Propeptide Processing during Factor IX Biosynthesis

EFFECT OF POINT MUTATIONS ADJACENT TO THE PROPEPTIDE CLEAVAGE SITE

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Factor IX is synthesized in a precursor form with a propeptide that contains the \(\gamma\)-carboxylation recognition site, an element which directs the post-translational \(\gamma\)-carboxylation of adjacent glutamic acid residues. After protein synthesis, the propeptide is cleaved to yield the mature Factor IX. To study propeptide processing, anti-proFactor IX antibodies were prepared using a synthetic peptide based upon the sequence of the Factor IX propeptide. Immunosaffinity-purified anti-proFactor IX antibodies were reactive with Factor IX Cambridge, a mutant form of Factor IX containing the propeptide, but were not reactive with Factor IX. These antibodies were used to examine the proteolytic processing of forms of Factor IX containing point mutations at P6, P3, P2, P1, P1', P2', and P3 adjacent to the propeptide cleavage site. Furthermore, the hierarchy of different pairs of basic residues at positions P1 and P2 was analyzed. The mutated cDNA constructs were expressed in Chinese hamster ovary cells. Propeptide processing was examined using intrinsically labeled Factor IX immunoprecipitated with either anti-proFactor IX antibodies or anti-Factor IX:total antibodies, and the Factor IX species were separated by SDS-gel electrophoresis. Under the expression conditions employed, the propeptide of wild type Factor IX was almost completely removed, whereas Factor IX mutated to threonine at P1 was not cleaved. The percentage of propeptide cleaved varied with the amino acid sequences of residues P2 and P1, respectively: Lys-Arg (93%), Arg-Arg (66%), Thr-Arg (33%), Arg-Lys (19%), Lys-Lys (10%), and Lys-Thr (<1%). Apart from alterations of basic amino acids at P1 and P2, nonconservative mutations at P6 and P3 decreased propeptide cleavage, whereas conservative mutations at P3, P1', P2', or P3' resulted in cleavage efficiencies approximately equal to that for wild type Factor IX. These results indicate that the preference of paired basic residues at P1 and P2 is similar to other endopeptidases active toward proteins secreted through the constitutive pathway and that the propeptide residues NH2-terminal to these paired basic residues are important in defining enzyme-substrate binding.

Factor IX is a vitamin K-dependent blood coagulation protein which undergoes several post-translational modifications during its synthesis and secretion as a zymogen into plasma. These modifications include signal peptide cleavage, \(N\)- and \(O\)-linked glycosylation, \(\beta\)-hydroxylation of Asp44, \(\gamma\)-carboxylation of the first 12 glutamic acid residues in the amino terminus, and cleavage of the 18-amino acid propeptide (for review, see Ref. 1). The propeptides of the vitamin K-dependent clotting proteins, including prothrombin, Factor IX, Factor X, Factor VII, protein C, and protein S, demonstrate marked sequence homology (2). These propeptides have been shown to be a necessary and sufficient recognition element to direct the \(\gamma\)-carboxylation of glutamic acid residues adjacent to the propeptide (3-6). The \(\gamma\)-carboxylation recognition site is located at the amino-terminal region of the propeptide, a polypeptide that includes a 10-residue amphipathic \(\alpha\)-helix (7), whereas the propeptidase recognition element is proposed to be located in the carboxyl-terminal region of the propeptide, adjacent to the scissile bond (8). Factor IX from some hemophilia B patient plasmas has been characterized by mutations that preclude propeptide cleavage (9-11). For example, Factor IX Cambridge (9) contains a mutation from Arg4 to serine, whereas Factor IX Oxford 3 (10) and Factor IX San Dimas (11) contain mutations from Arg4 to glutamine. Both of these mutations prevent propeptide cleavage during biosynthesis.

Many plasma proteins are synthesized in a precursor form, with their propeptides removed proteolytically as a late processing event (12, 13). These propeptides contain recognition elements that are critical for protein synthesis and trafficking. Most proproteins have a pair of basic amino acids at positions P1 and P2 adjacent to the processing site. This basic pair of residues has been shown both in vivo and in vitro to be a necessary recognition element for efficient propeptide cleavage of several plasma proteins, including albumin (14), Factor IX (9), and von Willebrand factor (15). In addition, an arginine at P4 has been included in an extended cleavage motif, Arg-X-(Lys/Arg)-Arg, which is recognized by a different class of endopeptidases. The requirement for Arg\(^+\) has been demonstrated in vivo in the case of Factor IX Oxford 3 (10) and Factor IX San Dimas (11). The first eukaryotic endopeptidase identified which cleaves propeptides after paired basic amino acids was Kex2, the yeast enzyme which processes the propeptides of \(\alpha\)-mating factor and cell wall toxin (17). Recently, candidate mammalian propeptidases have been identified and cloned by PCR (15, 18-21). When co-expressed in cultured mammalian cells with specific proproteins, these peptidases can process proproteins properly and efficiently. Such processing is dependent upon the secretion pathway for a

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\(^{1}\)The abbreviations used are: PCR, polymerase chain reaction; furin, fes/fip upstream region open reading frame; PACE, paired basic amino acid cleavage enzyme; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; CHO, Chinese hamster ovary.
given protein. For example, furin/PACE is expressed in most tissues (22, 23) and is active toward proteins secreted via the constitutive pathway. Furin/PACE is specific for protein substrates with basic residues at P1, P2, and P4. In contrast, PACE and PC3, PC2, and PC4 have been detected only in neural and endocrine cells (18, 21, 24) and appear active toward proteins secreted via the regulated pathway (25, 26, 28). These proteases also require a pair of basic amino acids at P1 and P2. The recently described rabbit liver endopeptidase purified by Kawabata and Davie (20) cleaved peptides modeled after the proteolytic cleavage sites of the vitamin K-dependent blood coagulation proteins pro-thrombin, Factor IX, and Factor X but showed a substrate specificity different from Kex2 and furin/PACE. This novel endopeptidase is a metalloprotease stimulated by CoCl2 and has a pH optimum of 8.7, properties in marked contrast to Kex2 and furin/PACE. The latter two endopeptidases are calcium-dependent subtilisin-like serine proteases and have pH optima of 5.5-7.5 (27-30).

In this report, we describe the preparation of anti-proFactor IX antibodies that specifically recognize the propeptide of Factor IX. This antibody reacts with proFactor IX but not with Factor IX. Using this antibody, the intracellular cleavage of proFactor IX and propeptide cleavage point mutants expressed in Chinese hamster ovary cells has been studied to determine the specificity and efficiency of the endogenous endopeptidase expressed in Chinese hamster ovary cells, which secrete proteins via the constitutive pathway.

**EXPERIMENTAL PROCEDURES**

**Preparation and Purification of Anti-proFactor IX Antibodies—** A peptide, KGGTVFLDHENANKILNRPKRYNS, corresponding to the Factor IX propeptide plus the first 3 residues of the mature molecule (residues -18 to +3), was synthesized on an Applied Biosystems 430A peptide synthesizer (31). The peptide was synthesized with a Lys-Gly-Gly amino-terminal linker arm to facilitate coupling to both BSA (Sigma) and agarose. After purification, the sequence of the peptide was verified by automated Edman degradation using an Applied Biosystems 470A Protein Sequencer (32). The peptide was coupled to BSA using glutaraldehyde. The proFactor IX peptide (150 pL; 5 mM), dissolved in PBS (10 mM sodium phosphate, pH 7.4, 140 mM NaCl), was mixed with 500 ml of 1% BSA dissolved in PBS, and 350 ml of PBS was added to bring this volume to 1 ml. Glutaraldehyde (1 ml; 2.5 mM; Sigma) was added dropwise to the peptide:BSA solution and stirred for 2 h at room temperature. The final molar ratio of peptide:BSA:glutaraldehyde was 30:1:10. The peptide:BSA conjugate was dialyzed against PBS and stored at -20 °C. A New Zealand White rabbit subsequently received monthly injections of 200 ng of the peptide:BSA conjugate in Freund's incomplete adjuvant (1 ml). The rabbit subsequently received monthly injections of 200 mg of the peptide:BSA conjugate in Freund's incomplete adjuvant (1 ml). Anti-serum was prepared 10-14 days after each immunization according to Harlow and Lane (33). Anti-proFactor IX antibodies were purified by immunoaffinity chromatography using the synthetic propeptide or BSA linked to cyanogen bromide-activated Sepharose-4B (Pharmac LKB Biotechnology Inc.) according to the manufacturer's instructions. Approximately 15 ml of antiserum was applied to a BSA-Sepharose column (7.5 mg BSA/ml Sepharose-4B; 1 x 5-cm column) at 25 °C to remove anti-BSA antibodies. Antibodies that failed to bind were applied to a propeptide-Sepharose column (6 mg of propeptide/ml of Sepharose-4B; 1 x 5-cm column). The column was washed with approximately 50 column volumes of 10 mM Tris-HCl (pH 7.4), 1 mM NaCl, 0.02% Tween 20, equilibrated with TBS (10 mM Tris (pH 7.4), 140 mM NaCl), and then eluted with 4 M guanidine HCl. Protein elution was monitored by absorbance at 280 nm on a Beckman DU-70 spectrophotometer. The guanidine HCl eluate was immediately dialyzed against TBS at 4 °C and then stored at -20 °C. Anti-Factor IX total antibodies were prepared as described previously (34).

**Enzyme-linked Immunosorbent Assay—** Purified antibodies were tested in an enzyme-linked immunosorbent assay. Factor IX-related antigens were bound at varying concentrations (100-0.1 nm) to 96-well microtiter plates (Nunc Immuno MaxiSorp; InterMed) at 4 °C overnight, then washed and blocked to a final volume of 90 ml. A solution of 5% non-fat dry milk (5% Carnation) made in TBS was added to the coated wells for 30 min at room temperature to block nonspecific protein binding sites. Plates were incubated with either the purified rabbit anti-proFactor IX antibodies (50 ml; 2 ng/ml in 5% milk) or purified goat anti-Factor IX total antibodies (50 ml of 2 ng/ml in 5% milk) for 1 h at room temperature. Plates were then washed three times with TBS, then incubated with either anti-rabbit or rabbit anti-goat immunoglobulin-alkaline phosphatase conjugates (Sigma) for 1 h at room temperature. The assay was developed with 50 ml of p-nitrophenyl phosphate (Sigma) in 0.5 M sodium carbonate buffer (pH 9.8) containing 0.1 M MgCl2. Plates were read at 405 nm on a microtiter plate reader (Molecular Devices) after an approximately 30-40 min incubation at 37 °C.

**Immunoprecipitation and Western Blotting of proFactor IX and Factor IX—** Goat polyclonal anti-Factor IX total antibody (50 ml) was used to immunoprecipitate Factor IX from a partially purified plasma-derived Factor IX Cambridge fraction (9) or from purified plasma-derived Factor IX (American Red Cross). After a 1-h incubation on ice, 25 ml of a 1:40 dilution of antiserum from donkey anti-goat IgG (Pel-Freeze) was added, and the reaction mixtures incubated for an additional hour on ice. The immune complexes were isolated by binding them to Staphylococcus aureus protein A-Sepharose (Sigma, 10% vv in TBS) during a 1-h incubation on ice with intermittent mixing. The Sepharose beads were sedimented in an Eppendorf microcentrifuge and washed twice with TBS containing 1 M NaCl and 1 mM CaCl2 with 0.5 M LiCl, suspended in gel loading buffer (35) containing 5% 2-mercaptoethanol (Sigma). The suspensions were boiled for 5 min, loaded onto 7% polyacrylamide gels containing 0.1% SDS, and the proteins were separated by electrophoresis (35). The separated proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore, Immobilon) at 70 V (1 h) in Laemmlin running buffer (35) containing 10% methanol. Nonspecific protein binding sites on the membrane were blocked by soaking it for 15 min at room temperature in 5% milk. The Factor IX species were detected with either rabbit anti-proFactor IX antibodies or goat anti-Factor IX total antibodies. The antibodies were diluted to 5 ng/ml in the 5% milk solution, and the membranes were incubated with the antibody solutions for 1 h at 37 °C. The membranes were washed three times with TBS and then incubated with the appropriate second antibody conjugated to alkaline phosphatase (Sigma) in a 1:1000 dilution in the 5% milk solution for 1 h at 37 °C. Blots were developed with 50 ml of p-nitrophenyl phosphate (Sigma) in 1 ml of 0.1 M sodium carbonate buffer (pH 9.8) containing 1 mM MgCl2 according to the manufacturer's protocol (Bio-Rad).

**In Vitro Mutagenesis of Human Factor IX cDNA—** Point mutations were generated by the oligonucleotide polymerase chain reaction method (30) using PCR. All mutations were made in the FIX/pMT2 expression plasmid. Briefly, two mutagenic oligonucleotides were made which each contained the mutation, overlap each other, and are antiparallel. Also, two antiparallel oligonucleotides flanking the mutation site and containing two unique restriction sites (BglII in pMT2,AscI in Factor IX cDNA) were made (Table I). All oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. For each mutation, two reactions, each containing one mutagenic primer, one flanking primer, and the Factor IX/pMT2 plasmid, were used in the standard PCR reaction as suggested by the manufacturers (Perkin-Elmer Cetus, Tag polymerase PCR kit). As an example, one reaction generated a 250-base pair fragment (Flanking Primer 1: FIX/RK-1 (+)-strand) and the other generated a 600-base pair fragment (Flanking Primer 2: FIX/RK-1 (-)-strand). After the first round of PCR, 5-ml aliquots of each of the products were mixed together in a second round of PCR with only the flanking primers added to the reaction. After a phenol/chloroform extraction, the 850-base pair fusion product was digested with BglII and AscII (New England Biolabs), gel-purified, and subcloned into Factor IX/pMT2 previously digested with BglII and AscII. The digested plasmid was ligated in XL-1 Blue cells (Strategene), purified by CsCl gradient centrifugation, and sequenced (37) by the double-stranded DNA dideoxynucleotide chain termination method (United States Biochemical Corp. Sequenase) to verify the mutations.

**Cell Lines and Tissue Culture Methods—** Maintenance of Chinese hamster ovary cells and the mammalian cell expression vector used, pMT2, have been described (40). Cells (1 x 106) were transfected by electroporation with a Bio-Rad Gene Pulser (300 mV; 960 microfarads) using 15 pl of DNA in 500 pl of media as indicated in the manufacturer's instructions. Cells were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v:v) fetal bovine serum (FBS) and 1% (v:v) antibiotics (penicillin, streptomycin, and amphotericin B).
To study the biosynthesis of recombinant Factor IX expressed in Chinese hamster ovary cells, we generated a polyclonal antibody directed against the Factor IX propeptide to examine propeptide cleavage during the conversion of pro-Factor IX to Factor IX. This antibody was generated in a rabbit using a synthetic peptide modeled after proFactor IX residues -18 to +3 that was coupled to BSA as a carrier protein. The anti-proFactor IX antibodies were purified from rabbit antiserum by sequential affinity chromatography. Antibody that failed to bind to BSA-Sepharose was applied to the propeptide-Sepharose column. The antibody that bound was eluted with 4 M guanidine HCl and is referred to as anti-proFactor IX antibody.

As shown in Fig. 1A, when tested in an enzyme-linked

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immunosorbent assay the anti-proFactor IX antibodies are reactive with either the synthetic propeptide (−18 to +3) or a partially purified fraction of plasma-derived Factor IX Cambridge. However, the anti-proFactor IX antibody did not react with plasma-derived Factor IX which lacks the propeptide. In comparison, an immunoadfinity-purified goat polyclonal antiFactor IX antibody, referred to as anti-Factor IX:total antibody which is directed against epitopes unaffected by bound calcium ions (34), bound to both plasma-derived Factor IX Cambridge and plasma-derived Factor IX, but not to the synthetic propeptide (Fig. 1B). Because of possible differences in the binding of the peptide antigens to the solid phase, quantitative comparisons of the properties of the antigen-antibody interactions are not possible from these data.

The interaction of the anti-proFactor IX antibody with proFactor IX and Factor IX was also evaluated by Western blotting. Fig. 2A shows that the anti-proFactor IX antibodies react with Factor IX Cambridge (lane 1) but not with Factor IX (lane 2). Fig. 2B emphasizes differences in the electrophoretic mobility between proFactor IX (lane 1) and Factor IX (lane 2), where the Factor IX species are detected with the anti-Factor IX:total antibody. These differences in electrophoretic migration have been attributed to the propeptide extension on proFactor IX (9).

To define the substrate specificity of the endopeptidase that cleaves the propeptide of Factor IX when expressed in Chinese hamster ovary cells, we mutated the residues around the scissile bond of the Factor IX propeptide. The requirement for a pair of basic residues at P1 and P2 as well as the hierarchy of pairs of basic residues at P1 and P2 was tested. Further mutations were selected to test the hypothesis that amino acids on either side of the scissile bond, in addition to P1, P2, and P4, are important for recognition by the propeptidase. These include P6, P3, P1', P2', and P3'. We made a series of conservative and nonconservative changes at these positions (Fig. 3). Mutations at P1' are of particular interest, since Factor IX contains a tyrosine at position P1', whereas the other vitamin K-dependent coagulation proteins contain alanine at this position. Likewise, Factor IX contains a proline at P3 which may play a special role in cleavage by the propeptidase machinery, since β-turns are frequently predicted to be adjacent to endopeptidase processing sites (39).

Requirement and Hierarchy of Basic Residues at P1 and P2—To analyze the requirement for basic amino acids at residues P1 and P2 for propeptide cleavage, point mutations in the Factor IX cDNA were made and expressed in Chinese hamster ovary cells which secrete biologically active Factor IX when transfected with the cDNA for wild type Factor IX (40, 41). Arg<sup>2</sup> or Lys<sup>2</sup> were individually mutated to threonine (FIX/RT-1 and FIX/KT-2, respectively) based on the observation that a related vitamin K-dependent blood clotting protein, Factor X, contains a threonine at P2 and that the naturally occurring variant, Factor IX Cambridge, contains a mutation from Arg<sup>2</sup> to serine. This mutation in Factor IX Cambridge precludes propeptide cleavage but does not affect secretion into the blood (9). Additional mutations were made which probe the hierarchy of preferred basic pairs of amino acids at positions P1 and P2. Factor IX with cleavage sites mutated to Arg-Arg (FIX/KR-2), Arg-Lys (FIX/RK-1, KR-2), or Lys-Lys (FIX/RR-1, RR-2) was also expressed (Fig. 3). Propeptide processing of Factor IX secreted by heterologous cells was evaluated by examining the [35S]cysteine-labeled Factor IX species secreted into CHO cell supernatants. The Factor IX species for each mutant and wild type Factor IX are shown in Fig. 4. Radiolabeled Factor IX was immunoprecipitated from the cultured CHO cell media with either the anti-

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**Fig. 3. Construction of Factor IX propeptide cleavage mutants.** Mutations were introduced by the site overlap extension method of the polymerase chain reaction (SOE-PCR) as described under "Experimental Procedures." Boldface letters represent mutated amino acids. Numbers above the amino acids indicate position in the propeptide or mature NH<sub>2</sub>-terminus. Mutants were generated in the Factor IX/pMT2 expression vector. The mutated fragments were subcloned into Factor IX/pMT2 and sequenced before transfection into CHO cells.

**Fig. 4. Propeptide cleavage pattern of wild type, P1, and P2 mutants of Factor IX.** CHO cells expressing wild type or propeptide cleavage mutants as P1 or P2 of Factor IX were radiolabeled with [35S]cysteine. The Factor IX species were immunoprecipitated from the cell supernatants with either anti-proFactor IX antibodies or anti-Factor IX:total antibodies. Protein samples were loaded onto 7% polyacrylamide gels and electrophoresed. Gels were dried and scanned with a PhosphorImager. A, anti-proFactor IX antibodies. B, anti-Factor IX:total antibodies. Lanes 1, molecular mass standards; lanes 2, mock-transfected CHO cells; lanes 3, FIX/wild type; lanes 4, FIX/KR-2; lanes 5, FIX/KT-2; lanes 6, FIX/RK-1, KR-2; lanes 7, FIX/RK-1; lanes 8, FIX/RT-1.
proFactor IX antibodies (Fig. 4A) or the anti-Factor IX:total antibodies (Fig. 4B) and subjected to SDS-gel electrophoresis. Protein bands were visualized by scanning on a PhosphorImager. Both anti-proFactor IX and anti-Factor IX:total antibodies specifically immunoprecipitated Factor IX species from CHO cell supernatants, since immunoprecipitation of a mock-transfected CHO cell line failed to detect any cross-reacting proteins (lane 2). Cells expressing wild type Factor IX (lane 3) processed proFactor IX to Factor IX with high efficiency since the amount of proFactor IX immunoprecipitated with either anti-proFactor IX or anti-Factor IX:total antibodies was barely detected. However, when a conservative mutation, Lys-2 to arginine, was introduced at P2 (FIX/KR-2), proFactor IX processing was less efficient, since more proFactor IX was immunoprecipitated by the anti-proFactor IX antibody (lane 4). Also, proFactor IX was visible when immunoprecipitated with the anti-Factor IX:total antibodies. When a nonconservative mutation at P2, Lys-2 to threonine, was expressed (FIX/HT-2), proFactor IX propeptide cleavage efficiency was further impaired (lane 5) as evidenced by the observation that the anti-proFactor IX antibodies immunoprecipitated more proFactor IX. Likewise, more proFactor IX was immunoprecipitated with anti-Factor IX:total antibodies (lane 5). The interchange of basic amino acids at P1 and P2 (FIX/RK-1, KR-2) resulted in poorly cleaved Factor IX species. This is demonstrated in lane 6 where most of the immunoprecipitated Factor IX species migrated as proFactor IX. When substitutions at Arg-1 were introduced, the ratio of proFactor IX to Factor IX immunoprecipitated with either anti-proFactor IX or anti-Factor IX:total antibodies increased. Lane 7 (FIX/RK-1) and lane 8 (FIX/RT-1) show barely detectable bands of Factor IX, and most of the Factor IX species migrates as proFactor IX.

The relative amounts of proFactor IX and Factor IX were quantitated using a PhosphorImager by integrating the volume of the protein bands represented by each species immunoprecipitated with anti-Factor IX antibodies from supernatants of FIX/LF-6, FIX/PV-3, FIX/PD-3, and FIX/LD-6. Values represent the average of at least two labeling experiments using three different cell lines of each mutant or wild type. 

**Effect of Mutation of Residues P6 and P3—Mutations at Leu-6 were made either to introduce a charged amino acid at P6 (FIX/LF-6) or an amino acid with a bulkier side chain (FIX/LF-6). These residues influence propeptidase recognition or catalytic efficiency, since the secreted mutant Factor IX species were only partially cleaved (Fig. 6, A and B).** Significantly more proFactor IX was immunoprecipitated by anti-proFactor IX antibodies from supernatants of FIX/LF-6 (lane 4) and FIX/LD-6 (lane 5) compared with wild type Factor IX (lane 3). By quantitation using the digitized imaging data, FIX/LD-6 was processed 31 ± 6%, and FIX/LF-6 was processed 30 ± 12% (Fig. 7). The proline at P3 was mutated to valine (FIX/PV-3) to disrupt any secondary structure that might be defined by an imino acid. Pro-3 was also mutated to aspartate (FIX/PD-3) to introduce a charged amino acid at this position. The results of these mutations are shown in Fig. 6, A and B. The FIX/PV-3 mutation resulted in 97 ± 4% propeptide cleavage, while the FIX/PD-3 mutation resulted in 97 ± 6% propeptide cleavage.
in efficient cleavage, since no proFactor IX is detected (lane 6). The introduction of an acidic amino acid adjacent to the basic pair at P2 and P1 reduced propeptide cleavage, since both Factor IX and proFactor IX species were immunoprecipitated with the anti-Factor IX:total antibody (lane 7). The efficiencies of propeptide processing for FIX/PV-3 and for FIX/PD-3 were 99 ± 6% and 58 ± 14%, respectively. These results indicate that although substitution of Pro-3 with valine does not alter propeptide cleavage efficiency, the introduction of an acidic residue adjacent to the basic pair at P2 and P1 can decrease Factor IX propeptide cleavage.

**Effect of Mutations at Positions P1’, P2’, and P3’**—The presence of tyrosine at P1’ is not homologous with the other vitamin K-dependent blood coagulation proteins. We mutated this residue in Factor IX to conform to the other clotting factors (FIX/YA+1). Meulien et al. (42) found that when this identical construct was expressed in BHK21 cells, the biological activity of the secreted Factor IX species increased 2-fold, presumably because the propeptide was removed more effectively. We confirmed that this mutated Factor IX is processed more efficiently than the wild type Factor IX, since proFactor IX was not immunoprecipitated with the anti-Factor IX antibody (Fig. 6A, lane 8). This mutant was completely processed (99 ± 2%; Fig. 7).

The conserved asparagine at P2’ and serine at P3’ were each changed to either a similar neutral amino acid (FIX/NV+2 and FIX/SK+3) or a charged amino acid (FIX/ND+2 and FIX/SK+3) to probe their effects on propeptide recognition or cleavage efficiency. The charge change at P2’ (FIX/NV+2) was associated with a modest decrease in processing, 84 ± 6%, as shown in Fig. 6, lane 9. The conservative mutation at P2’ (FIX/NV+2) and either the conservative (FIX/SA+3) or nonconservative mutation (FIX/SK+3) did not influence processing efficiency compared with the wild type Factor IX, as demonstrated in lanes 10, 11, and 12, respectively, where proFactor IX was barely detected with the anti-proFactor IX antibody. All the secreted Factor IX from these three mutants co-migrated with processed Factor IX immunoprecipitated with the anti-Factor IX:total antibodies. Quantitatively, FIX/NV+2 was processed 96 ± 4%, FIX/SA+3 was processed 89 ± 4%, and FIX/SK+3 was processed 93 ± 3%. These results indicate that the specificity of the propeptidase does not extend to residue P2’ or residue P3’ adjacent to the scissile bond.

**DISCUSSION**

Factor IX is synthesized in a precursor form, preproFactor IX, that includes a signal peptide, a propeptide, and the mature protein. The propeptides of the vitamin K-dependent blood coagulation proteins, including Factor IX, play a special role in post-translational processing inasmuch as this region contains a recognition element, termed the “γ-carboxylation recognition site,” that directs γ-carboxylation of the first 12 glutamic acid residues adjacent to the propeptide in the mature amino terminus of Factor IX (3). Between the time of carboxylation and the secretion of Factor IX from the cell into the plasma, the propeptide is cleaved, thus converting proFactor IX to Factor IX. Although the fully carboxylated proFactor IX has not been isolated and characterized, there is reason to believe that the propeptide attached to the NH₂ terminus of the protein Precudes folding of the Gla domain into its active conformation. The NH₂-terminal alanine of prothrombin forms ion pairs with Glal γ, Glal δ, and Glnγ which appear critical for the maintenance of the structure of the Gla domain and its interaction with phospholipid membranes (43). Acetylation of the NH₂ terminus precludes prothrombin-

-lipid interaction (44), so we might logically predict that the presence of the propeptide attached to the NH₂ terminus would also preclude membrane interaction of proFactor IX in the presence of calcium ions. Thus, the cleavage of the propeptide is critical to the generation of an active Factor IX species, and the presence of the propeptide may prevent proFactor IX from binding to internal cell membranes.

In this study, we have examined the role of amino acids on either side of the scissile bond in the cleavage of the propeptide from proFactor IX. Antibodies were raised against a synthetic peptide based upon the sequence of the Factor IX propeptide. These antibodies are presumably conformation-specific for the random format of the propeptide when, within the proFactor IX molecule, the propeptide likely contains significant α-helical structure (7). For these reasons, binding of the antibody to proFactor IX species was facilitated by use of certain assay systems in which the proFactor IX is likely unfolded. For example, Western blot analysis exposes the proFactor IX to SDS. Other assays involved attachment of the antigen to a solid phase. In these cases, it might be expected that the propeptide of proFactor IX would lose most secondary structure, thus resembling the structure of the peptide immunogen. Other workers (45) have also prepared antibodies that are specific for proalbumin and are not reactive with mature albumin. These antibodies allowed the rapid isolation of proalbumin and the analysis of the biosynthesis and processing of albumin in cultured rat hepatocytes.

We have examined the effect of mutations at residues -2 and -1 on propeptide cleavage of human Factor IX expressed in Chinese hamster ovary cells. We have shown previously that wild type Factor IX expressed in this heterologous system undergoes post-translational processing, including cleavage of the propeptide (3, 41). Our current results confirm this observation with the use of anti-propeptide antibodies, a more sensitive assay for propeptide than the gel electrophoretic shift or amino-terminal sequencing assays employed previously. We now demonstrate a hierarchy of preference for certain amino acids at P1 and P2. Arginine is the preferred basic amino acid at P1. However, the presence of a basic residue at P2 enhances propeptide cleavage, it is not essential since the Thrγ mutant is partially cleaved. This is in agreement with reports of the specificity of furin/PACE (16, 46) and Kex2 (27, 47) for paired basic amino acids at P1 and P2. In contrast to the other forms of proFactor IX tested, proFactor IX with Lysγ and Thrγ remains uncleaved. This form is analogous to Factor IX Cambridge, a naturally occurring mutant Factor IX in which Argγ is replaced by a serine (9). These results emphasize that a basic residue at P1 is strictly required for propeptide processing. On the basis of these results, the efficiency of cleavage of the propeptide varied in the following pattern of single or paired basic amino acids: Lys-Arg > Arg-Arg > Thr-Arg > Arg-Lys > Lys-Lys >> Lys-Thr.

Previous work has demonstrated the importance of other amino acids in the Factor IX propeptide which contribute to propeptidase recognition. Bentley et al. (10) and Ware et al. (11) identified Factor IX species from hemophilia B patient plasmas which contained propeptide. These aberrant Factor IX molecules both had Arg¹⁴ replaced with glutamine, a mutation which precluded propeptide cleavage. In addition, Leu²⁸ has been mutated in vitro to aspartate and the mutant Factor IX was found not to be cleaved, as determined by gel electrophoretic mobility and NH₂-terminal sequencing (8). We confirmed this result and showed quantitatively that either a charge change (FIX/LD-6) or a substitution with a
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bulky hydrophobic amino acid (FIX/LF-6) both significantly reduce propeptide processing.

Although there is no highly conserved amino acid at P3 among the vitamin K-dependent clotting factors, Factor IX contains a proline at this position. Rhomel et al. (39) reported that β-turns frequently are predicted to form at, or adjacent to, proprotein processing sites, flanked on either side by an α-helix or a β-sheet. Since the propeptide of prothrombin is predicted to form an α-helix from residues −13 to −3 (7), we speculated that if the homologous propeptide of Factor IX forms an α-helix, then the Pro-3 might introduce a β-turn at the COOH-terminal end of the α-helix, thus contributing to the propeptide recognition element. A mutation to disrupt a putative β-turn (FIX/PV-3) did not affect cleavage efficiency, a result which confirms recent work by Meullen et al. (42). The introduction of an acidic residue (FIX/PD-3) adjacent to the basic pair at P2 and P1 had a marginal effect on cleavage efficiency (58 versus 93% for the wild type). We conclude that the Pro-3 of Factor IX is not important for propeptide cleavage but is probably important for the spacing of arginines at P4 and P1.

Meullen et al. (42) also altered Tyr1 to alanine (42), a mutation in Factor IX that rendered the identical amino terminus as the other vitamin K-dependent coagulation proteins. When expressed in BHK21 cells, they found processing efficiency increased from 70% for wild type to 90% for the Ala1 mutant as detected by amino-terminal sequencing. In CHO cells, we found that the same mutation increased processing efficiency from 93% for wild type to 99% for the Ala1 mutant as determined by immunoprecipitation of radiolabeled Factor IX by anti-proFactor IX antibodies or quantitation using the anti-Factor IX-total antibodies. The differences observed are most likely due to the fact that processing is incomplete in BHK21 cells compared with CHO cells at comparable levels of expression of recombinant Factor IX. It is thought that at higher expression levels, the intracellular processing machinery becomes saturated with substrate, resulting in secretion of incompletely processed Factor IX (40).

It is possible to compare the CHO cell endopeptidase that cleaves proFactor IX with other propeptidases known to participate in proteolytic processing of polypeptides during intracellular biosynthesis. Furin/PACE, a processing enzyme localized to the trans-Golgi compartment (48–50), is involved in precursor cleavage of substrates containing the Arg-X-Lys/Arg-Arg consensus sequence motif (46). Rehentulla and Kaufman (16) have recently demonstrated that prorenin Wilberland factor containing an aspartic acid at P2 was partially cleaved in COS cells co-transfected with the furin/PACE cDNA. Using a mouse prorenin cDNA expressed in CHO cells, Watanabe et al. (51) demonstrated an endopeptidase activity within the constitutive pathway of secretion in CHO cells, with a preference for lysine over arginine at P2 and a preference for arginine over lysine at positions P4 and P1. However, the requirement for a basic residue at P2 was absolute, insofar as mutation of Lys2 to glutamine abolished propeptide cleavage. Furthermore, Kowabata and Davie (20) have reported a rabbit liver endopeptidase capable of cleaving synthetic peptides modeled after the sequence of the propeptide and mature NH₂ terminus of the vitamin K-dependent blood clotting proteins prothrombin, Factor IX, and Factor X. This enzyme had a preference for threonine at P2 and arginine at P3, but also cleaved paired basic amino acids at P2 and P1. In addition, this activity preferred an arginine at P4, but this was not absolute with the peptides employed in the assays. Moreover, this propeptidase was distinct from furin/PACE with regard to pH optimum, protease family, and primary sequence. The substrate recognition preferences of this enzyme appear slightly different than the constitutive endopeptidase activity in CHO cells examined in the current study. Furthermore, the intracellular distribution of this putative propeptidase is not known at present, so it remains uncertain whether it is located in organelles consistent with its role in proteolytic processing. In this study, mutation of Lysy to threonine decreased propeptide cleavage efficiency, but cleavage efficiency was nonetheless within 3-fold of the wild type. Another vitamin K-dependent blood clotting protein, Factor X, contains threonine at the P2 site. Expression in CHO cells leads to fully processed cleaved protein (52). Thus, it remains unclear whether a CHO cell homologue of one of these previously identified propeptidases or a different propeptidase is operative in the constitutive pathway during the expression of proteins such as Factor IX and Factor X.

In summary, we have shown the importance of certain amino acid residues surrounding the scissile bond of the Factor IX propeptide and their role in propeptide cleavage. The preparation of a proFactor IX-specific antibody proved useful for cleavage analysis. Our results allow us to neither confirm nor disprove that Factor IX processing in CHO cells is mediated by the serine protease furin/PACE or by a metalloprotease described recently (20). Moreover, the number of propeptidases involved in propeptide processing remains unknown. Further study of the sequences recognized in vivo by these propeptidases, the localization of propeptidases within intracellular compartments, the identification of related endopeptidases, and the purification and characterization of propeptide forms of the vitamin K-dependent proteins will facilitate understanding of the biosynthesis of this unique class of proteins containing γ-carboxyglutamic acid.

REFERENCES

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