The \( \gamma \)-Carboxylation Recognition Site Is Sufficient to Direct Vitamin K-dependent Carboxylation on an Adjacent Glutamate-rich Region of Thrombin in a Propeptide-Thrombin Chimera*

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Barbara C. Furie, Jennifer V. Ratcliffe‡, Jonathan Tward, Maria J. Jorgensen§, Lawrence S. Blaszkowsky†, Donna DiMichele¶, and Bruce Furie**

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

The propeptides of the vitamin K-dependent proteins contain a \( \gamma \)-carboxylation recognition site that is required for \( \gamma \)-glutamyl carboxylation. To determine whether the propeptide is sufficient to direct carboxylation, two mutant prothrombin species were expressed and characterized with regard to posttranslational \( \gamma \)-carboxylation. A double point mutant, in which serine substituted for cysteines 17 and 22 disrupted a conserved loop formed by a disulfide bond, was fully carboxylated when expressed in Chinese hamster ovary cells. A propeptide/thrombin chimeric protein, constructed by deleting the Gla, aromatic amino acid stack, and kringle domains of prothrombin, has the signal peptide and propeptide juxtaposed to a glutamate-rich COOH-terminal region of prothrombin, residues 249–530. Of the 8 glutamic acid residues contained within the first 40 residues of the NH\(_2\) terminus adjacent to the propeptide, at least seven were fully carboxylated as demonstrated by direct \( \gamma \)-carboxyglutamic acid analysis of the alkaline hydrolysate and by NH\(_2\)-terminal sequence analysis. These results indicate that the \( \gamma \)-carboxylation recognition site within the prothrombin propeptide in a prothrombin propeptide-thrombin chimeric protein is sufficient to direct \( \gamma \)-carboxylase-catalyzed carboxylation of adjacent glutamic acid residues in a glutamate-rich region of thrombin that is not normally \( \gamma \)-carboxylated. Furthermore, the disulfide loop in the Gla domain of prothrombin is not required for complete carboxylation.

The vitamin K-dependent blood coagulation and regulatory proteins belong to a class of calcium-binding proteins that play an essential role in the initiation and regulation of blood coagulation. These proteins, which include prothrombin, Factor VII, Factor IX, Factor X, protein C, and protein S, are synthesized in the liver in precursor forms (for review, see Ref. 1). These precursors are comprised of a signal peptide and a propeptide NH\(_2\)-terminal to the mature protein sequence (2). The precursor proteins undergo extensive posttranslational modification, including \( \gamma \)-carboxylation of the 10–12 most NH\(_2\)-terminal glutamic acid residues (3, 4). In addition to the polypeptide substrate, \( \gamma \)-carboxylation requires the vitamin K-dependent carboxylase, molecular oxygen, CO\(_2\), and reduced vitamin K (5). Although the carboxylase resides in both the endoplasmic reticulum and the Golgi apparatus, carboxylation takes place in the endoplasmic reticulum (6, 7). The \( \gamma \)-carboxyglutamic acid residues confer metal-binding properties on the proteins (1). In the presence of metal ions, these proteins undergo conformational changes essential to the expression of membrane-binding properties and coagulant activity (8–12).

The propeptides of all mammalian proteins containing \( \gamma \)-carboxyglutamic acid exhibit sequence homology (13–15). Within the propeptide is a \( \gamma \)-carboxylation recognition site that directs \( \gamma \)-carboxylation (16, 17) by binding directly to the carboxylase. In addition a \( \gamma \)-carboxylation recognition site is found within the mature sequence of matrix Gla protein, a \( \gamma \)-carboxylated protein found in bone (18). The interaction of the carboxylase with the propeptide of a vitamin K-dependent protein has been used as a strategy for \( \gamma \)-carboxylase purification (19) and has led to the isolation (20–22) and cloning (23, 24) of this enzyme. The vitamin K-dependent carboxylase, comprised of 758 amino acids in a single polypeptide chain, contains the carboxylase active site (25) and propeptide-binding site (26) within the NH\(_2\)-terminal third of the protein. However, a recent report suggests that the propeptide-binding site on the carboxylase is located on the COOH-terminal two-thirds of the enzyme (27), while a preliminary study based on the yeast two-hybrid system concludes, as does Yamada et al. (26), that the propeptide binds to the NH\(_2\)-terminal third of the enzyme (28). Synthetic oligopeptides and polypeptides incorporating the propeptide sequences are low \( K_a \) substrates for \textit{in vitro} carboxylation, in contrast with substrates lacking the propeptide (29–31). Expression of mutants containing point mutations within the \( \gamma \)-carboxylation recognition site has shown this recognition element in prothrombin or Factor IX to be defined by residues –18, –17, –16, –15, and –10, with phenylalanine –16 playing a critical role (16, 32). The propeptide, an amphipathic \( \alpha \)-helix between residues –13 and –3 (33), stimulates carboxylation of FLEEL in the \textit{in vitro} carboxylase assay (34).

Others have proposed that a second recognition site might reside within the mature sequence of the nascent vitamin K-dependent protein polypeptide. Castellino and colleagues pro-
posed a Glu domain consensus sequence, E16XXE29XXC22, common to the vitamin K-dependent proteins (35). Disruption of cysteine 22 by site-specific mutagenesis was associated with the expression of incompletely carboxylated recombinant protein C in a mammalian expression system (35). In their expression system, Zhang et al. observed incomplete carboxylation of recombinant protein C in which glutamic acid residues in the Glu domain were systematically mutated (35, 36, 64). This necessitated purification of the fully carboxylated mutant protein C subpopulation prior to functional characterization. In contrast, we prepared ten mutants of prothrombin in which each of the glutamic acid residues in the Glu domain were modified to aspartic acid (37). Expression of these mutant prothrombins generated fully carboxylated protein. These results may relate to differences in the synthesis of the vitamin K-dependent proteins or differences in the heterologous mammalian expression systems employed.

The γ-carboxylation recognition site and the Glu domain are encoded by a single exon within the genes of the vitamin K-dependent proteins (38–46). Although the propeptide and the Glu domain may be considered as separate albeit functionally related elements, it is not known whether the propeptide of a vitamin K-dependent protein appended to the mature amino terminus of an irrelevant polypeptide will direct γ-carboxylation of glutamic acid residues in the adjacent sequence. To begin to address this question, we generated two mutant forms of prothrombin. In the double point mutant, a loop defined by a disulfide bond is disrupted. In a chimeric protein, the prothrombin propeptide and the first three amino-terminal residues of the prothrombin sequence are linked directly to a glutamate-rich region of thrombin, from residue 249 to 530 of the prothrombin sequence. In this construct, the Glu, aromatic amino acid stack, and kringle domains of prothrombin are deleted. Expression of these proteins in Chinese hamster ovary cells yielded fully carboxylated proteins. We conclude that the γ-carboxylation recognition site on the propeptide is sufficient to direct in vivo carboxylation of adjacent glutamic acid residues in the absence of the disulfide loop of the Glu domain and within the context of an amino acid sequence within thrombin that is not normally γ-carboxylated.

EXPERIMENTAL PROCEDURES

**Construction of the cDNA pMT2-PT/((34,248))**—The cDNA encoding a deletion mutant of prothrombin lacking residues 4–248 was constructed in pMT2-PT (47, 48) by site-directed mutagenesis (49). A heteroduplex of pMT2-PT, containing a single-stranded deletion of the first 1316 base pairs in the 5′-coding sequence of prothrombin was created. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and were gel-purified prior to use. The 27-base synthetic mutagenic primer, 5′-GGCGAGCCAACACCGAGGAGGC-GCGTG-3′, was composed of blocks of 14 and 13 bases complementary to the regions to be joined in the desired 735-base pair-deleted mutant. The oligonucleotide was 5′-phosphorylated, and 10 µl (50 pmol) of the oligonucleotide was mixed with 30 µl (0.1 pmol) of the heteroduplex mixture, and 3.3 µl of 5 M NaCl was added. The annealing reaction was carried out by heating to 68 °C for 10 min and cooling slowly to room temperature. The primer was extended and joined to the existing partial second strand during overnight incubation at room temperature. After inactivating the enzymes 2 µl of this DNA was used to transform the TG1 strain of Escherichia coli and plasmids containing the desired mutation were identified by colony hybridization using 32P-labeled mutagenic oligonucleotide. Successive transformations produced a single positive colony that was selected as the source of plasmid DNA and used for CHO cell transfection.

**Construction of the cDNA pMT2-PT/((34,17,22))**—The cDNA encoding the prothrombin mutant containing the cysteine to serine substitutions at positions 17 and 22 was constructed using the same heteroduplex described for pMT2-PT/((34,248)). The mutagenesis was carried out as above using the 28-base oligonucleotide, 5′-GGCGAGCCAACACCGAGGAGGC-GCGTG-3′. The presence of the desired mutations was verified by DNA sequence analysis (50).

**Cell Culture, DNA Transfection, and Cell Line Selection**—The expression plasmids containing the wild-type, chimeric or mutant cDNAs were linearized at the ClaI site in the plasmid. DNA (20 µg) was transfected by electroporation into 2.6 × 106 dihydrofolate reductase-deficient CHO cells, CHODUKX-B11 (51), previously shown to carboxylate recombinant wild-type prothrombin under the expression conditions employed (48). Visible colonies were isolated using cloning rings and reestablished in cell culture. Cell supernatants were analyzed for expression of the recombinant protein by dot blot analysis using polyclonal immunofluorescence (52) and positive clones expanded.

**Purification of Recombinant Wild-type Prothrombin, PT/((CS17,22)), and PT/((34,248))**—Recombinant wild-type prothrombin, PT/((CS17,22)), and PT/((34,248)) were isolated from 2 liters of cell supernatant. The cell supernatants were applied to an immunofluorescence column derivatized with rabbit anti-prothrombin antibodies directed against the metal-dependent conformation of prothrombin (10). Each mutant was prepared on a dedicated column that was not used to isolate prothrombin species. Chromatography was performed at 4 °C. After loading the matrix and washing with borate-buffered saline/0.1% Tween 20, the prothrombin species were eluted with 4 mM guanidine HCl and dialyzed at 4 °C against Tris-buffered saline. The concentrations of purified recombinant prothrombin species were measured by radioimmunoassay (53) and the purity of the preparations established by polyacrylamide electrophoresis in the presence of dodecyl sulfate under reducing conditions (54).

**Phospholipid Binding**—Unilamellar phospholipid vesicles were prepared by the method of Barenholz et al. (55). The final concentration of phospholipid was determined by elemental phosphorus (56). The phospholipid vesicles were stored under nitrogen and used within a week. The binding of the prothrombin species to phospholipid vesicles was determined by gel filtration using a Pharmacia S-12 FPLC column (1 × 30 cm). The column was equilibrated in 0.5 M NaCl, 0.1% bovine serum albumin, 5 mM CaCl₂, 0.05 M Tris-HCl (pH 7.4), and 0.025% NaN₃. The prothrombin species were labeled, where indicated, with 125I using the lactoperoxidase method. The radiolabeled proteins and phospholipid vesicles were applied to the column and the column developed with the equilibration buffer. The effluent was monitored by phosphor analysis and/or assay of 125I.

**Prothrombin Coagulation Assay**—Prothrombin coagulant activity was measured using prothrombin-deficient plasma in a two-stage assay (53). Purified plasma-derived prothrombin of known concentration was used as a standard.

**Amino-terminal Sequence Analysis and Molecular Mass Determination**—Recombinant proteins were desalted by high pressure liquid chromatography prior to analysis. Automated Edman degradation was performed on a Milligan model 6600 ProSequence (Milligen/Biosearch) or an Applied Biosystems Procise HT protein sequencer. In sequencing experiments in which γ-carboxyglutamic acid residues were identified and quantitated, γ-carboxyglutamic acid was directly identified as the methyl ester by the method of Cairns et al. (57). All peptide carbonyl groups were methyl-esterified using methanolic HCl. Phenylthiohydantoin-Gla(OMe)₃ was identified by HPLC of the phenylthiohydantoin-derivatives. Mass spectral analysis was performed on a Voyager Linear matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Perspective Biosystems) in positive ion mode. Under these conditions, γ-carboxyglutamic acid-containing proteins are deacylated.

**γ-Carboxyglutamic Acid Analysis**—Determination of the γ-carboxyglutamic acid content of proteins was performed by the method of Kotow (58) as a modification of the method of Kudwas and Katayama (59). The high pressure liquid chromatography system consisted of a Beckman model 126 pump and an Applied Biosystems Spectrflow 980 fluorescence detector equipped with a 415-nm band-pass filter. The excitation monochromator of the fluorescence detector was set at 240 nm. Separations were performed isocratically at room temperature. The mobile phase consisted of equal volumes of 0.1 M sodium citrate (pH 5.28) and acetonitrile run at a flow rate of 1 ml/min. Protein samples were purified by HPLC using an Aquapore C4 300 A reversed phase cartridge (Brownlee Laboratories) prior to amino acid analysis. Lyophilized plasma-derived and recombinant protein samples were sub-
RESULTS

Expression of a Prothrombin Mutant Lacking the NH2-terminal Disulfide Loop—To test whether a consensus sequence required for the process of in vivo γ-carboxylation exists within the amino terminus of the Gla domain of the vitamin K-dependent proteins, point mutations in the wild-type prothrombin cDNA were made to mutate cysteine to serine at positions 17 and 22. The desired outcome of these mutations was a disruption in the six-membered disulfide loop that is highly conserved among proteins that undergo posttranslational γ-carboxylation and alteration of the consensus sequence, E10XXE20X22, hypothesized to play a role in directing γ-carboxylation (35). The cDNA encoding the mutant protein, PT/(CS17,22), was ligated into the expression vector pMT2 and the plasmid used to transfect CHO cells. The mutant prothrombin, purified by immunoaffinity chromatography, migrated as a single band on SDS-PAGE (Fig. 1). The migration of the band was consistent with a molecular weight identical to that of prothrombin at 72,000. NH2-terminal amino acid sequencing of the first 25 residues verified that propeptide cleavage had occurred and that the cysteine to serine mutations at amino acid positions 17 and 22 were present. Direct γ-carboxyglutamic acid analysis of this protein revealed it to be completely carboxylated when compared with plasma-derived and wild-type recombinant prothrombin (Table I). This mutant protein, despite complete γ-carboxylation, did not bind to phospholipid vesicles using assay conditions under which plasma-derived and wild-type recombinant prothrombin bind completely (Table II). In contradistinction to recombinant wild-type prothrombin, PT/(CS17,22) had no coagulant activity. These results indicate that the disulfide loop and the conserved sequence E10XXE20X22 are not components of the γ-carboxylation recognition site in prothrombin but are required for protein function.

Expression of a Prothrombin Propeptide/Thrombin Chimera Lacking the Gla Domain and Kringle Domains—The propeptide, containing the γ-carboxylation recognition site, is required for posttranslational carboxylation. To determine whether this recognition element is sufficient to direct carboxylation in a model system, we expressed a mutant form of prothrombin in which the Gla domain, aromatic amino acid stack domain, and the kringle domains (residues 4–248) were deleted, thus placing the prothrombin propeptide adjacent to a series of 8 glutamic acid residues within the first 40 residues of the serine protease domain (Fig. 2). These glutamic acids are not normally carboxylated and are distant from the propeptide and γ-carboxyglutamic acid-containing domain in prothrombin. The sequence context of these glutamic acids is unrelated to that of the Gla domain of prothrombin or other known vitamin K-dependent proteins. The cDNA encoding PT/(Δ4,248) was ligated into the expression vector pMT2, the plasmid transfected into Chinese hamster ovary cells and the mutant protein expressed. The protein, purified by immunoaffinity chromatography and high pressure liquid chromatography, was analyzed by SDS-gel electrophoresis. A dominant band (>90%) with a molecular weight of about 44,000 and a minor band of about 39,000 were observed (Fig. 3). The molecular mass of the protein, determined by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy, is 40,169 ± 400 daltons. The protein is decarboxylated during analysis so that the molecular mass of the carboxylated protein (see below) is 40,477 or 40,521 ± 400 daltons. Western blot analysis of PT/(Δ4,248), using antiprothrombin antibodies, shows that both bands on the SDS-gel expressed antigenic determinants common to prothrombin, as expected. The major band of 44,000 and a second band of lower intensity and molecular weight were stained.

Direct γ-carboxyglutamic acid analysis of the alkaline hydrolysate of the purified protein revealed the presence of 7.5 ± 0.2 mol of γ-carboxyglutamic acid/mol of protein (Table I). Eight glutamic acid residues are located within the first 40 residues of the propeptide in this mutant protein.

The amino-terminal sequence of the methyl-esterified protein was determined by automated Edman degradation and revealed the expected amino acid sequence (Table III). γ-Carboxyglutamic acid residues were observed at cycles 4, 5, 8, 9, 10, 17, and 24, thus indicating the carboxylation of 7 glutamic acid residues. This carboxylation appears complete in that no glutamic acid was detected. Given the amount of material available and the repetitive yield of the Edman degradation, we were unable to directly analyze the protein sequence beyond cycle 24. Thus, we are unable to comment on the carboxylation status of the glutamic acids at residue 31 and residue 45 of the chimeric protein.

Table I
| γ-Carboxyglutamic acid content of prothrombin mutants | Theoretical | Obtained |
|--------------------------------------------------------|-------------|----------|
| PT/(CS17,22)                                           | 10          | 10.5 ± 1.5 |
| PT/(Δ4,248)                                            | 8           | 7.5 ± 0.2 |

Table II
| Properties of PT/(CS17,22) |
|---------------------------|
| Clotting activity (%)      | 94          | 8        | 100 |
| Phospholipid binding (%)   | <1          | 8        | 0.02 |
| Binding to conformation-specific antibodies (%) |  

<sup>a</sup> Percent of activity compared with plasma-derived prothrombin.
<sup>b</sup> ([μg/ml antigen determined using antibodies specific for the metal-dependent conformer]/[μg/ml antigen determined using conformation-independent antibodies]) × 100.
<sup>c</sup> Recombinant wild-type prothrombin.
The residue numbers of prothrombin are indicated and represent potential substrates for the vitamin K-dependent carboxylase. The glutamic acid residues (Glu) are shaded and represent potential substrates for the vitamin K-dependent carboxylase.

**FIG. 2. Structure of PT/(Δ4,248).** The mutant prothrombin species PT/(Δ4,248) lacks the Gla domain, aromatic amino acid stack domain, and the two kringle domains of prothrombin and contains the signal peptide and propeptide of prothrombin adjacent to a glutamic acid-rich region of the serine protease domain. The black bar indicates the propeptide sequence. The glutamic acid residues (Glu) are shaded and represent potential substrates for the vitamin K-dependent carboxylase.

**FIG. 3. SDS-PAGE and Western blot analyses of the PT/(Δ4,248).** Lanes A and B, SDS-PAGE stained with Coomassie Blue. Lanes C and D, Western blot developed with antiprothrombin: part A, plasma derived prothrombin. All protein samples were reduced with β-mercaptoethanol.

**TABLE III**

| PT/(Δ4,248), residue predicted | Residue observed | Picomoles |
|--------------------------------|-----------------|----------|
| 1 Ala                          | Ala             | 36.4     |
| 2 Asn                          | Asn             | 29.8     |
| 3 Thr                          | Thr             | 24.6     |
| 4 Glu                          | Glu             | 13.0     |
| 5 Glu                          | Glu             | 15.1     |
| 6 Ala                          | Ala             | 25.4     |
| 7 Val                          | Val             | 25.5     |
| 8 Glu                          | Glu             | 10.5     |
| 9 Glu                          | Glu             | 12.3     |
| 10 Glu                         | Glu             | 13.2     |
| 11 Thr                         | Thr             | 10.8     |
| 12 Gly                         | Gly             | 30.2     |
| 13 Asp                         | Asp             | 12.4     |
| 14 Gly                         | ?               |          |
| 15 Leu                         | ?               |          |
| 16 Asp                         | Glu             | 8.8      |
| 18 Asp                         | Asp             | 12.2     |
| 19 Ser                         | Ser             | 12.8     |
| 20 Asp                         | ?               |          |
| 21 Arg                         | Arg             | 16.5     |
| 22 Ala                         | Ala             | 12.5     |
| 23 Ile                         | Ile             |          |
| 24 Glu                         | Glu             | 4.6      |

The extinction coefficient for phenylthiohydantoin-Gla was assumed to be equivalent to phenylthiohydantoin-Glu.

**DISCUSSION**

The prothrombin propeptide/thrombin chimera PT/(Δ4,248), which contains a glutamate-rich region of thrombin directly adjacent to the γ-carboxylation recognition site of the prothrombin propeptide, allowed us to test the hypothesis that the propeptide could direct the in vivo carboxylation of a polypeptide chain containing glutamic acid residues within close proximity of the propeptide. Indeed, this mutant protein was well carboxylated, containing 7–8 γ-carboxyglutamic acid residues per protein molecule. The theoretical number of carboxylatable glutamic acid residues in PT/(Δ4,248) was uncertain since the distance from the γ-carboxylation recognition site to a substrate glutamate has not been defined for the vitamin K-dependent carboxylase. In the naturally occurring vitamin K-dependent blood clotting and regulatory proteins, glutamic acid residues included between residue 6 and residue 40 of the mature NH₂ terminus are carboxylated. There are no glutamic acid residues between positions 1 and 5 or beyond 40 that are modified. In the precursor form of PT/(Δ4,248), the first two glutamic acids appear at positions 4 and 5, and there is a glutamic acid at position 45. Therefore, the expected number of γ-carboxyglutamic acid residues in PT/(Δ4,248) was between five and nine. Thus, our demonstration that the mutant contains 7–8 γ-carboxyglutamic acid residues and that residues 4, 5, 8, 9, 10, 17, and 24 are fully carboxylated, within experimental error, indicates that three adjacent glutamic acid residues can undergo full carboxylation in vivo and that a glutamic acid at residue 4 is also a substrate for the carboxylase. Given the presence of an uncarboxylated glutamic acid residue at residue 2 in conantokin G (60) and a partially carboxylated glutamic acid in matrix Gla protein that is NH₂-terminal of the carboxylation recognition site (14), additional work is necessary to understand the other details of the specificity of the carboxylase for substrate peptides containing glutamic acid.

It has been previously established that the propeptides of vitamin K-dependent proteins are required for γ-carboxylation (16, 17). The current studies were undertaken to determine whether the propeptide of these proteins was sufficient to direct carboxylation. It has been proposed that in addition to conserved structures in the propeptide a conserved sequence in the mature amino terminal of these proteins, E₁₆XXXE₂₀XC₂₂, may be necessary as a recognition element in designating the vitamin K-dependent proteins as substrates for γ-carboxylation. Zhang and Castellino (35) found that mutations in the consensus sequence, E₁₆XXXE₂₀XC₂₂, glutamic acid 19 and 20 to aspartic acid or cysteine 22 to serine in protein C yielded mutant protein C species in which only about 20% of the former and about 15% of the latter molecules were well carboxylated when expressed in 293 cells, a human embryonal kidney cell line. They suggest that the E₁₆XXXE₂₀XC₂₂ may act as a second recognition element for the carboxylase in addition to the γ-carboxylation recognition site in the propeptide. In our experiments, we demonstrate that the propeptide alone is sufficient to direct complete carboxylation of two prothrombin mutants. The prothrombin mutant, PT/(Δ4,248), does not contain the consensus sequence E₁₆XXXE₂₀XC₂₂ proposed as a second recognition element for carboxylation and yet it is well carboxylated. In addition, PT/(CS17,22) was fully carboxylated when expressed in Chinese hamster ovary cells. It lacks the disulfide loop proposed as a component of this second recognition element.

Based on the above results, we conclude that the propeptide may be sufficient to direct carboxylation of adjacent glutamate...
residues regardless of the sequence context of these residues. The efficiency of carboxylation may vary with different proteins, but we do not currently understand the motifs necessary for the most efficient carboxylation. For example, we have observed efficient carboxylation of human prothrombin (48) but less efficient carboxylation of human Factor IX (47). We have created chimeric vitamin K-dependent proteins in which domains remote from the Gla region of the proteins have been either deleted or exchanged. Replacement of the second epidermal growth factor domain and serine protease domains of Factor IX with those from Factor X leads to a chimera in which the Gla domain of Factor IX is well carboxylated (62). In contrast, Factor X in which the first epidermal growth factor domain has been replaced with the first epidermal growth factor domain of Factor IX is only about 15% carboxylated. Prothrombin mutants in which either the first kringle domain or both kringle domains are deleted are only 40–50% carboxylated (63). We suggest that the phenomena observed in the protein C mutations is related to our observation of the relationship of structures in the mature proteins to the efficiency of carboxylation.

While the efficiency with which substrates containing the propeptide are carboxylated may be dependent upon complex structural aspects of the substrate, the propeptide containing the γ-carboxylation recognition site may be sufficient to designate a protein as a substrate for the vitamin K-dependent carboxylase and to direct the synthesis of γ-carboxyglutamic acid. Analysis of the propeptide-mediated in vivo carboxylation of protein substrates containing sequences irrelevant to the vitamin K-dependent proteins should further understanding of the γ-glutamyl carboxylase-substrate interaction.

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