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Factor IX San Dimas

SUBSTITUTION OF GLUTAMINE FOR ARG^{-4} IN THE PROPEPTIDE LEADS TO INCOMPLETE γ-CARBOXYLATION AND ALTERED PHOSPHOLIPID BINDING PROPERTIES*

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DNA sequence analysis of the Factor IX gene from a hemophilia B patient (98% Factor IX antigen; <0.01 unit/ml clotting activity) has identified a point mutation in exon II. A guanine to adenine transition causes the substitution of a glutamine codon for an arginine codon at −4 in the propeptide of Factor IX. This variant, termed Factor IX San Dimas, circulates in the plasma as proFactor IX with a mutant 18-amino acid propeptide still attached. Like Factor IX Cambridge (Arg^{-1} → Ser), Factor IX San Dimas is unable to express metal-induced epitopes recognized by conformation-specific polyclonal antibodies. Amino acid analysis of the alkaline hydrolysate indicates that purified Factor IX San Dimas contains a reduced number of γ-carboxyglutamyl residues compared to Factor IX. However, this protein undergoes metal-induced quenching of the intrinsic fluorescence. In addition, Factor IX San Dimas is unable to interact with phospholipid vesicles. The absence of coagulant activity in Factor IX San Dimas can be attributed to impaired calcium-induced conformational changes and loss in the ability to bind phospholipid vesicles in the presence of calcium ions.

Factor IX is a critical protein in the intrinsic pathway of blood coagulation (1). Dysfunctional or absent Factor IX molecules result in the heterogeneous sex-linked bleeding disorder hemophilia B. Structural analyses of mutant Factor IX molecules have identified several aspects of synthesis and processing which are important for normal Factor IX function (2–6). One essential feature of synthesis is the role of an 18-residue propeptide in directing the selective modification of the first 12 glutamic acid residues of the Factor IX zymogen to γ-carboxyglutamyl (Gla)[1] residues (2, 4).

The Gla residues confer metal-binding properties characteristic of the vitamin K-dependent blood-clotting proteins (7, 8). Binding of metal ions induces conformational changes that are required for normal activity. These conformational changes, which allow Factor IX to interact with membrane surfaces that are required during blood clotting (9, 10), have been monitored by the quenching of intrinsic fluorescence (11, 12) and the expression of *neo*-antigenic determinants using immunologic assays (13–15). A three-state model of the Factor IX metal-induced conformational changes has been recently proposed (10). This model depicts Factor IX conformational changes proceeding through an intermediate metal-dependent conformer. The first metal-dependent transition is nonselective for divalent cations and is followed by a second transition which is selective for Ca(II) or Sr(II) cations. The second transition results in a conformer capable of binding membrane surfaces.

The role of specific domains in Factor IX that direct γ-carboxylation has been investigated with mutant Factor IXs constructed by site-directed DNA mutagenesis (4–6). These results suggest that the 18-residue Factor IX propeptide contains a recognition element, the γ-carboxylation recognition site, that directs this post-translational modification. Two naturally occurring Factor IX variants, Factor IX Cambridge (Arg^{-1} → Ser) (2) and Factor IX Oxford3 (Arg^{-4} → Gln) (3), have point mutations in their propeptide sequence that are phenotypically expressed as severe hemophilia B. Factor IX Cambridge contains a reduced number of Gla residues, is unable to undergo metal-induced fluorescence quenching, and does not bind to lipid membranes. Factor IX Cambridge is unable to assume the metal-stabilized conformation that is required for phospholipid binding and coagulant activity. Through an unknown mechanism, the point mutation at residue −1 interferes with normal γ-carboxylation (2). Factor IX Oxford3 also circulates as a prozymogen with the variant 18-amino acid propeptide still attached. However, despite a mutation at residue −4 in the propeptide, Factor IX Oxford3 was initially reported to contain a normal number of Gla residues and to interact with a monoclonal antibody that recognizes a metal ion-induced epitope (3). More recently, an analog of Factor IX Oxford3 has been prepared by site-specific mutagenesis and *in vitro* expression in a heterologous dog kidney expression system (6). The recombinantly synthesized Factor IX Oxford3 failed to bind to barium citrate suggesting

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[1] The abbreviations used are: Gla, γ-carboxyglutamic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

that the extent of γ-carboxylation was overestimated in the initial report.

The observation that Factor IX Cambridge and Factor IX Oxford3 both have mutations in the propeptide region but that only the mutation at −1 in Factor IX altered carboxylation is not in accord with the demonstration of the role of the propeptide in the carboxylation of Factor IX (4, 5). Furthermore, the observation that recombinant mutagenized Factor IX ($Arg^{-4} \rightarrow Gln$) is not fully carboxylated, in contrast to the naturally occurring hemophilia B mutant Factor IX Oxford3 ($Arg^{-4} \rightarrow Gln$), suggested that reexamination of the properties of Factor IX containing a glutamine in place of arginine at residue −4 might resolve this discrepancy. In the current paper, we report on the properties of Factor IX San Dimas, a naturally occurring mutant Factor IX with the same defect as Factor IX Oxford3. We demonstrate that Factor IX San Dimas is not fully carboxylated and that it is unable to bind to phospholipid vesicles.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of the Mutant Factor IX Gene—Genomic DNA was isolated by the standard procedure (16) from the blood of a severe hemophilia B patient who synthesized a mutant Factor IX termed Factor IX San Dimas. *Eco*RI genomic DNA fragments from a complete digest were gel-purified. These fragments represent the eight Factor IX San Dimas exons according to the normal Factor IX sequence (17). The purified fragments were used to construct a recombinant DNA bacteriophage library. Without prior amplification, the library was screened using nick-translated (18) subclones containing exons II through VIII of the Factor IX Alabama gene (19). Another recombinant plasmid containing Factor IX sequences from exon I was kindly provided by C. Shoemaker (Genetics Institute) and was used to isolate exon I of the Factor IX San Dimas gene (20).

Plaque-purified Factor IX San Dimas bacteriophage DNA was prepared by a DEAE purification scheme (21). Factor IX San Dimas DNA was subcloned into M13mp18 or M13mp19 using a strategy which minimized the sequencing of intron sequences (22). Several synthetic oligonucleotides were also synthesized to match intron sequences near the Factor IX intron/exon boundary. The DNA sequence analysis utilized the Klenow fragment of DNA polymerase I in a dideoxy chain termination reaction with [α-^{35}S]dATP (23, 24).

DNA from the patient with Factor IX San Dimas and a normal individual was restricted with *Hae*III and analyzed by Southern blotting (25). The nitrocellulose filter was hybridized with a nick-translated fragment of normal Factor IX as described and washed under stringent conditions (68 °C in 0.1 × SSC (3 M NaCl, 0.3 M sodium citrate)). The filter was monitored by autoradiography at −70 °C using a Cronex Lightning Plus intensifying screen and Kodak X-Omat film.

Immunoassays—The interactions of the mutant and normal Factor IX proteins with conformation-specific polyclonal antibodies specific for the metal-stabilized conformers were evaluated in a competition radioimmunoassay. Normal and hemophilic plasma were serially diluted and incubated with anti-Factor IX:total polyclonal antibodies[2] which recognize Factor IX in the presence or absence of metal ions (15). For the identification of *neo*-antigens induced by metal ions, anti-Factor IX:Mg(II) antibodies were used (10). The interaction between the antibody and Factor IX was assessed as previously described (2).

Protein Purification and Characterization—Factor IX San Dimas was isolated from plasma using DEAE-Sephacel and immunoaffinity chromatography (2). Briefly, Factor IX San Dimas plasma (200 ml) was applied to a DEAE-Sephacel (Pharmacia LKB Biotechnology Inc.) column equilibrated with 0.1 M potassium phosphate, 1 mM benzamidine (pH 7.5). The column was eluted batchwise with 0.5 M potassium phosphate, 1 mM benzamidine (pH 7.4), 8 mM $MgCl_2$, and fractions containing Factor IX antigen were dialyzed against 0.05 M Tris-HCl (pH 7.4), 0.5 M NaCl, 0.5% Tween 20, 1 mM benzamidine (pH 7.4), 8 mM $MgCl_2$. Fractions containing Factor IX antigen were further purified using an anti-Factor IX:Mg(II) antibody-Sepharose column equilibrated as above to remove exogenous Factor IX from normal plasma transfused into the patient. The flow-through material, containing the purified mutant Factor IX San Dimas, was made 10 mM with regard to EDTA and repurified on a column of anti-Factor IX:total antibodies coupled to Sepharose and equilibrated in 0.05 M Tris-HCl (pH 7.4), 0.5 M NaCl, 0.05% Tween 20, 1 mM benzamidine, 1 mM EDTA. The Factor IX San Dimas was eluted with 4 M guanidine HCl and applied to a C4 reverse-phase high pressure liquid chromatography column (4.6 × 30 mm) equilibrated in 0.1% aqueous trifluoroacetic acid. The proteins were eluted with a linear gradient of acetonitrile from 0 to 60% in 0.1% trifluoroacetic acid, and the protein peaks were monitored by optical density at 214 and 280 nm. Factor IX San Dimas fractions were concentrated by lyophilization.

To allow direct comparison, Factor IX from normal plasma was purified by the identical procedure employed for Factor IX San Dimas. Purified Factor IX and Factor IX San Dimas were subjected to electrophoresis in 10–15% gradient SDS-PAGE gels (Phastgel, Pharmacia) and visualized by staining with Coomassie Blue (26). The amino-terminal amino acid sequence of Factor IX San Dimas was determined by electrotransferring Factor IX San Dimas onto polyvinylidene difluoride paper (27), followed by automated Edman degradation of the stained band on the blot using an Applied Biosystems model 470A gas-phase protein sequenator (28). The γ-carboxyglutamic acid analysis of the alkaline hydrolysate of 1 nmol of Factor IX San Dimas was performed as previously described (5). Amino acid standards (Pierce Chemical Co.) and DL-γ-carboxyglutamic acid (Sigma) were available from commercial sources. The derivatized amino acids were detected with a sensitivity of 10 pmol. Factor IX coagulant activity was assayed in a two-stage clotting assay using Factor IX-deficient plasma (29).

Fluorescence Quenching—Factor IX and Factor IX San Dimas were purified as described above. The Factor IX and Factor IX San Dimas peaks were lyophilized and, when redissolved, Factor IX retained full specific coagulant activity. Fluorescence quenching was evaluated on a Perkin-Elmer MPF-3 fluorescence spectrophotometer using the method of Nelsestuen *et al.* (11) and Prendergast and Mann (12). Factor IX and Factor IX San Dimas were made metal-free by dialysis against 0.1 M NaCl, 0.05 M Tris-HCl (pH 8.0), Chelex 100 (1 g/liter). A 1.0-ml aliquot of 0.5 μM Factor IX or Factor IX San Dimas was added to a fluorescence cuvette (Hellma) and the emission spectrum obtained using an excitation wavelength of 280 nm. Aliquots of 0.05 M $CaCl_2$ were added to the cuvette; following a 2-min equilibration the emission spectrum was determined at each concentration of $CaCl_2$.

Phospholipid Binding—Unilamellar phospholipid vesicles were prepared by the method of Barenholz *et al.* (30). Folch Fraction III (100 mg; brain extract, 85% phosphatidylserine) and 100 mg of phosphatidylcholine (egg yolk) were dissolved in chloroform:methanol (9:1) and dried under nitrogen. The precipitate was redissolved in benzene and lyophilized. This powder was suspended in 0.1 M NaCl and sonicated in a 4 °C water bath for 60 min. The suspension was sedimented by centrifugation at 100,000 × *g* for 25 min and at 159,000 × *g* for 3 h. The supernatant was aspirated, quantitated for phospholipid concentration by the method of Chen *et al.* (31), and used as unilamellar phospholipid vesicles.

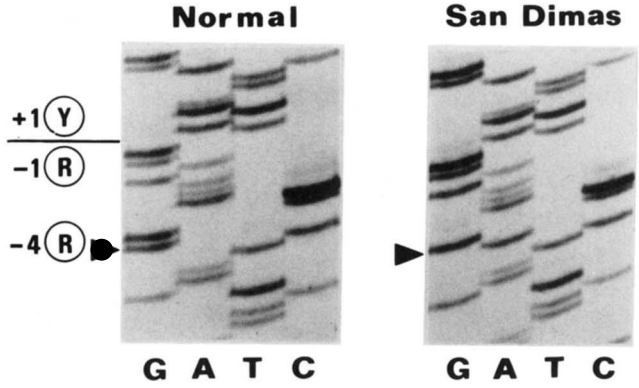
Factor IX and Factor IX San Dimas were iodinated using the Enzymobead reagent (Bio-Rad) according to the manufacturer's protocol. The iodinated protein was separated from free iodine by gel filtration and immunoaffinity chromatography using anti-Factor IX:total antibodies coupled to Sepharose. The binding of Factor IX to phospholipid vesicles was determined by gel filtration using a Pharmacia S-12 FPLC column (10 × 300 mm). The column was equilibrated in 0.15 M NaCl, 0.1% bovine serum albumin, 5 mM $CaCl_2$, 0.05 M Tris-HCl (pH 7.4). The proteins (^{125}I-Factor IX, ^{125}I-Factor IX San Dimas) and phospholipid vesicles were applied to the column at the indicated concentrations, and the column was developed with the equilibration buffer. Fractions (0.5 ml) were collected. The ef-

[2] Antibodies to Factor IX that bind to Factor IX regardless of the presence or absence of metal ions are termed *anti-Factor IX:total* antibodies. The term *anti-Factor IX:Mg(II)* antibodies is used to describe a population of conformation-specific antibodies that bind to Factor IX in the presence of metal ions, including magnesium. In contrast, *anti-Factor IX:Ca(II)-specific* antibodies only bind to Factor IX in the presence of calcium ions and not in the presence of most other metal ions, including magnesium. These two populations are derived by fractionation of *anti-Factor IX:Ca(II)* antibodies, which have characteristics nearly identical to *anti-Factor IX:Mg(II)* antibodies.

fluent was monitored by absorbance at 280 nm, phosphorus analysis, and assay of ^{125}I.

RESULTS

Factor IX San Dimas is present in the plasma of a hemophilia B patient with normal levels of Factor IX antigen (98%). However, this plasma contains less than 0.01 unit/ml of coagulant activity, as determined in a two-stage clotting assay. This analysis suggests a molecular defect at a site which is critical to normal Factor IX function. To identify the genetic defect associated with Factor IX San Dimas, recombinant bacteriophage clones containing the Factor IX San Dimas gene were constructed and isolated. The sequences of 1383 nucleotides comprising the eight Factor IX San Dimas exons were determined as were approximately 1700 nucleotides of intron sequences which flank the eight exons. This analysis demonstrated a single base substitution at nucleotide 6365 (numbers according to Ref. 17) in exon II of the Factor IX San Dimas gene. A guanine to adenine transition causes the substitution of a glutamine codon (CAG) for an arginine codon (CGG) at −4 in the propeptide of Factor IX San Dimas (Fig. 1). The presence of the nucleotide substitution in the patient genome was confirmed by Southern blot analysis. The guanine to adenine transition destroys a *Hae*III restriction site (GGCC) (Fig. 2). The autoradiograph revealed fragments at 700 base pairs, as predicted for a normal Factor IX sequence, and at 1900 base pairs, as predicted for the DNA of the Factor IX San Dimas gene. This result confirmed the presence of the mutation in the Factor IX San Dimas genome and assures that the observed mutation is not a cloning or sequencing artifact.



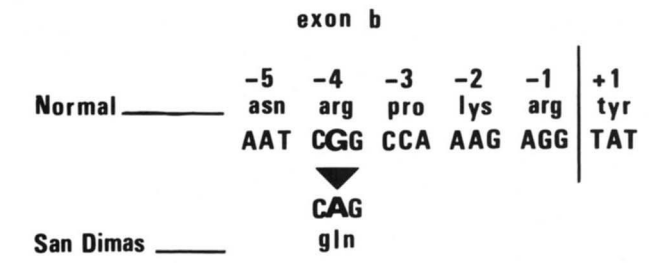


FIG. 1. **Molecular defect resulting in Factor IX San Dimas.** An autoradiograph demonstrating the transition mutation at residue 6365 (number according to Ref. 17) in exon II of the Factor IX San Dimas gene is shown. The substituted base is highlighted at the level of the *arrowhead* for normal sequence (*left*) and Factor IX San Dimas (*right*). The mutation results in a predicted change of arginine (*arg*) to glutamine (*gln*) at residue −4 of the Factor IX San Dimas propeptide and is schematically represented at the *bottom*.

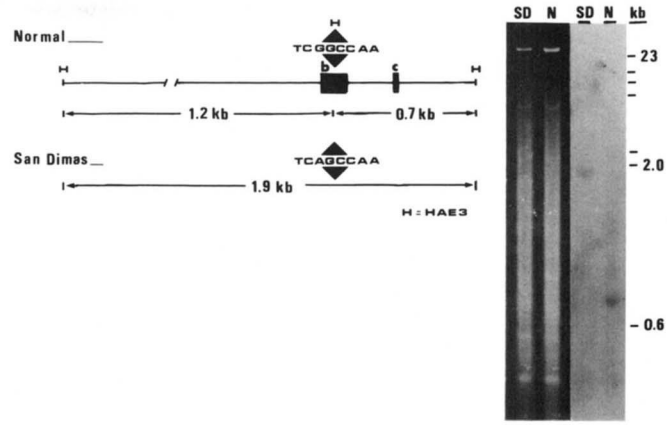


FIG. 2. **Identification of molecular defect in genomic DNA.** The guanine to adenine transition in the Factor IX San Dimas gene results in the removal of a naturally occurring *Hae*III restriction site (GGCC). Ten micrograms of DNA from a normal individual and from the patient with Factor IX San Dimas was digested to completion with *Hae*III and analyzed by Southern blotting. A cloned 0.7-kilobase pair (*kb*) restriction fragment of genomic DNA spanning Factor IX exon II was labeled and used to probe a Southern blot (*right*) of a 0.8% agarose gel (*left*). The Factor IX San Dimas autoradiograph reveals a signal at 1.9 kilobase pairs (*lane SD*) as predicted, whereas the normal genomic DNA contains a 0.7-kilobase pair fragment (*lane N*). The standard length markers are shown at the appropriate positions.

The ability of Factor IX San Dimas to undergo a metal-induced conformational transition was evaluated using a competition radioimmunoassay and polyclonal immunoaffinity-purified conformation-specific antibodies. These experiments were performed in plasma, without purification of either Factor IX or Factor IX San Dimas from their respective plasmas. In addition, the Factor IX San Dimas plasma used for this experiment was obtained from the patient when he had not had a recent Factor IX transfusion. Therefore, minimal amounts of exogenous Factor IX will be present in the plasma sample. As shown in Fig. 3*A*, both normal plasma and Factor IX San Dimas plasma contain Factor IX antigen that competes with ^{125}I-labeled Factor IX for anti-Factor IX:total antibodies. These results indicate that the plasma concentration of Factor IX antigen is similar in both normal and Factor IX San Dimas plasmas. The ability of Factor IX and Factor IX San Dimas to express metal-induced epitopes was determined using a radioimmunoassay employing conformation-specific antibodies and plasma supplemented with magnesium ions. As demonstrated in Fig. 3*B*, plasma containing Factor IX San Dimas was not significantly reactive with the anti-Factor IX:Mg(II) antibodies. In contrast, normal plasma was fully reactive with the anti-Factor IX:Mg(II) antibodies. These results indicate that Factor IX San Dimas, prior to purification, is unable to assume a metal-stabilized conformation characteristic of Factor IX. The inability of the protein to undergo the metal-induced conformational changes is consistent with the complete absence of biological function of this protein.

To allow comparison of the properties of Factor IX San Dimas with Factor IX Cambridge and Factor IX Oxford3, Factor IX San Dimas was purified from patient plasma. Because of the chronic severity of the bleeding and neurologic disorders in this patient, this plasma could only be obtained during a period in which the patient was receiving transfusion with Factor IX preparations. Therefore, exogenous Factor IX contaminated the Factor IX San Dimas in the patient plasma. Given the absence of interaction of anti-Factor IX:Mg(II) antibodies with Factor IX San Dimas (Fig. 3*B*), immunoaf-

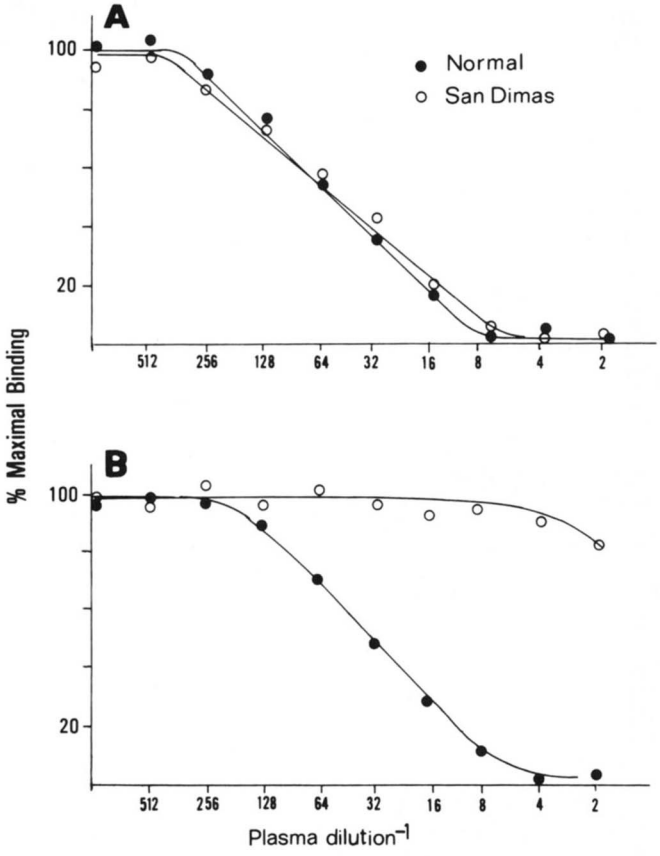


FIG. 3. **Competition radioimmunoassays with normal plasma and plasma from the Factor IX San Dimas patient.** Diluted plasma samples were reacted with anti-Factor IX antibodies which recognize total Factor IX antigen (*panel A*). Percent maximal binding (0–100%) of a competing ^{125}I-Factor IX is shown with Factor IX present in normal plasma (*solid circles*) and Factor IX present in the patient's plasma (*open circles*). An identical experiment using conformation-specific anti-Factor IX antibodies (*panel B*) demonstrates only minimal competition of the diluted patient plasma (*open circles*) with the ^{125}I-Factor IX molecule. Factor IX present in normal plasma competes with the iodinated Factor IX for the conformational specific antibodies (*solid circles*).

finity chromatography using anti-Factor IX:Mg(II) antibody-Sepharose was employed to separate native Factor IX from Factor IX San Dimas. The starting plasma (200 ml) contained 4.2 μg/ml Factor IX antigen, of which 0.58 μg/ml (14%) was recognized by anti-Factor IX:Mg(II) antibodies. The latter was exogenous Factor IX. We isolated both endogenous and exogenous Factor IX species from the starting plasma by DEAE-Sephacel chromatography with 90% recovery of Factor IX antigen as measured by competition radioimmunoassay using anti-Factor IX:total antibodies. The DEAE-Sephacel eluate was dialyzed into buffer containing 8 mM $MgCl_2$ and applied to an anti-Factor IX:Mg(II)-Sepharose column. This resulted in the quantitative removal of the exogenous Factor IX. The fraction that failed to bind to anti-Factor IX:Mg(II) antibodies, Factor IX San Dimas, was isolated using an anti-Factor IX:total Sepharose column. From the starting plasma, we recovered 480 μg of Factor IX San Dimas. This represents 57% of the initial Factor IX antigen (endogenous and exogenous) and 66% of the initial Factor IX San Dimas. Factor IX purified from plasma by DEAE-Sephacel chromatography followed by immunoaffinity chromatography using an anti-Factor IX:total Sepharose column gave a yield of 65% of Factor IX antigen.

The purified mutant protein was characterized by SDS gel

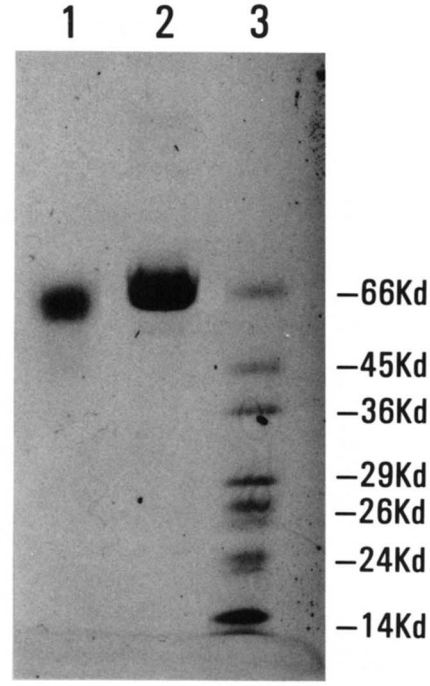


FIG. 4. **Gel analysis of Factor IX San Dimas.** Purified Factor IX and Factor IX San Dimas were analyzed in a 10–15% gradient SDS-PAGE gel in the presence of β-mercaptoethanol. *Lane 1*, purified normal Factor IX; *lane 2*, purified Factor IX San Dimas; *lane 3*, molecular weight standards. Factor IX San Dimas migrates more slowly than Factor IX, suggesting an increased molecular weight. Amino acid analysis confirmed the amino-terminal sequence Thr-Val-Phe-Leu of Factor IX San Dimas, demonstrating the presence of the 18-residue propeptide on the purified prozymogen.

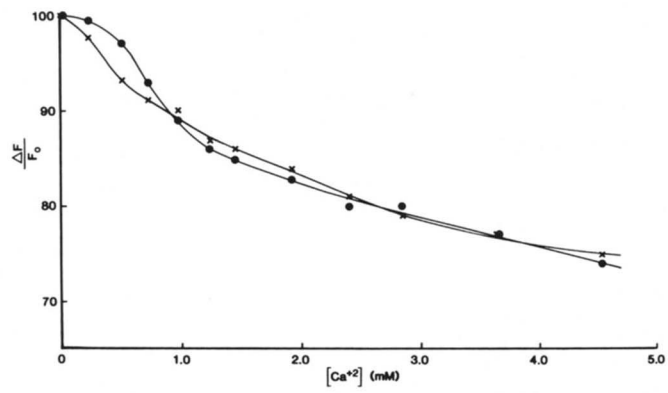


FIG. 5. **Fluorescence quenching of purified Factor IX and Factor IX San Dimas.** Aliquots of Ca(II) (0.05 M $CaCl_2$) were titrated to each purified metal-free protein and the emission spectrum was monitored using an excitation wavelength of 280 nm and an emission spectrum from 320 to 360 nm. Both Factor IX and Factor IX San Dimas undergo a similar decreased fluorescence in the presence of Ca(II) ions with half-maximal quenching at 1.0 mM Ca(II). ●, normal Factor IX; ×——×, Factor IX San Dimas.

electrophoresis. The migration, as shown in Fig. 4, is slightly retarded as compared to Factor IX. These results suggest a Factor IX species of higher molecular weight than Factor IX and are consistent with the results observed for Factor IX Cambridge and Factor IX Oxford3 due to the addition of a propeptide extension on the amino terminus. To confirm the presence of the propeptide on Factor IX San Dimas, we electrotransferred Factor IX San Dimas onto polyvinylidene difluoride paper and subjected the stained band to automated Edman degradation. The amino-terminal sequence was: Thr (46.8 pmol)-Val (6.8 pmol)-Phe (2.8 pmol)-Leu (2.6 pmol).

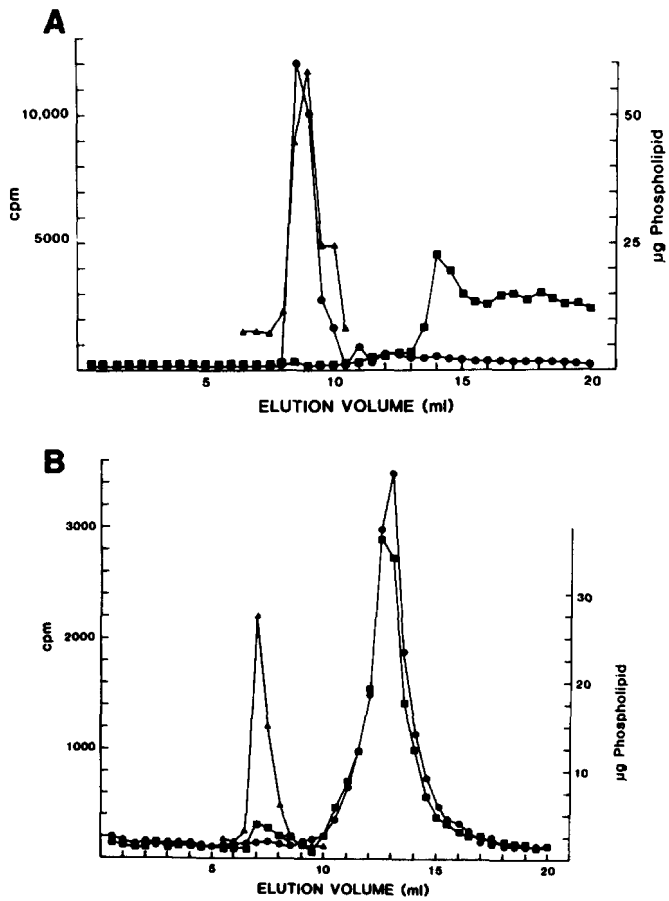


FIG. 6. **Phospholipid binding analysis of purified Factor IX and Factor IX San Dimas.** The interactions of Factor IX (*panel A*) and Factor IX San Dimas (*panel B*) with phospholipid vesicles in the presence of calcium ions were assessed by gel filtration. Under the conditions used, unilamellar vesicles elute at the void volume of 8.5 ml (*solid triangles*), while Factor IX elutes at 14-ml elution volume (*solid squares*). When Factor IX and phospholipid are run together in the presence of calcium ions, all of the Factor IX elutes in the void volume along with the phospholipid vesicles (*solid circles*). This signifies Factor IX's ability to bind the phospholipid. *Panel B* shows the results of a similar column in which phospholipid alone elutes at 7 ml (*solid triangles*) and Factor IX San Dimas elutes at 13 ml (*solid squares*). In contrast to normal Factor IX, when Factor IX San Dimas is run with phospholipid it fails to interact and continues to elute at 13 ml (*solid circles*).

This sequence is identical to that of Factor IX Cambridge, Factor IX Oxford3, and the predicted amino acid sequence of the propeptide derived from the Factor IX cDNA (32). These results indicate that, like Factor IX Cambridge and Factor IX Oxford3, Factor IX San Dimas includes the 18-residue propeptide extension characteristic of proFactor IX.

The extent of γ-carboxylation of Factor IX San Dimas was determined by direct amino acid analysis of the Factor IX San Dimas alkaline hydrolysate. Purified Factor IX San Dimas contained 7.6 ± 0.4 mol of Gla/mol of protein. In contrast, Factor IX contained 11.4 ± 0.6 mol of Gla/mol of protein, within the experimental error of the 12 residues in Factor IX. These results indicate that, like Factor IX Cambridge (2), Factor IX San Dimas is partially carboxylated.

The metal-induced conformational changes were examined by monitoring the effects of calcium on the quenching of intrinsic fluorescence. Factor IX and Factor IX San Dimas exhibited equivalent quenching of intrinsic fluorescence upon the addition of increasing concentrations of calcium ions (Fig. 5). Half-maximal transitions were observed at about 1 mM Ca(II). These results indicate that Factor IX San Dimas, in contrast to Factor IX Cambridge, is able to undergo the conformational change monitored by the quenching of intrinsic tryptophan fluorescence.

The phospholipid binding properties of Factor IX San Dimas were studied to compare the properties of this protein to Factor IX Cambridge. Because of the availability of small amounts of protein, the interaction of Factor IX San Dimas with phospholipid vesicles was studied by gel filtration analysis instead of light scattering, as previously employed (2). Fig. 6*A* demonstrates the ability of ^{125}I-labeled Factor IX to bind unilamellar phospholipid vesicles in the presence of Ca(II). In contrast to Factor IX, ^{125}I-labeled Factor IX San Dimas failed to interact with phospholipid vesicles in the presence of Ca(II) (Fig. 6*B*).

DISCUSSION

Two previously described Factor IX mutants, Factor IX Cambridge ($Arg^{-1} \rightarrow Ser$) and Factor IX Oxford3 ($Arg^{-4} \rightarrow Gln$), have point mutations in the propeptide of Factor IX that preclude normal post-translational cleavage of the propeptide in proFactor IX during biosynthesis. Both mutant proteins lack coagulant activity and circulate with the 18-amino acid propeptide attached. Factor IX Cambridge is incompletely γ-carboxylated (7 Gla residues), is unable to bind phospholipid vesicles, and fails to bind to conformation-specific polyclonal antibodies directed at the metal-stabilized conformer of Factor IX (2). In contrast, Factor IX Oxford3 is reported to be at least 90% carboxylated (3, 6) and is recognized by the metal ion-dependent monoclonal antibody A-7. These results could be intrinsically consistent since these two Factor IX mutants are not identical. However, the observation of the role of the propeptide in directing carboxylation (4, 5) and the presence of a carboxylation recognition site including residues −4 (6) and −1 (2) raised the possibility that Factor IX Oxford3 may not be fully carboxylated and that the A-7 monoclonal antibody may not be specific for the region that is defective in this mutant protein.

Factor IX San Dimas is genetically identical to Factor IX Oxford3. Therefore, study of this mutant protein allowed us the opportunity to determine the γ-carboxyglutamic acid content of this mutant protein, to evaluate metal-induced conformational transitions by the quenching of intrinsic fluorescence, and to characterize the phospholipid binding properties of a Factor IX species in which Arg^{-4} was replaced by a glutamine. As originally reported by Bentley *et al.* (3), we have confirmed that mutation at the −4 position of the propeptide precludes propeptide cleavage by a processing protease. However, Factor IX San Dimas is only partially γ-carboxylated. These results are consistent with the more recent observation that a genetically engineered recombinant Factor IX containing an arginine to glutamine mutation at −4 in the propeptide was carboxylated at very low levels (6). The failure of Factor IX San Dimas to undergo the conformational transition monitored by the conformation-specific antibodies employed and to bind to phospholipid vesicles is associated with the absence of functional activity of this protein.

Although Factor IX San Dimas and Factor IX Cambridge share many properties, at least one characteristic distinguishes these proteins. Like Factor IX, Factor IX San Dimas undergoes calcium-induced quenching of the intrinsic fluorescence. This quenching has been used to report a metal-induced transition in the vitamin K-dependent proteins (11, 12). In contrast, Factor IX Cambridge does not display calcium-induced fluorescence quenching, but like Factor IX San Di-

mas, fails to interact with the conformation-specific antibodies (2). The results for Factor IX San Dimas represent another example (33) among the vitamin K-dependent proteins of a partially carboxylated protein that undergoes complete metal-induced fluorescence quenching but does not bind to conformation-specific antibodies (34–36). These results may suggest that the distribution of Gla residues in Factor IX San Dimas differs from that of Factor IX Cambridge, but the actual distribution within these partially carboxylated proteins is not known. Further characterization of these proteins may give additional insight into the amino acids in the Gla-rich domain that are responsible for these conformational changes.

In summary, these results demonstrate that a mutation resulting in a glutamine at −4 in the Factor IX propeptide interferes with γ-carboxylation. This form of Factor IX cannot undergo the complete conformational changes characteristic of Factor IX in the presence of calcium ions and cannot bind to phospholipid vesicles in the presence of calcium ions. This mutant provides further evidence for the role of the Factor IX propeptide in post-translational carboxylation (2, 4) and confirms the role of the extended substrate binding site required by a trypsin-like propeptidase (6). The molecular basis for the absence of biological activity in Factor IX San Dimas, as well as Factor IX Cambridge, remains unclear. Some partially carboxylated prothrombins (34) and Factor IXs (20) retain significant activity, while other partially carboxylated proteins are devoid of functional activity (34, 37). Welsch and Nelsestuen (38) using prothrombin fragment 1 have implicated the amino-terminal alanine in calcium-dependent membrane interaction. The presence of the propeptide attached covalently to the Factor IX amino-terminal tyrosine, as in Factor IX San Dimas, may also inhibit calcium-dependent membrane interaction. The careful examination of the properties of fully carboxylated proFactor IX isolated via a heterologous expression system and the *in vitro* carboxylation of synthetic peptide substrates (39) should assist in identifying the basis for partial carboxylation and the basis for the absence of functional activity in these naturally occurring mutants.

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