenflo *et al*., 1974; Nelsestuen *et al*., 1974; Zytkovicz & Nelsestuen, 1975; Fernlund *et al*., 1975; Esmon *et al*., 1975). The Gla domains of vitamin K-dependent blood proteins are required for calcium-dependent phospholipid membrane binding (Nelsestuen, 1976). The factor IX Gla domain has further been implicated in endothelial cell binding and factor XIa recognition (Cheung *et al*., 1991, 1992; Liebman *et al*., 1987).

Three pairs of Gla residues are conserved among the Gla domains of coagulation and anticoagulation factors: residues 7 and 8, 20 and 21, and 26 and 27 (using factor IX numbering). From the crystal structure of the bovine prothrombin Gla domain, it is likely that the first pair of Gla residues, residues 7 and 8, aids in maintaining the proper N-terminal loop structure through stabilizing ligations with four calcium ions (Soriano-Garcia *et al*., 1992). The prothrombin structure indicates that the Gla residues in the second conserved pair of Gla's, residues 20 and 21, lie in significantly different environments. While both Gla 20 and Gla 21 bind two solvent-exposed calcium ions, which may be involved in a membrane-binding calcium bridge, Gla 21 additionally appears to stabilize the N-terminal loop structure through a calcium ligation and electrostatic interactions with the N-terminus.

Much attention has been given to the specific functions of individual Gla residues in the Gla domain. Zhang and Castellino (1992, 1993) and Zhang *et al*. (1992) mutated each glutamic acid residue of anticoagulation factor protein C to aspartic acid and reported the effects of each mutation on protein activity, calcium-dependent intrinsic fluorescence quenching, and phospholipid binding. They observed that mutating Gla 20 of protein C (equivalent to Gla 21 in human factor IX) decreased the activity and increased the calcium concentration required to achieve half-maximal intrinsic fluorescence quenching approximately 4-fold. They also observed that the dissociation constant of the Gla mutant to

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⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

[1] Abbreviations: Gla, γ-carboxyglutamic acid; FIXwt, factor IX wild type; FIX21D, recombinant factor IX containing aspartic acid substituted for glutamic acid at position 21; aPTT, activated partial thromboplastin time; PTT, partial thromboplastin time assay; PS/PC, phosphatidylserine/phosphatidylcholine; EGF, epidermal growth factor-like domain; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBS, Tris-buffered saline (20 mM Tris, 136 mM NaCl, pH 7.3); RMSD, root mean square deviation.

S0006-2960(96)00502-8 CCC: $12.00

phospholipid vesicles was 3-fold increased from that of wild-type recombinant protein C. Ratcliffe *et al.* (1993), likewise, observed a similar loss of activity and a 4-fold decrease in the affinity of prothrombin mutant E20D (equivalent to Gla 21 of human factor IX) to phospholipid vesicles. Additionally, Gla 21 is critical for *in vivo* function; a patient whose glutamic acid residue 21 is replaced with lysine has about 50% of the expected antigen level, with no intrinsic factor IX activity (Hamaguchi *et al*., 1991).

We investigated the structural and functional role of Gla residue 21 of factor IX. Using *in vitro* mutagenesis, we changed glutamic acid residue 21 to aspartic acid (FIX21D) and examined the effect on clotting activity, factor XIa activation, fluorescence quenching, phospholipid binding, and endothelial cell binding. We also examined the structural effects of this mutation on the factor IX Gla domain using monoclonal antibodies against the Gla domain of factor IX. Finally, we performed molecular dynamics simulations on models of the wild-type structure and on the mutant structure in order to compare the differences observed in these structures with those observed experimentally.

MATERIALS AMD METHODS

Proteins. Human factor IX was purified from human plasma as described (Smith & Ono, 1984). Human factor XIa, a gift from Dr. David Straight, was purified according to Braunstein *et al*. (1981).

Four factor IX-specific monoclonal antibodies were used in this study: A-1, A-5, A-7, and JK-IX-2 (Smith *et al*., 1987; Sugo *et al*., 1990). Monoclonal antibodies A-1 and A-5 recognize the factor IX activation peptide (residues 147−153) and the heavy chain (residues 180−310), respectively (Frazier *et al*., 1989). Antibody A-7 is conformation-specific and divalent metal ion-dependent, recognizing residues 33−40 of the factor IX Gla domain in the presence of calcium or magnesium ions (Cheung *et al*., 1995). Antibody JK-IX-2 is a calcium-specific monoclonal antibody which recognizes the N-terminal region of the factor IX Gla domain (Sugo *et al*., 1990; Cheung *et al*., 1996).

In Vitro Mutagenesis and Construction of the Expression Plasmid. Site-directed mutagenesis was accomplished as described by Kunkel (1985). The synthetic oligonucleotide used for mutagenesis (5′-GAGAGAGAATGCATGGAA-GATAAGTGTAGT-3′) (Oligos Etc. Inc., Guilford, CT) introduced the *Nsi*I restriction site into the factor IX cDNA. The mutated cDNA was sequenced by the dideoxy chain termination method using the Sequenase reagent kit (United States Biochemical, Cleveland, OH) (Tabor & Richardson, 1987). Site-directed mutagenesis and sequencing were carried out using the phagemid pMA254 (Kramer & Fritz, 1987), and factor IX mutant cDNA was ligated into the expression vector pCMV4 at the *Bam*HI site (Thompson *et al*., 1984).

Cell Culture and Transfection by the $CaPO_4$ Method. Human embryonic kidney 293 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 medium supplemented with 10% fetal calf serum. The transfection was performed using the calcium phosphate coprecipitation method described by Graham and van der Eb (1973). Ten micrograms of pCMV4 factor IX cDNA, 10 μg of pCMV5 carboxylase DNA (Wu *et al*., 1991), and 2 μg of pSV2-neo (Berk & Sharp, 1978) were used to transfect cells grown in 100 mm dishes. Cells were passaged 1:2 and grown in media supplemented with 500 μg/mL geneticin (G418) (Gibco) plus 1 μg/mL vitamin K (Phytonadione, Abbott Laboratories) 2 days after transfection. G418-selected colonies were isolated with cloning cylinders and placed into 12-well dishes. Each clone was grown to confluence and assayed for factor IX expression using an immunoradiometric assay. Clones with the highest levels of factor IX expression were expanded for further production.

Purification of Recombinant Factor IX. Initial purification of FIX21D was accomplished according to the method of Yan *et al*. (1990) using a Q Sepharose Fast Flow resin (1.5 cm column diameter) with a 2 mL bed size per liter of media. Protein was eluted with TBS (20 mM Tris, 136 mM NaCl, pH 7.3) and a calcium chloride gradient from 0 to 30 mM in 60 mL. FIX21D eluted from the Q column between 9 and 11 mM calcium chloride. Peak fractions of factor IX determined by SDS−PAGE were pooled and concentrated. Q Sepharose-purified FIX21D was then affinity purified using the metal-dependent, conformation-specific monoclonal antibody, A-7, coupled to Affi-Gel 10 (Bio-Rad). After extensive washing with TBS containing 20 mM $MgCl_2$ and 5 mM $CaCl_2$, FIX21D was eluted from the A-7 affinity column with 20 mM EDTA in TBS and concentrated with a Centricon 30 ultrafilter (Amicon), and the concentration was determined using the Bio-Rad assay.

Gla Analysis. Gla analysis was performed according to the procedure of Przysiecki *et al*. (1987), with alkaline hydrolysis following the method of Price *et al*. (1983).

Clotting Assay. One-stage activated partial thromboplastin time (aPTT) assays were performed according to the manufacturer's instructions (Sigma). Partial thromboplastin time (PTT) assays, in which FIXwt or FIX21D was activated prior to the clotting assay, were also performed. Briefly, FIXwt or FIX21D was incubated for 2 h at 37 °C with factor XIa and checked for full activation by SDS−PAGE. Aliquots were then subjected to a clotting assay similar to the aPTT assay, except without preincubation of the active proteins with the factor IX-deficient plasma. The ability of the factor IX proteins to correct the clotting time of factor IX-deficient plasma was compared to a standard curve using plasma-purified factor IX.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970).

Activation by Factor XIa. FIXwt or FIX21D (0.877 μM) was incubated with 9.43 nM factor XIa in TBS, pH 7.5, containing 5 mM $CaCl_2$ at 37 °C. Aliquots of the ongoing reaction were withdrawn and subjected to analysis after treatment with SDS and 2-mercaptoethanol. Gels were quantitated using densitometry analysis after silver staining.

Intrinsic Fluorescence Quenching. Intrinsic calcium-dependent fluorescence quenching was performed according to the method of Nelsestuen *et al*. (1976) and Prendergast and Mann (1977) using a Shimadzu RF-5000 spectrofluorometer with excitation and emission wavelengths of 280 and 340 nm, respectively.

Phospholipid Binding. Phospholipid vesicles were prepared using variations of the method of Barenholtz *et al*. (1977). Two milligrams of phosphatidylserine (PS) and 6 mg of phosphatidylcholine (PC) (25%/75%) dissolved in chloroform were mixed and dried under nitrogen, resuspended in TBS, and sonicated at room temperature for 30

min using 10 s pulses followed by 20 s of cooling. The suspension was sedimented by centrifugation at 140000*g* for 1 h. The upper third of the supernatant was removed, assayed for phosphorus content by the method of Chen *et al*. (1956), and used as phospholipid vesicles within 24 h of preparation. The phospholipid binding assay was performed and analyzed according to the method of Nelsestuen and Lim (1977).

Endothelial Cell Binding. Bovine aortic endothelial cells were seeded at 3×10^4 cells/cm^2 and grown to confluence in 48-well microtiter plates in Dulbecco's modified Eagle's medium containing penicillin and streptomycin and supplemented with 20% fetal calf serum. Confluent wells (1 cm^2) contained an average of 2.2×10^5 cells. Binding assays and analysis of data were performed as previously described (Cheung *et al*., 1991).

Iodination of Protein. One hundred micrograms of monoclonal antibody was labeled with 1 mCi of $Na^{125}I$ (Amersham) using Iodobeads according to the manufacturer's directions (Pierce). Labeled antibody was purified from free $Na^{125}I$ using a Sephadex G-25 column.

Immunoradiometric Assay. One hundred microliters of the factor IX-specific antibody, A-5, was coated onto a 96-well microtiter plate at 50 μg/mL. Factor IX samples, diluted in TBS containing 0.1% BSA, were added to the wells and left at either 4 °C overnight or 37 °C for 4 h. ^{125}I-radiolabeled antibody diluted in TBS containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added to the wells at 1×10^5 cpm/well. After 4 h at room temperature, unbound A-7 antibody was removed and each well was measured for radioactivity.

Molecular Modeling of Factor IX Gla Domain. The modeling of the Gla domain of human coagulation factor IX was reported previously (Li *et al*., 1996). The wild-type model of factor IX (1−47) was based on the crystal structure of calcium-bound bovine prothrombin fragment 1 (Soriano-Garcia *et al*., 1992). The mutant structure was constructed from the model of the wild type through computational mutation of Gla 21 to Asp. Molecular dynamics simulations (160 ps) on both proteins were carried out using the protocol reported previously (Li *et al*., 1995), except that an advanced technique, the Particle−Mesh−Ewald method, was employed to evaluate the Coulombic forces without truncation (Darden *et al*., 1993).

RESULTS

Gla Analysis. Purified FIX21D was measured to have 9.35 ± 0.05 Gla/molecule of an expected 11 Gla/molecule, one Gla residue less than human FIXwt which was measured to have 10.5 ± 0.2 Gla/molecule out of a total possible 12 Gla/molecule (Table I). The total yield of carboxylated FIX21D was approximately 208 μg/L of serum-free culture media.

Clotting Activity Assays. FIX21D had reduced clotting activity (9.7 ± 4%) when compared to plasma-purified factor IX in a standard one-step activated partial thromboplastin time (aPTT) assay (Table I). A clotting assay using activated recombinant factor IX (PTT) indicated that the activated form of FIX21D had 7.1 ± 0.9% activity compared to plasma-purified FIXwt.

Activation by Factor XIa. FIX21D exhibited a 3-fold reduced rate of activation by factor XIa compared to FIXwt as visualized through SDS−PAGE (5.5×10^{-4} s^{-1} and 1.65×10^{-3} s^{-1}, respectively).

Table 1: Gla Analysis, Clotting Activity, Fluorescence Quenching Data, PS/PC Binding, Endothelial Cell Binding, and Antibody Binding Results

Gla analysis	FIXwt	FIX21D
Gla analysis	10.5 ± 0.2/12	9.35 ± 0.05/11
aPTT activity (%)	100	9.7 ± 4.0
PTT activity (%)	100	7.1 ± 0.9
ΔF_{max} (%)	40	37
C_{50} of Gla domain quenching (mM)	0.5	0.4
PS/PC binding (μM)	0.97 ± 0.55	8.67 ± 0.63
endothelial cell binding (nM)	2.8	ND[a]
A-7 binding (%)	100	100
JK-IX-2 binding (%)	100	ND

[a] ND, no binding detected.

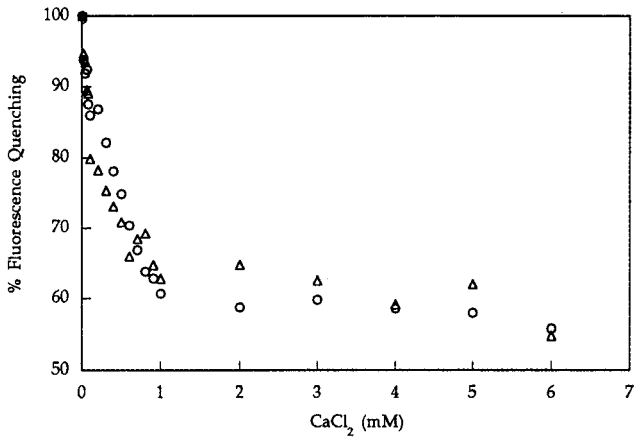

FIGURE 1: Intrinsic calcium-dependent fluorescence quenching. FIXwt (○) and FIX21D (△) were titrated with sequential additions of calcium chloride, and the fluorescence intensity was measured. Percent fluorescence quenching was determined by $F/F_0 \times 100$.

Fluorescence Quenching. FIX21D underwent two-stage calcium-dependent intrinsic fluorescence quenching at calcium concentrations similar to those of FIXwt (Figure 1). Initial rapid quenching, characteristic of a calcium binding event outside of the Gla domain, occurred at low calcium concentrations for both plasma-purified factor IX and FIX21D. The total percent change in fluorescence intensity as well as the calcium concentration required for half-maximal quenching (C_{50}) of the Gla domain dependent phase of the quenching response for FIX21D was similar to FIXwt (Table I).

Phospholipid Binding Assay. Phospholipid binding assays indicated that FIX21D had a reduced affinity for phospholipid vesicles compared to FIXwt. The K_d of binding of FIXwt to PS/PC vesicles was 0.97 ± 0.55 μM, similar to the previously reported K_d (Burri *et al*., 1987; Sugo *et al*., 1990) (Table I). FIX21D bound PS/PC vesicles with a K_d of 8.67 ± 0.63 μM. Interestingly, a factor IX construct in which glutamic acid residue 20 was changed to aspartic acid, FIX20D, bound PS/PC vesicles with a K_d only 4-fold above that of FIXwt (2.63 ± 0.06 μM). We were, however, unable to purify FIX20D to its fully carboxylated form.

Endothelial Cell Binding. The endothelial cell binding assay used radiolabeled FIXwt with cold recombinant factor IX as a competitor. The K_d of binding of FIXwt to endothelial cells was 2.8 nM. FIX21D showed no competition with radiolabeled FIXwt in binding endothelial cells even using a 100-fold higher concentration of FIX21D competitor

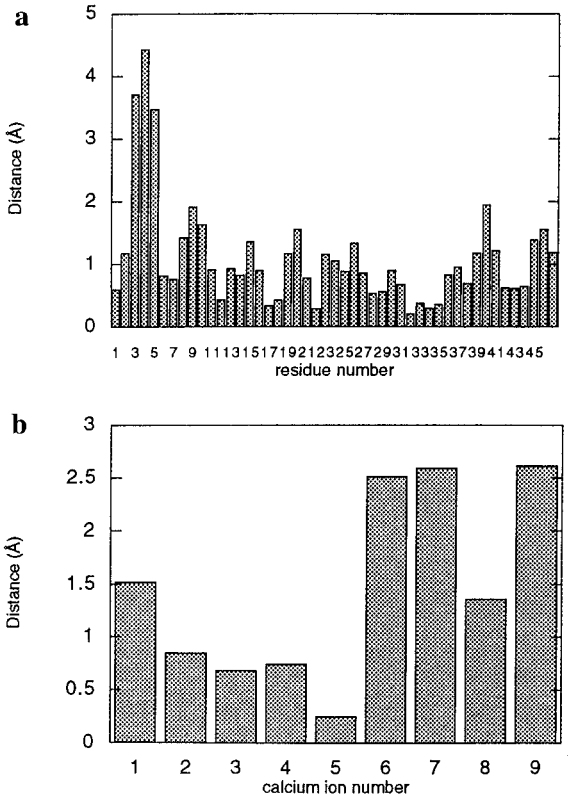

FIGURE 2: Alignment based on lowest RMSD in residues 13−47: (a) distances between α-carbons in the aligned FIXwt and FIX21D structures; (b) distances between the calcium ions in the aligned FIXwt and FIX21D structures.

relative to FIXwt (Table I). The incompletely purified protein FIX20D, conversely, displaced radiolabeled FIXwt on endothelial cells (K_d of 8.1 nM).

Radioimmunoassay. Radioimmunoassays were conducted using the metal-dependent monoclonal antibody, A-7, and the calcium-specific antibody, JK-IX-2. Antibody A-7 bound FIX21D with 100% affinity relative to FIXwt. The antibody JK-IX-2 had no affinity toward FIX21D (Table I).

Molecular Modeling. Molecular dynamics simulations were continued on both the FIXwt and FIX21D models until the systems reached equilibrium. An average structure of the final 16 ps of each simulation was constructed and used for analysis. Coordinations between calcium ions and Gla residues remained fairly consistent between the FIXwt and FIX21D structures. In the FIX21D simulation model, calcium ions 1, 3, and 6 (Figure 3) lost 1 protein coordination, calcium ions 2 and 5 lost 2 protein coordinations, and calcium ion 7 gained 1 coordination to residue 20, compared to FIXwt. The FIX21D simulation model was less stable than FIXwt based on the root mean square deviation (RMSD) of the backbone atoms of the two proteins (1.7 *versus* 1.1 Å, respectively) when compared to their initial model structures (data not shown). Alignment of the two molecules based on the lowest RMSD in residues 13−47 was used to compare the differences in the positions of the α-carbons and calcium ions of the two models (Figures 2 and 3). Residues 3−5 exhibited significantly larger than average structural changes in the FIX21D model compared to the FIXwt model (3.7, 4.4, and 3.4 Å between the aligned FIXwt and FIX21D α-carbons, respectively, *versus* an average distance of 1.1 Å) (Figure 4). Neither residues 1 nor 21 exhibited a significant change from the FIXwt structure. The

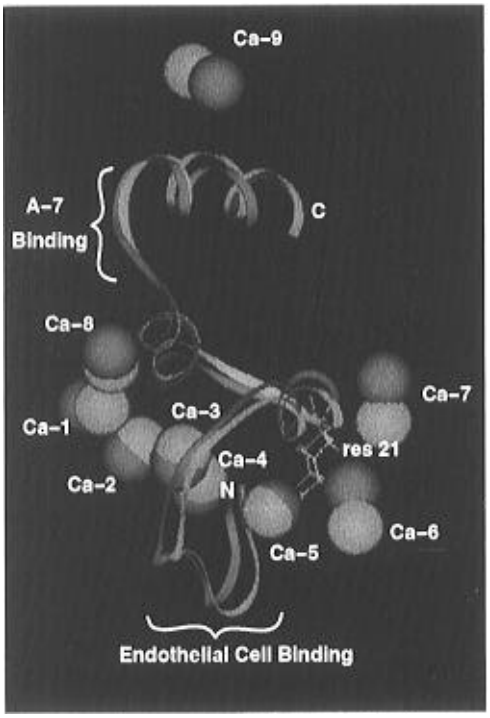

FIGURE 3: Model of the human factor IX Gla domain (overlay based on residues 13−47). FIXwt and associated calcium ions are shown in cyan; FIX21D and associated calcium ions are shown in magenta. The endothelial cell (FIXwt) and A-7 (FIXwt and FIX21D) binding regions are indicated.

highly coordinated calcium ions 2−5 remained stable in the FIX21D simulation; however, the less coordinated calcium ions, including calcium ion 7 and calcium ion 6, which is the calcium ion proposed to be involved in an ion bridge to the phospholipid membrane (Zhang & Castellino, 1993), exhibited considerable movement in the FIX21D structure, relative to FIXwt.

DISCUSSION

γ-Carboxylation of glutamic acid residues in the Gla domains of the clotting factors is an interesting, physiologically significant posttranslational modification. The Gla residues are critical for calcium binding, proper structure formation, phospholipid binding, and physiological activity. The functional significance of individual Gla residues in prothrombin and protein C has been examined by mutating the normally carboxylated glutamic acid residue to the noncarboxylated, but similar residue, aspartic acid. From these earlier studies, it has been shown that Gla residue 21 (factor IX numbering) is important for activity in both protein C and prothrombin (Zhang & Castellino, 1992, 1993; Zhang *et al*., 1992; Jhingan *et al*., 1994; Ratcliffe *et al*., 1993). Additionally, a patient having the natural mutation to lysine at this position in factor IX has no detectable factor IX activity (Hamaguchi *et al*., 1991). We therefore mutated residue 21 of factor IX to aspartic acid to examine the functional significance of this residue in factor IX.

FIX21D had reduced aPTT and PTT activity compared to FIXwt, and SDS−PAGE analysis of an activation time course of FIX21D indicated that FIX21D was activated by factor XIa more slowly than was FIXwt. Thus FIX21D possesses reduced enzymatic activity as well as a reduced ability to be recognized or cleaved by factor XIa. The factor IX binding region of factor XIa has been mapped to a site

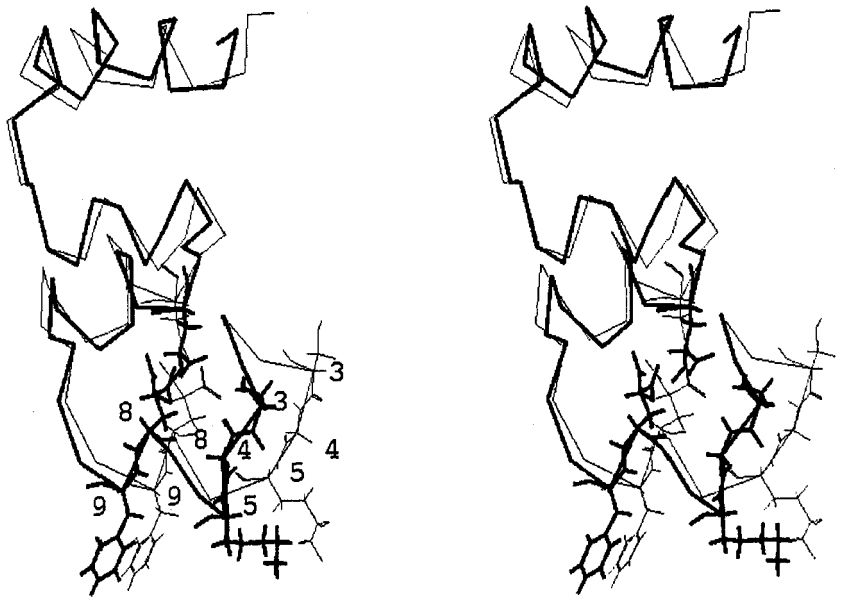

FIGURE 4: Stereoview of a C_α trace of the FIXwt (black) and FIX21D (gray) Gla domains (overlay based on residues 13−47). Residues 3, 4, 5, 8, 9, and 21 are shown.

distal to the factor XIa active site (Baglia *et al*., 1991). Thus it is likely that factor IX similarly possesses a factor XIa binding region spatially distinct from the activation sites. It is not surprising, therefore, that mutating residue 21 within the Gla domain disrupts the factor IX/factor XIa interaction and reduces the activation rate. Lu *et al*. (1994) observed that a mutation to alanine at the homologous residue of protein C affects the ability of activated protein C to cleave factor V, further implicating residue 21 in protein−protein recognition affecting regions distal to the Gla domain. Mutating residue 21 (factor IX numbering) to aspartic acid similarly reduces the activity of both protein C and prothrombin (Zhang & Castellino, 1992; Ratcliffe *et al*., 1993).

Several of the vitamin K-dependent proteins exhibit a calcium-dependent intrinsic fluorescence quenching event contingent on calcium binding sites both within and external to the Gla domain (Nelsestuen *et al*., 1976; Morita *et al*., 1984; Schwalbe *et al*., 1989). Titrating factor IX with calcium ions causes an initial drop in fluorescence intensity at low calcium concentrations (below 0.4 mM $CaCl_2$), resulting from a calcium-dependent binding event outside of the Gla domain (Morita *et al*., 1984). Factor IX subsequently undergoes Gla domain-dependent fluorescence quenching at higher calcium concentrations. The Gla domain-dependent fluorescence quenching response is believed to result from the changing environment of Trp 42 relative to the disulfide bridge between residues 18 and 23, which occurs upon folding to the calcium-dependent structure (Persson *et al*., 1991). In our studies, FIX21D exhibited maximal fluorescence quenching and a C_{50} similar to those of FIXwt, indicating that the calcium ion-induced conformational changes surrounding Trp 42 in FIX21D are similar to those observed in FIXwt. Because a lack of calcium-dependent fluorescence quenching indicates structural defects in the Gla domain, the similarity in calcium-dependent fluorescence quenching behavior between FIX21D and FIXwt suggests that the conformation of the FIX21D Gla domain, at least in the region near Trp 42 and the disulfide bridge, approximates that of the native factor IX structure. Our simulation model of FIX21D supports this conclusion; the disulfide bridge (residues 18 and 23) and Trp 42 do not significantly deviate in structure in the FIX21D model compared to that of FIXwt (Figures 2 and 3). Zhang and Castellino (1992) noticed a 3.8-fold increase in the calcium concentration required for half-maximal quenching in their Gla to Asp mutation at the equivalent position in protein C (rPC20D). The intrinsic calcium-dependent fluorescence quenching event differs between factor IX and protein C; we observed a ΔF_{max} for the Gla domain dependent quenching of factor IX of approximately 26%, while the ΔF_{max} for protein C is only approximately 15% (Zhang & Castellino, 1992). This difference may indicate a variation in the environment of Trp 42 in the protein C Gla domain from the factor IX Gla domain. The lower calcium requirement for intrinsic fluorescence quenching and activity of FIX21D compared to rPC20D, *versus* each of their wild-type counterparts, may result from a difference in calcium binding in the C-terminal regions of the two Gla domains, possibly involving the three additional Gla residues present in the C-terminal region of the factor IX Gla domain relative to protein C.

We used both calcium-specific and metal-dependent monoclonal antibodies to evaluate FIX21D for its ability to bind metal ions in the same conformationally specific manner as FIXwt. FIX21D bound A-7 normally, indicating normal folding and epitope expression in residues 33−40. In conjunction with the normal calcium-dependent fluorescence quenching observed in FIX21D and molecular modeling observations, these results imply that the C-terminal region of the FIX21D Gla domain through the aromatic stack region, including residue 42 and likely the disulfide bond between residues 18 and 23, approximates the native factor IX structure.

FIX21D failed to bind antibody JK-IX-2, however, indicating the loss of this epitope which includes the N-terminal region of factor IX (Cheung *et al*., 1996). FIX21D also failed to bind bovine aortic endothelial cells, the binding of which is mediated in residues 3−11 (Cheung *et al*., 1991), in spite of the fact that FIX20D bound endothelial cells with K_d only 4-fold increased from that of

FIXwt. Additionally, the binding of FIX21D to phospholipid vesicles was reduced approximately 10-fold compared to FIXwt. Previously, Ryan *et al*. (1989) observed that phospholipid binding and endothelial cell binding by factor IX are independent phenomena; however, residue 21 appears to be required for both endothelial cell binding and phospholipid binding.

It has been suggested that Gla domain-containing proteins participate in phospholipid binding by providing a negative charge for coordination of a calcium ion in a bridge with the phospholipid vesicle surface (Nelsestuen *et al*., 1976). The requirement of specific phospholipid molecules (phosphatidylserine and not phosphatidylcholine) for binding of the Gla domain-containing proteins (Nelsestuen, 1976) supports this theory. In studies involving the phospholipid binding protein, annexin V, with phosphatidylserine and phosphatidylethanolamine, only the negatively charged phosphatidylserine was able to properly function as a surface for annexin V; phosphatidylserine coordinated calcium ions in a conformationally specific manner, which then functioned as a bridge between the protein and phospholipid surface (Swairjo *et al*., 1995). In the Gla domain-containing coagulation proteins, Gla residue 21 (factor IX numbering) has specifically been implicated in this potential bridging interaction because it coordinates an exposed calcium ion (calcium ion 6) in the crystal structure of bovine prothrombin fragment 1; thus this calcium ion may be available for formation of a bridge with the phospholipid surface (Soriano-Garcia *et al*., 1992). In contrast to this theory, it has been suggested that the binding of Gla domain-containing proteins to phospholipid is based primarily on a residue−surface interaction between hydrophobic residues which become surface exposed upon calcium binding in the Gla domain and subsequently buried in the hydrophobic lipid membrane (Christiansen *et al*., 1994; Sunnerhagen *et al*., 1995). Indeed, calcium binding to the N-terminal region of the factor X Gla domain directs the formation of such a potential phospholipid binding structure by burying the N-terminus and exposing hydrophobic residues on the ω loop (residues 1−11) (Sunnerhagen *et al*., 1995). In support of this hypothesis, surface-exposed hydrophobic residues 5, 6, 9 (factor IX numbering) on the ω loop of protein C have recently been implicated in phospholipid binding (Zhang & Castellino, 1994; Christiansen *et al*., 1995).

In the bovine prothrombin fragment 1 structure, as well as in our model of human factor IX, residue 21 contacts the N-terminus and calcium ions 6 and 7. Mutating this residue from Gla to Asp therefore shortens the length of the side chain and reduces the number of negative charges provided for calcium ion coordination. In our model, this amino acid substitution results in a significant structural change in residues in the ω loop, as well as in calcium ions 6 and 7 (Figures 2 and 3). The movement observed in calcium ions 6 and 7 likely results from the changes in coordinations provided by Gla residues in FIX21D *versus* FIXwt. Since the salt bridge between residues 21 and the N-terminus is maintained in the FIX21D model, the high structural deviation observed in residues 3−5 and 8−10 in FIX21D is likely a secondary effect in response to the movement of calcium ion 6 away from the ω loop. The general trend in movement observed for calcium ion 6 is in a direction away from the proposed orientation of the phospholipid membrane surface relative to the Gla domain, which may reduce the phospholipid binding potential of the FIX21D mutant in the calcium bridging mechanism. Calcium ion 6 does, however, appear to remain coordinated with the FIX21D structure and is not lost to the solvent during the simulation despite the reduced coordination provided by the protein; thus it might still be able to contact the phospholipid surface in the event of a calcium bridging situation. Two hydrophobic residues which have been implicated in protein C binding to phospholipid vesicles, residues 6 and 9 (leucine, leucine and leucine, phenylalanine in protein C and factor IX, respectively), are substantially shifted with respect to the FIXwt model which may reduce the phospholipid binding potential in the hydrophobic residue burial mechanism. Thus the greatest deviation observed in the FIX21D model *versus* FIXwt occurs in the regions previously implicated in endothelial cell binding (residues 3−5, 9, and 10) and phospholipid binding (residues 6 and 9 and calcium ion 6). Indeed, it appears from these data that the reduced phospholipid binding observed for the FIX21D molecules results from a *combined* effect of the shifted calcium ion 6 and residues 3−11. The reduced coordination provided to calcium 6 may affect structure within residues 3−5 and 8−10 by an indirect mechanism, such as a change in the solvent structure or long-range electrostatic potentials in these regions. Since N-acetylation of the bovine prothrombin N-terminus also causes reduced binding to phospholipid vesicles (Welsch *et al*., 1988), it is possible that changes at the N-terminus of the Gla domain affect the phospholipid binding ability of the protein by causing a localized structural effect in the ω loop region.

Therefore, we hypothesize that the diminished binding ability of FIX21D to the calcium-specific antibody, phospholipid vesicles, and endothelial cells results from the structural changes observed in the modeling simulation. That is, residue 21 may be specifically involved in the binding epitopes of JK-IX-2, endothelial cells, and the phospholipid surface, perhaps participating through a calcium bridging mechanism via calcium ion 6. The Gla to Asp mutation therefore may alter the epitope or calcium bridging properties and directly eliminate binding. Alternatively, the Gla to Asp mutation may reduce binding to JK-IX-2, phospholipid vesicles, and endothelial cells through a localized conformational change in the ω loop. Since the mutation appears to disrupt both a potential calcium bridge and structure within residues 3−11, the reduced binding of FIX21D to phospholipid, endothelial cells, and the calcium-specific antibody likely occurs via a combined event involving both mechanisms or by differing mechanisms for each of the lost functions.

In conclusion, our study suggests that the mutation at residue 21 from Gla to Asp destabilizes residues in the ω loop and calcium ions 6 and 7, resulting in improper folding of the surface loop and loss of affinity to JK-IX-2, phospholipid vesicles, and the endothelial cell receptor. The mutation does not, however, appear to disrupt either structure or function in residues in the C-terminal region of the Gla domain. We suggest that Gla 21 is important for recognition of factor IX by factor XIa and that Gla 21 also appears to be involved in the epitope of at least one factor IX specific calcium-specific antibody, but is not required for binding of the nonspecific metal ion-dependent antibody, A-7.

ACKNOWLEDGMENT

We thank William J. VanDusen of Merck and Co. for Gla analysis. We also thank Pola Berkowitz for technical assistance and Dr. David Straight for his helpful discussions and critical reading of the manuscript. We have benefitted substantially from a computer time grant from the North Carolina Supercomputing Center.

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BI960502I