Effect of Propeptide Mutations on Post-translational Processing of Factor IX

EVIDENCE THAT $\beta$-HYDROXYLATION AND $\gamma$-CARBOXYLATION ARE INDEPENDENT EVENTS

Marie-Joséphie Rabiet, Maria J. Jorgensen, Bruce Furie, and Barbara C. Furie

From the Center for Hemostasis and Thrombosis Research, Division of Hematology-Oncology, Department of Medicine, New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts 02111

Post-translational processing of Factor IX includes glycosylation, cleavage of the signal peptide and propeptide, vitamin K-dependent carboxylation of specific glutamic acid residues to form $\gamma$-carboxyglutamic acid, and $\beta$-hydroxylation of aspartic acid at residue 64 to form $\beta$-hydroxyaspartic acid. The human Factor IX cDNA coding sequence was modified in the propeptide region (residue -18 to -1) using oligonucleotide-directed site-specific mutagenesis. The altered Factor IX cDNA was expressed in Chinese hamster ovary cells. The effects of the mutations on proteolytic processing, $\gamma$-carboxylation, and $\beta$-hydroxylation were assessed by direct structural analysis. After purification, the molecular weight of each of the recombinant Factor IX species and its NH$_2$-terminal amino acid sequence were shown to be identical to those of plasma Factor IX. $\gamma$-Carboxyglutamic acid and $\beta$-hydroxyaspartic acid analyses revealed that recombinant wild-type Factor IX contained 9.2 $\gamma$-carboxyglutamic acid and 0.3 $\beta$-hydroxyaspartic acid residues/molecule, compared with 11.4 $\gamma$-carboxyglutamic acid and 0.39 $\beta$-hydroxyaspartic acid residues in plasma Factor IX. When the 18-residue propeptide was deleted or when the cells were grown in the presence of sodium warfarin, secreted Factor IX contained no detectable $\gamma$-carboxyglutamic acid but 0.36 and 0.40 residues of $\beta$-hydroxyaspartic acid, respectively. Point mutations leading to substitution of alanine for phenylalanine at residue -16 or glutamic acid for alanine at residue -10 contained 0.2 and 1.7 $\gamma$-carboxyglutamic acid residues, respectively, and 0.2 residues of $\beta$-hydroxyaspartic acid. These data confirm that the propeptide mutations made do not interfere with proteolytic processing and that the Factor IX propeptide contains a recognition site that designates the adjacent glutamic acid-rich domain for $\gamma$-carboxylation. In contrast, $\beta$-hydroxylation of aspartic acid 64 is an independent process which does not require vitamin K and is mediated through a hydroxylation recognition site in the mature Factor IX, not in the propeptide.

Factor IX is a vitamin K-dependent protein ($M_r$, 56,000) that participates in an intermediate phase of the intrinsic pathway of blood coagulation. This protein contains two modified amino acids. $\gamma$-Carboxyglutamic acid, discovered by Stenflo (1) and Nelsestuen (2) in 1974, is a metal-binding amino acid (3) that plays a fundamental role in stabilizing a biologically active conformer of Factor IX that interacts with membrane surfaces (4). Factor IX contains 12 $\gamma$-carboxyglutamic acid residues near the NH$_2$ terminus of the protein (5). $\beta$-Hydroxyaspartic acid, initially discovered by Stenflo in protein C (6), has been found subsequently in other vitamin K-dependent proteins including protein S, protein Z, Factor X, and Factor IX (7). $\beta$-Hydroxyaspartic acid is located at residue 64 in Factor IX. The function of this amino acid is unknown. The synthesis of both amino acids involves post-translational modification. In the case of $\gamma$-carboxylation, specific glutamic acid residues in the precursor form of the protein undergo carboxylation in the presence of a vitamin K-dependent carboxylase, CO$_2$, and reduced vitamin K (8). $\beta$-Hydroxyaspartic acid is formed post-translationally by the enzymatic hydroxylation of a single aspartic acid residue in Factor IX.

The translation product of the Factor IX cDNA includes a prototypic signal peptide from residue -46 to residue -19, a propeptide from residue -18 to -1, and the mature Factor IX zymogen from residue +1 to 416 (9, 10). The length of the propeptide was deduced by the discovery of a mutant Factor IX, Factor IX Cambridge, that, due to a substitution of serine for arginine at residue -1, circulates with the propeptide attached to the mature Factor IX (11). The nature of the signal that directs post-translational modification of a specific protein has been a long-standing question. Based upon marked sequence homology among the propeptide regions of the vitamin K-dependent proteins (12) and our observation that Factor IX Cambridge, containing a point mutation in the propeptide, remains only partially carboxylated (11), we have previously examined the hypothesis that the propeptide designates Factor IX for $\gamma$-carboxylation (13). We demonstrated that the propeptide of Factor IX contains a $\gamma$-carboxylation recognition site. Upon alteration of this site by oligonucleotide-directed mutagenesis and the expression of Factor IX lacking the propeptide or containing either of two point mutations replacing highly conserved residues, the Factor IX $\gamma$-carboxylation was impaired. These observations were monitored using conformation-specific antibodies directed against the metal-stabilized $\gamma$-carboxyglutamic acid-dependent conformation (13).

In the current study these mutant recombinant Factor IX species have been isolated, structurally characterized, and the role of the propeptide in $\beta$-hydroxylation examined. We demonstrate that $\gamma$-carboxylation and $\beta$-hydroxylation are independent post-translational processes. Although $\gamma$-carboxylation is directed by the $\gamma$-carboxylation recognition site on the propeptide, $\beta$-hydroxylation is directed by a hydroxylation
Acid–Acid and alkaline hydrolyses were performed on 200 pmol to were identified by high performance liquid chromatography using an temperature in 20 ml vitamin K1 or 0.074 µg/ml sodium warfarin. Culture media were centrifuged to remove cellular debris and stored at −20°C. Hydrolysis was performed under vacuum for 24 h at 110 °C. After alkaline hydrolysis, the samples were treated according to the method of Hauschka (18). The dried acid or alkaline hydrolysates were dissolved in 10 µl of a 7:1:1 solution of methanol:water:triethylamine (Pierce Chemical Co.). The samples were reduced to dryness in a Savant Speed Vac concentrator. Samples were dissolved in 200 µl of 0.1 M sodium acetate, 0.2% triethylamine (pH 4.0). Phenylthiohydantoin derivatives were identified by high performance liquid chromatography using an Applied Biosystems model 120 phenylthiohydantoin analyzer.

Determination of γ-Carboxyglutamic Acid and β-Hydroxyaspartic Acid—Acid and alkaline hydrolysates were performed on 200 pmol to 2 ml of Factor IX. Constant boiling HCl (6 M, 0.2 ml) or freshly prepared potassium hydroxide (2 M, 0.2 ml) was added to each sample. Hydrolysis was performed under vacuum for 24 h at 110 °C. After alkaline hydrolysis, the samples were treated according to the method of Hauschka (18). The dried acid or alkaline hydrolysates were dissolved in 10 µl of a 2:2:1 mixture of methanol:water:triethylamine and redried. Derivatization was performed for 20 min at room temperature in 20 µl of a 7:1:1 solution of methanol:water:triethylamine (Pierce Chemical Co.). The samples were reduced to dryness in a Savant Speed Vac concentrator. Samples were dissolved in 200 µl of 0.1 M sodium acetate, 0.2% triethylamine (pH 4.0). Phenylthiohydantoin amino acid separation was performed on a Waters PICO-TAG reverse-phase HPLC column at 38°C. Solvent A was 0.1 M sodium acetate, 0.2% triethylamine (pH 4.0); solvent B was 60% acetonitrile. After an isocratic step of 5 min, linear gradients from 0 to 100% B in 12 min, from 10 to 50% B in 15 min, and from 50 to 100% B in 2 min were applied with a flow rate of 1 ml/min. The column development was monitored at 254 nm using a Beckman model 160 absorbance detector. Under these conditions the sensitivity of the system was about 10 pmol for each derivatized amino acid. Amino acid standards (Pierce Chemical Co.), including DL-γ-carboxyglutamic acid (Sigma) and DL-three-β-hydroxyaspartic acid (Sigma), were obtained commercially. The number of moles of Factor IX was determined by amino acid analysis of an acid hydrolysate. The number of moles of ethyrl-three-β-hydroxyaspartic acid in Factor IX was quantitated using a DL-three-β-hydroxyaspartic acid standard. The β-hydroxyaspartic acid present in each of the recombinant molecules was measured from the ratio of β-hydroxyaspartic acid and aspartic acid in a given sample and compared to the ratio obtained for a plasma Factor IX sample hydrolyzed and derivatized at the same time, assuming 1 mol of Factor IX to contain 0.39 mol of β-hydroxyaspartic acid. For γ-carboxyglutamic acid content analysis, a prothrombin standard was employed. Prothrombin contains 10 mol of γ-carboxyglutamic acid/mol of protein.

The abbreviations used are: SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography.

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MATERIALS AND METHODS

Mutagenesis and Expression of Factor IX cDNA—Construction of the Factor IX expression plasmid (pMT2-IX/wt) has been described (13). Alterations were made in the Factor IX coding sequence to obtain a deletion of the entire propeptide and the desired amino acid changes (FA-16, phenylalanine to alanine at residue −16; AE-10, alanine to glutamic acid at residue −10). DNA transfection, cell culture, and cell line selection were performed as previously described (14). The dihydrofrolate reductase-deficient Chinese hamster ovary cell line CHO DUKX-B11 was transfected with the expression plasmid by electroporation. Cells were grown in media containing 5 µg/ml vitamin K1 or 0.074 µg/ml sodium warfarin. Culture media were centrifuged to remove cellular debris and stored at −20°C.

Purification of Proteins—Human plasma Factor IX was purified from Factor IX concentrate by immunoadfinity chromatography using anti-Factor IX:Ca(II) antibodies immobilized on Sepharose (15). The recombinant Factor IX species contained in the cell culture supernatants were purified by immunoadfinity chromatography using anti-Factor IX:total antibodies immobilized on Sepharose (4). The EDTA or 4 M guanidine hydrochloride eluates were desalted on a 30 × 4.6-mm Aquapore 300A butyl MPLC analytical cartridge. Fractions containing the Factor IX species were collected and dried in a Savant Speed Vac concentrator.

Protein Analysis—SDS1-gel electrophoresis was performed on 10–15% polyacrylamide gradient gels in the presence of SDS and β-mercaptoethanol using a Pharmacia Biotechnology, Inc. PhastSystem (16). NH2-terminal amino acid sequences were determined by automated Edman degradation using an Applied Biosystems model 470A gas-phase protein sequenator (17). Phenylthiohydantoin derivatives were identified by high performance liquid chromatography using an Applied Biosystems model 120 phenylthiohydantoin analyzer.

RESULTS

Expression of Factor IX Species in Chinese Hamster Ovary Cells—The Factor IX expression vector pMT2-IX was used to introduce the Factor IX cDNA sequence or modified Factor IX coding sequences into Chinese hamster ovary cells (13). A deletion mutant, FIX/d(−18,−1), lacked the coding sequence for the entire 18-residue propeptide. Two substitution mutants, FIX/FA−16 and FIX/AE−10, contained point mutations replacing alanine for phenylalanine −16 or glutamic acid for alanine −10, respectively. All of the Factor IX molecules were expressed in the presence of vitamin K, and the wild type was also expressed in the presence of warfarin, an inhibitor of vitamin K. Culture media containing secreted Factor IX were assayed by competition radioimmunoassay using anti-Factor IX:total antibodies, which bind to Factor IX regardless of the extent of γ-carboxylation. The amount of Factor IX antigen expressed varied in concentration between 0.09 and 3.2 µg/ml.

Isolation and Characterization of the Recombinant Factor IX Species—Antibodies specific for Factor IX (anti-Factor IX:total antibodies) or for the metal-stabilized conformation of Factor IX (anti-Factor IX:Ca(II) antibodies) were immobilized on Sepharose and used for the immunoaffinity purification of the recombinant Factor IX species secreted in the cell culture supernatants or plasma Factor IX, respectively. The eluates were then chromatographed by reverse-phase HPLC on an Aquapor 300 butyl MPLC analytical cartridge equilibrated in 0.1% aqueous trifluoroacetic acid. A linear gradient of acetonitrile was used to elute the proteins, with the absorbance monitored at 214 and 280 nm. In all cases a single protein peak was observed. The fractions containing Factor IX were collected, pooled, and subsequently aliquoted for analysis by SDS-gel electrophoresis, NH2-terminal protein sequence analysis, and amino acid analysis.

The recombinant Factor IX species were analyzed for purity and molecular weight by electrophoresis in the presence of SDS. The proteins were stained with Coomassie Blue. As shown in Fig. 1, the wild-type recombinant Factor IX secreted when the cells were grown in the presence of vitamin K (FIX/wt, lane 3) or in the presence of sodium warfarin (FIX/wt + warfarin, lane 4) had the same electrophoretic migration as plasma Factor IX (lane 2). Similarly, the propeptide deletion mutant (FIX/d(−18,−1), lane 5), the point mutant at residue −10 (FIX/AE−10, lane 6), and the point mutant at residue −16 (FIX/FA−16, lane 7) migrated equivalently to plasma Factor IX. These results confirm our prior results in which these mutant Factor IX forms in cell culture supernatants were analyzed using Western blotting techniques (13). These data indicate the apparent homogeneity of the protein preparations and the apparent absence of a leader sequence (e.g. signal peptide, propeptide) associated with the secreted Factor IX.

To firmly establish that propeptide processing is not altered by mutations in the propeptide, automated Edman degradation was performed on each of the purified recombinant Factor IX molecules. As shown in Table I, the NH2-terminal sequences obtained were identical to the NH2-terminal sequence of plasma-derived Factor IX. There were no secondary sequences observed that corresponded to the NH2 terminus of the signal peptide or the propeptide. These results indicate that the secreted recombinant Factor IX species, including the one containing the deleted propeptide, two containing single propeptide point mutations, and the wild type form expressed in the presence of warfarin, were normally processed with regard to proteolytic cleavage. In addition, the absence of glutamic acid at residues 7 and 8 in the wild-type

1 The abbreviations used are: SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography.
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**Table I**

| Residue number | wt | wt + warfarin | d(-18,-1) | FA-16 | AE-10 |
|----------------|----|---------------|-----------|-------|-------|
| 1              | Tyr (51) | Tyr (15) | Tyr (66) | Tyr (21) | Tyr (6.7) |
| 2              | Asn (14) | Asn (8.5) | Asn (72) | Asn (6.4) | Asn (7.3) |
| 3              | Ser (5.7) | Ser (8.2) | Ser (38) | Ser (3.5) | Ser (1.4) |
| 4              | Gly (22) | Gly (19) | Gly (52) | Gly (21) | Gly (6.3) |
| 5              | Lys (53) | Lys (22) | Lys (23) | Lys (18) | Lys (6.5) |
| 6              | Leu (46) | Leu (18) | Leu (60) | Leu (18) | Leu (7.5) |
| 7              | [Glu] | Glu (2.9) | Glu (19) | Glu (3.5) | Glu (3.2) |
| 8              | [Glu] | Glu (2.7) | Glu (18) | Glu (4.2) | Glu (4.0) |
| 9              | Phe (29) | Phe (6.6) | Phe (30) | Phe (12) | Phe (7.5) |
| 10             | Val (47) | Val (6.7) | Val (35) | Val (18) | Val (2.1) |

**Table II**

| $\gamma$-Carboxyglutamic acid | $\beta$-Hydroxyaspartic acid | mol/mol Factor IX |
|-------------------------------|-------------------------------|------------------|
| Plasma FIX                    | 11.4 ± 0.6                    | 0.39 ± 0.12      |
| FIX/wt                        | 9.4 ± 0.9                     | 0.30             |
| FIX/wt + warfarin             | 0.0                           | 0.40             |
| FIX/d(-18,-1)                 | 0.0                           | 0.36             |
| FIX/AE(-10)                   | 1.7                           | 0.22             |
| FIX/FA(-16)                   | 0.2                           | 0.23             |

**Fig. 1.** Electrophoretic analysis of purified recombinant Factor IX species. Recombinant Factor IX species present in the culture supernatant of cells transfected with the wild-type Factor IX cDNA or mutated Factor IX cDNA containing deletion or point mutations in the proFactor IX were purified by immunoaffinity chromatography. The purified recombinant proteins and plasma Factor IX were analyzed on a 10-15% dodecyl sulfate-polyacrylamide gradient gel in the presence of sodium dodecyl sulfate. The purified recombinant proteins and plasma Factor IX were analyzed on a 10-15% dodecyl sulfate-polyacrylamide gradient gel in the presence of sodium dodecyl sulfate. The plasma-derived Factor IX contained no detectable $\gamma$-carboxyglutamic acid. However, the amount of $\beta$-hydroxyaspartic acid recovered at each cycle. No secondary sequences, representing uncleaved propeptide or signal peptide, were observed.

The $\gamma$-carboxyglutamic acid content of their $\gamma$-carboxyglutamic acid disclosed that when glutamic acid was substituted for alanine at residue -10, a perfectly conserved position, almost no $\gamma$-carboxyglutamic acid was detectable. However, in both cases, the amount of $\beta$-hydroxyaspartic acid was within experimental error of that of plasma Factor IX. The results indicate that sodium warfarin inhibits the $\gamma$-carboxylation of glutamic acid residues in Factor IX but does not inhibit $\beta$-hydroxylation of aspartic acid. Unlike the wild-type Factor IX, the Factor IX expressed and secreted when the cells were transfected with the propeptide deletion mutant pMT2-IX/d(-18,-1) contained no detectable $\gamma$-carboxyglutamic acid. However, the amount of $\beta$-hydroxyaspartic acid was normal, within experimental error. These results, direct confirmation of our previous results (13), indicate that deletion of the propeptide interferes with vitamin K-dependent $\gamma$-carboxylation. Two single amino acid substitutions were also made in the propeptide. The analysis of their $\gamma$-carboxyglutamic acid content disclosed that when glutamic acid was substituted for alanine at residue -10, a well conserved position in the amino acid sequence of the vitamin K-dependent proteins, approximately 1.7 mol of $\gamma$-carboxyglutamic acid/mol of Factor IX was detected. When alanine was substituted for phenylalanine at residue -16, a perfectly conserved position, almost no $\gamma$-carboxyglutamic acid was detectable. However, in both cases, the amount of $\beta$-hydroxyaspartic acid was within experimental error of that of plasma Factor IX. The normal $\beta$-hydroxyaspartic acid contents of the propeptide deletion mutant, the point mutants, and the wild type Factor IX expressed in the presence of warfarin indicate that $\beta$-hydroxylation is an event mechanistically distinct from $\gamma$-carboxylation.

**DISCUSSION**

Examination of the full-length Factor IX cDNA predicts a signal sequence coded by the 5' end of this cDNA. However, based upon the known NH$_2$ terminus of human Factor IX and the typical specificities of the signal peptidase, Kurachi
and Davie (9) postulated the presence of a propeptide between the signal peptide and mature Factor IX. The role of a propeptide in the post-translational modification of vitamin K-dependent proteins has been observed for Factor IX and for other secreted mammalian proteins. We have previously shown by immunochemical analysis of a naturally occurring mutant Factor IX (11) and recombinant Factor IX modified by site-directed mutagenesis (13) that changes in the propeptide disrupt γ-carboxylation during protein synthesis. This work has demonstrated, for the first time, a specific function for the propeptide of a protein.

In the current study we have completed the chemical characterization of the recombinant Factor IX species secreted from mammalian host cells transfected with the wild-type Factor IX cDNA and Factor IX cDNA mutated in the propeptide coding sequence. Our results, based upon direct amino acid analyses of γ-carboxyglutamic acid and β-hydroxyaspartic acid, demonstrate that a recognition element located in the propeptide sequence is required for γ-carboxylation of these proteins. Several observations suggested that the propeptide comprises the γ-carboxylation recognition site. Analysis of sequence homologies among the vitamin K-dependent proteins reveals that the amino-terminal portions of these regions are highly conserved (12). Furthermore, Factor IX Cambridge, with a point mutation in the propeptide, is only partially γ-carboxylated (11). Most importantly, we have shown that mutation of residue -16 or -10 greatly impairs carboxylation of Factor IX, as monitored using conformation-specific antibodies. These results indicate that these residues specifically and the propeptide more generally play an essential role in defining a functional carboxylation recognition site (13). The finding that the carboxylation of a protein C precursor, synthesized by recombinant techniques in bacteria, is better carboxylated (albeit incompletely) in an in vitro carboxylation system if a portion of the propeptide, from -10 to -1, is attached to the NH2-terminal end of the mature protein C is consistent with the role of the propeptide in carboxylation (19).

In contrast, the propeptide plays no role in β-hydroxylation. Mutants lacking the propeptide, Factor IX lacking γ-carboxyglutamic acid, and Factor IX synthesized in the presence of a vitamin K antagonist contain β-hydroxyaspartic acid. These results indicate that a hydroxylation recognition site located on the mature Factor IX molecule, and not on the Factor IX precursor, proFactor IX, is responsible for direct β-hydroxylation. These results are in complete agreement with those of Dahlback et al. (20). These investigators have postulated that β-hydroxylation occurs in domains homologous to the epidermal growth factor precursor in certain of the vitamin K-dependent proteins as well as proteins outside of this family, including the complement protein C3. A consensus sequence for β-hydroxylation is the four epidermal growth factor domains of bovine protein S and the single epidermal growth factor domains of bovine secreted mammalian human Factor IX, bovine Factor X, human Factor X, and bovine protein Z has been noted (20):

\[ X-X-Cys-X-Asp/Asn-X-X-X-X-Phe/Tyr-Cys-X-Cys-X \]

The aspartic acid or asparagine residue is the substrate for β-hydroxylation. Epidermal growth factor domains in these or other proteins that lack this consensus sequence do not contain β-hydroxylated aspartic acid or asparagine.

These results indicate two separate mechanisms for post-translational modification of glutamic acid residues and aspartic acid residues in Factor IX. On one hand, γ-carboxylation is directed by a recognition site adjacent to, but nonetheless outside of, the γ-carboxyglutamic acid-rich region. We hypothesize that an intracellular protein, perhaps a domain of the vitamin K-dependent carboxylase or alternatively a docking protein with recognition elements for both the carboxylation recognition site and the carboxylase, binds to the carboxylation recognition site. Based on the evidence that the carboxylating activity is associated with the endoplasmic reticulum (21), this recognition protein would also need to be located in the rough endoplasmic reticulum. The substrate for this reaction is proFactor IX, containing the propeptide. After carboxylation, the propeptide is cleaved. Whether this cleavage also occurs in the endoplasmic reticulum or whether it occurs in the Golgi or post-Golgi is not known. In contrast, β-hydroxylation presumably occurs by direct substrate recognition of either the proFactor IX or Factor IX molecule. This reaction appears inefficient since less than half of the Factor IX molecules contain β-hydroxyaspartic acid. Furthermore, the role of this amino acid, if any, remains obscure. In the absence of specific data, hydroxylation may occur in either the endoplasmic reticulum or the Golgi apparatus. Further studies involving in vitro post-translational processing and purification of the enzyme and recognition elements involved will be useful to understand the molecular basis of protein trafficking during the synthesis of the vitamin K-dependent proteins.

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