Molecular basis of hemophilia B: A defective enzyme due to an unprocessed propeptide is caused by a point mutation in the factor IX precursor

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ABSTRACT A mutant factor IX, designated factor IX^{Cambridge}, was isolated from a patient with hemophilia B. This protein includes an 18-residue propeptide attached to the NH₂ terminus of factor IX. A point mutation at residue -1, from an arginine to a serine, precludes cleavage of the propeptide by a processing protease and interferes with γ -carboxylation of the factor IX, indicating the importance of the leader sequence in substrate recognition by the vitamin K-dependent carboxylase. This represents an example of an enzyme defect due to the presence of a point mutation in a precursor protein (preproenzyme) that is the cause of a human hereditary disease. This defect will serve as a prototype for understanding the molecular basis of some forms of hemophilia and other hereditary enzyme deficiencies.

Factor IX is a glycoprotein of M_r 56,000 whose presence in plasma is essential for the efficient progression of blood coagulation (1, 2). This protein is synthesized in hepatocytes as a single polypeptide chain. As predicted from the factor IX cDNA nucleotide sequence, there is a 46-amino acid leader/propeptide sequence attached to the amino terminus of the factor IX molecule (3, 4). After cleavage of the signal peptide, the factor IX precursor (profactor IX) undergoes post-translational modifications prior to secretion of the zymogen into the plasma. These include glycosylation, vitamin K-dependent carboxylation of the γ -carbon of the first 12 glutamic acid residues of the protein (5-7), β -hydroxylation of aspartic acid at residue 64 (8), and cleavage of the propeptide. The 46-amino acid sequence can be divided into two domains: a signal peptide that is necessary for the protein to be transported across plasma membranes, and a propeptide whose function is unknown. The propeptide shows marked structural homology with that of other γ -carboxyglutamic acid-containing proteins, suggesting that this region may be involved in vitamin K-dependent carboxylation (9, 10).

The vitamin K-dependent blood coagulation proteins in general, and factor IX specifically, undergo a metal-induced conformational transition (1) that can be monitored by spectroscopic (11, 12) and immunochemical (13) techniques. We have prepared a series of immunochemical reagents that bind to factor IX only in the presence of metal ions and are conformation specific for the metal-stabilized three-dimensional structure of factor IX (13). As such, these antibodies recognize antigenic markers on the metal-stabilized conformer of factor IX that are related to its membrane-binding properties and coagulant activity.

Absence or functional deficiency of factor IX results in the X chromosome-linked recessive disease hemophilia B. Two mutant factor IX molecules have been characterized at a structural level (14, 15). Factor IX^{Chapel Hill} contains a histi-

dine at position 145 instead of an arginine (14). This mutation prevents cleavage of the activation peptide and thus the expression of factor IXa enzymatic activity. Factor IX^{Alabama} contains a glycine instead of an aspartic acid at position 47 (15). This form is inactive for reasons that are not fully understood. A defective factor IX gene causing hemophilia B has been shown to be due to a point mutation in a splice junction (16). Gross deletions of regions of the factor IX gene have also been implicated in hemophilia B and are likely the cause of absent circulating factor IX in some patients (17).

We have searched for a mutant factor IX with a defect near the NH₂-terminal γ -carboxylated domain that would impair the membrane-binding properties essential for coagulant activity. We have developed anti-factor IX antibodies that are directed against a conformationally defined antigenic determinant on factor IX that is expressed only in the presence of metal ions. Using these antibodies, we have discovered a mutant factor IX, designated factor IX^{Cambridge}, in the plasma of a patient with severe hemophilia B. This patient has <1% of the normal factor IX activity in his plasma. In the presence of metal ions, factor IX^{Cambridge} does not react with the conformation-specific antibodies that recognize this metal-induced conformational change. In this study, we describe a point mutation in the factor IX precursor that precludes normal post-translational processing. Factor IX^{Cambridge} is homologous to factor IX except for the presence of an 18-residue extension on the NH₂ terminus.*

METHODS

Protein Purification. Factor IX^{Cambridge} and factor IX were isolated from plasma using DEAE-Sephacel chromatography and immunoaffinity chromatography. Plasma (225 ml) was applied to a DEAE-Sephacel (Pharmacia) column (2.5 $\times$ 10 cm) equilibrated in 0.1 M potassium phosphate/1 mM benzamidinium, pH 7.5. The column was developed with 0.1 M potassium phosphate/1 mM benzamidinium, pH 7.5, and eluted batchwise with 0.5 M potassium phosphate/1 mM benzamidinium, pH 7.5. The factor IX-containing fraction was dialyzed against 0.05 M Tris-HCl, pH 7.4/0.5 M NaCl/0.05% Tween 20/0.05% NaN₃/1 mM benzamidinium for 18 hr at 4°C. This material was applied to an anti-factor IX antibody-Sepharose column equilibrated in 0.05 M Tris-HCl, pH 7.4/0.5 M NaCl/0.05% Tween 20/0.05% NaN₃/1 mM benzamidinium/1 mM EDTA. The protein that bound factor IX^{Cambridge} was eluted with 4 M guanidine-HCl. The purified factor IX^{Cambridge} and factor IX were analyzed by NaDod-SO₄/PAGE in a 10% gel (18).

Immunochemical Analysis of Factor IX with Conformation-Specific Antibodies. The interaction of two anti-factor IX antibody populations, anti-factor IX antibodies and anti-factor IX:Mg(II) antibodies, with factor IX and factor IX^{Cambridge} was evaluated in a competition radio-

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*A preliminary report of this work has been published (39).

immunoassay. Antibody (2 nM) was incubated with either factor IX^{Cambridge} or factor IX (as indicated) in 0.1 M Tris-HCl, pH 7.4/0.15 M NaCl/0.01% Tween 20/0.1% bovine serum albumin/3 mM CaCl₂ in a volume of 200 μ l; ¹²⁵I-labeled factor IX (100 μ l) was added to a final concentration of 0.1 nM. After incubation at 23°C for 1 hr, the bound antibody was separated from the free antibody using goat anti-rabbit immunoglobulin, as described (19). The bound factor IX was determined by displacement of ¹²⁵I-labeled factor IX. Anti-factor IX antibodies represent an immunoaffinity-purified polyclonal anti-factor IX antibody population that binds equivalently to carboxylated and uncarboxylated factor IX. Anti-factor IX:Mg(II) antibodies represent a conformation-specific antibody population directed against a metal-stabilized determinant on factor IX. These antibodies are immunoaffinity-purified from rabbit anti-factor IX antibody populations on the basis of their ability to bind to factor IX in the presence of Mg(II), but not in the presence of EDTA. These antibodies were purified in a manner analogous to that used for the anti-prothrombin:Ca(II) antibodies (20, 21).

Quenching of Intrinsic Fluorescence. Factor IX^{Cambridge} or factor IX (0.5 μ M) was prepared metal-free in 0.1 M NaCl/0.05 M Tris-HCl, pH 8.0. Protein solutions (2.2 ml) were equilibrated at 25°C. Using the procedure of Prendergast and Mann (22) and Nelsestuen *et al.* (23), aliquots of 0.05 M CaCl₂ were added and the fluorescence spectrum was obtained by using a Perkin-Elmer MPF-3 fluorescence spectrophotometer. The excitation wavelength was 280 nm; the emission maximum was at 340 nm.

Phospholipid Binding Properties of Factor IX. The interaction of factor IX^{Cambridge} and factor IX with phospholipid micelles was evaluated by using the 90° light-scattering technique (24–26). Phospholipid vesicles (phosphatidylcholine/phosphatidylserine, 1:1) were prepared by the procedure of Barenholz *et al.* (27). Light scattering was performed on a Perkin-Elmer MPF-3 fluorescence spectrophotometer. Proteins were dialyzed in 0.02 M Tris-HCl, pH 7.4/0.15 M NaCl/5 mM CaCl₂. Aliquots of the protein solution (factor IX, 40 μ M; factor IX^{Cambridge}, 50 μ M) were added to the fluorescence cuvette containing 0.96 ml of 0.02 M Tris-HCl, pH 7.4/0.15 M NaCl/5 mM CaCl₂ with or without phospholipid. The scattering intensity obtained was corrected for the scattering intensity of the protein in the absence of phospholipid. Data were plotted as the light intensity versus the ratio (μ g of protein)/(μ g of lipid).

Amino Acid Sequence Analysis. Purified factor IX^{Cambridge} (100 pmol) in 0.05% NaDodSO₄ was subjected to automated Edman degradation using an Applied Biosystems Model 470A gas phase sequencer (28). All phenylthiohydantoin-derivatized amino acids were identified by high-performance liquid chromatography using the Applied Biosystems Model 120 PTH analyzer. The protein recovery was $\approx$ 50%, and a repetitive yield of 94.5% was observed.

RESULTS

We purified factor IX^{Cambridge} by using ion-exchange chromatography followed by immunoaffinity chromatography using antibodies to factor IX bound to Sepharose 4B. This resulted in the isolation of 1.2 mg of factor IX^{Cambridge} from 225 ml of the patient's plasma. This protein had a specific activity of 3.5 units/mg, as compared to 150 units/mg for factor IX purified by the same method, or $\approx$ 2% of the coagulant activity of factor IX. Factor IX^{Cambridge} migrated as a single band on both reduced and nonreduced NaDodSO₄/polyacrylamide gel electrophoresis (Fig. 1). Factor IX^{Cambridge} has a slightly reduced electrophoretic mobility as compared to factor IX, indicating a higher molecular weight than the normal protein.

FIG. 1. NaDodSO₄ gel electrophoresis of factor IX^{Cambridge}. The purified factor IX^{Cambridge} and factor IX were analyzed by NaDodSO₄/PAGE in a 10% gel. Factor IX^{Cambridge} migrated as a single band in both the presence (lane 2) and absence (lane 3) of 2-mercaptoethanol. Its electrophoretic mobility was decreased compared to factor IX, suggesting that factor IX^{Cambridge} has a molecular weight 2000–5000 greater than factor IX. The purified factor IX^{Cambridge} had a specific coagulant activity of 3.5 units/mg. Lane 1, factor IX (reduced); lane 2, factor IX^{Cambridge} (reduced); lane 3, factor IX^{Cambridge} (unreduced); lane 4, factor IX (unreduced).

Competition radioimmunoassays were performed to evaluate the ability of factor IX^{Cambridge} to compete for binding to anti-factor IX antibodies. As shown in Fig. 2A, factor IX and factor IX^{Cambridge} competed equivalently with ¹²⁵I-labeled factor IX for anti-factor IX antibodies. However, when the competition radioimmunoassay was done by using conformation-specific factor IX:Mg(II) antibodies, factor IX bound tightly to these antibodies while factor IX^{Cambridge} demonstrated minimal reaction (Fig. 2B). These results indicate that purified factor IX^{Cambridge} does not express the metal-stabilized conformer characteristic of factor IX.

We studied metal-induced quenching of intrinsic fluorescence of factor IX^{Cambridge} to confirm that a conformational change does not occur in the presence of metal ions. Factor IX undergoes a decrease in fluorescence in the presence of increasing concentrations of calcium ions (Fig. 3). Half-maximal fluorescence quenching was observed at $\approx$ 1 mM CaCl₂. The initial fluorescence was quenched by $\approx$ 30% upon saturation of factor IX with CaCl₂. In contrast, factor IX^{Cambridge} undergoes no fluorescence quenching upon titration with CaCl₂. These results indicate the absence of a metal-induced conformation change in factor IX^{Cambridge}.

The binding of factor IX^{Cambridge} to phospholipid vesicles was evaluated to determine the membrane-binding properties of the mutant protein. By using vesicles composed of phosphatidylserine and phosphatidylcholine, the binding of factor IX to vesicles could be observed using the 90° relative light-scattering technique (Fig. 4). Factor IX, in the presence of 5 mM CaCl₂ and phospholipid vesicles, showed increasing light scattering with increasing protein concentration. These results are consistent with increasing particle size due to binding of protein to the vesicle surface. No such increase in light scattering was observed with factor IX and lipid vesicles in the presence of 5 mM EDTA. In the presence of 5 mM CaCl₂ and phospholipid vesicles, factor IX^{Cambridge} demonstrated no increase in light scattering, indicating that this protein does not bind to lipid surfaces, despite the presence of CaCl₂.

The structural defect in factor IX^{Cambridge} was determined by establishing the amino-terminal sequence by Edman degradation using a gas-phase sequencer (Fig. 5). This revealed an 18-amino acid polypeptide extension on the normal NH₂ terminus of factor IX. This polypeptide is identical at cycles 1–17 to the sequence of a portion of the

FIG. 2. Binding of anti-factor IX:Mg(II) antibodies to factor IX^{Cambridge}. The interaction of two anti-factor IX antibody populations, anti-factor IX antibodies and anti-factor IX:Mg(II) antibodies, with factor IX and factor IX^{Cambridge} was evaluated in a competition radioimmunoassay. The bound factor IX was determined by displacement of ¹²⁵I-labeled factor IX. (A) Anti-factor IX antibodies, an immunoaffinity-purified polyclonal anti-factor IX antibody population that binds equivalently to carboxylated and uncarboxylated factor IX. (B) Anti-factor IX:Mg(II) antibodies, a conformation-specific antibody directed against a metal-stabilized determinant on factor IX. These antibodies are immunoaffinity-purified from rabbit anti-factor IX antibody populations on the basis of their ability to bind to factor IX in the presence of Mg(II), but not in the presence of EDTA. These antibodies were purified in a manner analogous to that used for the anti-prothrombin:Ca(II) antibodies (20, 21). ■, Factor IX; ●, factor IX^{Cambridge}.

propeptide (residues -18 to -2) of the factor IX precursor predicted from the cDNA nucleotide sequence. Cycle 18 of factor IX^{Cambridge}, equivalent to residue -1 of the factor IX precursor, the normal processing point of this propeptide, has undergone mutation such that a serine has been substituted for the arginine at the cleavage site. This prevents cleavage of the propeptide. Since the signal peptide (-46 to -19) has been cleaved, the protein is secreted with the mutant propeptide attached. Amino acids at cycles 19-24 of the mutant protein correspond to the first 6 amino acids of factor IX. These results are consistent with a single base change in codon 46 in the factor IX precursor gene in which the codon AGG, coding for arginine, is mutated to either codon AGT or AGC, coding for serine.

The γ -carboxylation of factor IX^{Cambridge} is impaired because of the alteration in the NH₂ terminus of the protein. Amino acid analysis of the alkaline hydrolysate of factor IX^{Cambridge} indicated the presence of ≈ 7 γ -carboxyglutamic acid residues (29, 30). In contrast, factor IX contained 12 γ -carboxyglutamic acid residues. These results suggest that arginine -1 has a role in the recognition of the factor IX

FIG. 3. Metal-induced fluorescence quenching of factor IX^{Cambridge} and factor IX. Factor IX^{Cambridge} or factor IX (0.5 μ M) was prepared metal-free in 0.1 M NaCl/0.05 M Tris-HCl, pH 8.0. Protein solutions (2.2 ml) were equilibrated at 25°C. Aliquots of 0.05 M $CaCl_2$ were added and the fluorescence spectrum was obtained by using a Perkin-Elmer MPF-3 fluorescence spectrophotometer. The excitation wavelength was 280 nm; the emission maximum was at 340 nm. ●, Factor IX; ▲, factor IX^{Cambridge}.

precursor as a substrate for the vitamin K-dependent γ -carboxylase, or that the presence of a serine at position -1 in the precursor interferes with normal carboxylation, or that the propeptide extension on the NH₂ terminus prevents complete carboxylation.

Based on the above results, we propose the following model to explain the defect in the post-translational processing of factor IX^{Cambridge}. Like factor IX precursor, the factor IX^{Cambridge} precursor is cleaved between residues -19 and -18, with the release of the signal peptide. The factor IX^{Cambridge} signal peptide-less precursor is then γ -carboxylated at certain glutamic acid residues. However, this γ -carboxylation reaction is only partial, due to the amino acid substitution at position -1. Because of the mutation of the arginine to a serine at position -1, the propeptide cannot be cleaved between residue -1 and residue +1 by the trypsin-like processing peptidase to yield the mature zymogen protein, factor IX. Factor IX^{Cambridge}, with an 18-residue extension at the NH₂ terminus, is secreted into the circulation. Because of the presence of the mutant propeptide or because of the impaired γ -carboxylation, factor IX^{Cambridge}

FIG. 4. Phospholipid binding properties of factor IX^{Cambridge}. The interaction of factor IX^{Cambridge} and factor IX with phospholipid micelles was evaluated by using the 90° light-scattering technique. The scattering intensity obtained was corrected for the scattering intensity of the protein in the absence of phospholipid. Data were plotted as the light intensity versus the ratio (μ g of protein)/(μ g of lipid). ○, Factor IX^{Cambridge} containing 5 mM $CaCl_2$; ●, factor IX containing 5 mM $CaCl_2$; ▲, factor IX containing 5 mM EDTA.

Factor IX - cDNA ACA GTT TTT CTT GAT CAT GAA AAC GCC AAC AAA ATT CTG AAT CGG CCA AAG AGG TAT AAT TCA GGT AAA TTG

FIG. 5. Amino acid sequence of factor IX^{Cambridge}. Factor IX^{Cambridge} contains an 18-residue NH₂-terminal extension homologous to the predicted amino acid sequence of the propeptide of factor IX (stippled) extrapolated from the known cDNA sequence of factor IX (3, 4). Factor IX has an NH₂-terminal sequence of Tyr-Asn-Ser-. Residue 18 of factor IX^{Cambridge}, analogous to the arginine at residue -1 of the factor IX precursor, is mutated to a serine (solid). This point mutation (Arg⁻¹ → Ser⁻¹) in the factor IX propeptide precludes normal proteolytic processing between residues -1 and +1. The codon for arginine -1 in the factor IX precursor, AGG, is altered to either AGT or AGC (solid), codons that code for serine.

cannot assume the metal-stabilized conformation necessary for binding to membrane surfaces and for coagulant activity.

This patient's mutation also allows us to divide the leader sequence into two domains. The first domain is region -46 to -19, a signal peptide whose cleavage is necessary for transmembrane transport and secretion of the protein. The second domain is region -18 to -1, a propeptide whose function heretofore has been unknown. However, given the marked amino acid sequence homology in the propeptide of the vitamin K-dependent proteins (9, 10) and our direct evidence that a mutation in the propeptide impairs γ -carboxylation, there is reason to suspect that the propeptide plays a role in the recognition of these proteins for γ -carboxylation.

Factor IX^{Cambridge} is a unique mutant form of a human enzyme. The point mutation at residue -1 in the factor IX precursor leads to a peptide bond that is not susceptible to normal proteolytic processing during protein synthesis that occurs at paired basic amino acid residues, specifically after Lys⁻²-Arg⁻¹. This leads to a factor IX that contains an NH₂-terminal peptide extension, decreased numbers of γ -carboxylglutamic acids, and impaired membrane-binding properties necessary for the expression of coagulant activity.

Processing defects have been noted in other proteins, leading to minimal changes in the protein function. Hemoglobin^{Long Island} and Hemoglobin^{Marseille}, both with His_{β2} → Pro_{β2} substitutions, contain a methionine at position -1 (31, 32). Hemoglobin^{South Florida}, with a Val_{β1} → Met_{β1} substitution, also has the initiator methionine as residue -1 (33). The presence of a methionine at residue 1 or a proline at residue 2 in the β chain interferes with removal of the initiator methionine. Several families have been described with familial hyperproinsulinemia (34, 35). The junction between the C chain and A chain of proinsulin is normally cleaved between arginine-65 and glycine-66. However, point mutation of the arginine-65 to histidine blocks post-translational cleavage of proinsulin to insulin (35). In addition, two proalbumin variants have been described, one with an Arg-Glu and one with a His-Arg sequence replacing the Arg-Arg pair at the propeptide-albumin junction (36, 37). These mutations impair post-translational cleavage of the proalbumin to albumin.

In the examples cited, the absence of post-translational processing appears to have little physiologic importance. The mutant hemoglobins with the NH₂-terminal methionine extension and proalbumin function normally. Patients with hyperproinsulinemia are either normal or have mild hyperglycemia. In the case of factor IX^{Cambridge}, the absence

of post-translational processing of a pro-proenzyme leads to a hereditary deficiency of factor IX coagulant activity and causes hemophilia B. Factor IX^{Cambridge} is the first example of a point mutation in a human protein precursor that impairs proteolytic processing of the protein during synthesis and causes a hereditary human disease due to lack of enzyme activity.

Note Added in Proof. A similar mutant factor IX has recently been described (38).

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