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Molecular cloning of matrix Gla protein: Implications for substrate recognition by the vitamin K-dependent γ-carboxylase

(γ-carboxyglutamic acid/bone/protein carboxylation)

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ABSTRACT Matrix Gla protein (MGP), a low molecular weight protein found in bone, dentin, and cartilage, contains 5 residues of the vitamin K-dependent amino acid γ-carboxyglutamic acid (Gla). We have used antibodies raised against MGP and oligonucleotide probes to screen a λgt11 cDNA library constructed from the rat osteosarcoma cells (line ROS 17/2) that had been pretreated with 1α,25-dihydroxyvitamin D_3. By sequencing several cloned cDNAs, we established a 523-base-pair sequence that predicts an 84-residue mature MGP and a 19-residue hydrophobic signal peptide. The 84-residue mature rat MGP predicted from the cDNA sequence has an additional 5 residues at its C terminus (-Arg-Arg-Gly-Ala-Lys) not seen in the sequence of MGP isolated from bovine bone. The structure of rat MGP provides insight into the mechanisms by which the vitamin K-dependent γ-carboxylase recognizes substrate. The present studies show that MGP, unlike other vitamin K-dependent proteins, lacks a propeptide. The absence of an MGP propeptide demonstrates that γ-carboxylation and secretion of vitamin K-dependent proteins need not be linked to the presence of a propeptide or to its proteolytic removal. The propeptides of other vitamin K-dependent proteins are structurally homologous, and there is evidence that this homologous propeptide domain is important to substrate recognition by the γ-carboxylase. Mature MGP has a sequence segment (residues 15–30) that is homologous to the propeptide of other vitamin K-dependent proteins and probably serves the same role in γ-carboxylase recognition. Rat MGP also has a second sequence that has recently been identified in all known vitamin K-dependent vertebrate proteins, the invariant unit Glu-Xaa-Xaa-Xaa-Glu-Xaa-Cys (EXXXEXC). Since the glutamic residues in this unit are sites of γ-carboxylation, it has been suggested that the EXXXEXC unit could allow the γ-carboxylase to discriminate between substrate and product. The demonstration that two structures common to vitamin K-dependent proteins, the homologous propeptide domain and the invariant EXXXEXC unit, are in mature MGP indicates that des-γ-carboxy-MGP should be an excellent *in vitro* γ-carboxylase substrate for analysis of mechanisms involved in substrate recognition and product dissociation.

Matrix Gla protein (MGP) is a 79-residue protein, found in bone, dentin, and cartilage, that contains 5 residues of the vitamin K-dependent amino acid, γ-carboxyglutamic acid (Gla) (1–3). The C-terminal domain of bovine MGP (residues 31–79) has 20% sequence identity to the 49-residue structure of bone Gla protein (BGP), the only other vitamin K-dependent protein isolated from bone. Although this degree of sequence identity is at the threshold of statistical significance, the fact that specific amino acid residues conserved in BGP from all species examined to date are also found in MGP indicates that the C-terminal domain of MGP and the secreted form of BGP arose from a common ancestor by gene duplication and subsequent divergent evolution (2). MGP associates with the organic matrix of cartilage and bone and is virtually water-insoluble (<10 μg/ml) in the absence of denaturants, a property that is noteworthy given its high percentage of hydrophilic amino acids and its small size (1–4). Although the function of MGP is unknown, vitamin K-deficient rats develop excessive mineralization of growth-plate cartilage, which indicates that a vitamin K-dependent cartilage protein, possibly MGP, normally prevents cartilage mineralization (5). In clonal osteoblastic cells, MGP production is regulated by 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (6, 7). In the present study, we have undertaken the molecular cloning of rat MGP in order to generate probes for further investigation of its biosynthesis and hormonal regulation and in order to obtain the complete amino acid sequence of the initial translation product.† The results identified the probable structures involved in substrate recognition by the γ-carboxylase and the relationship between the γ-carboxylase binding site and the location of glutamic residues that are modified.

EXPERIMENTAL PROCEDURES

Antibody Screening. Antibodies raised in rabbits against bovine MGP (4) were purified by affinity adsorption to rat MGP-Sepharose 4B, elution with 4 M guanidine hydrochloride, and dialysis against phosphate-buffered saline at 4°C. This antibody preparation, which crossreacts with rat MGP (4), was used at a protein concentration of 1.5 μg/ml to screen our rat osteosarcoma (ROS) 17/2 λgt11 cDNA library (8) with the ProtoBlot immunoscreening system (Promega Biotec, Madison, WI). This library was prepared with mRNA from ROS 17/2 cells that had been treated with 1,25-$(OH)_2D_3$ at 1 ng/ml for 54 hr (8), a treatment time that we have shown is sufficient to induce MGP expression by ROS 17/2 cells (7). Positive clones were plaque-purified, subcloned into plasmid pUC8, and sequenced by the dideoxynucleotide chain-termination method on double-stranded DNA (9).

Screening with Labeled Probes. Additional MGP clones were obtained by screening (8) our ROS 17/2 library with MGP-1 DNA, the largest insert identified by screening with antibody; labeling by nick-translation, nitrocellulose filter preparation, hybridization, and washing have been described (8, 10, 11). In a separate approach to MGP cloning, we also probed our ROS 17/2 λgt11 cDNA library with oligonucleotides corresponding to segments of the N-terminal sequence of rat MGP. MGP was isolated from rat bone (1, 4) and

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Abbreviations: MGP, matrix Gla protein; BGP, bone Gla protein; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3.

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†This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03026).

subjected to N-terminal sequence analysis for 33 steps as described (2). The N-terminal sequence of rat MGP is Tyr-Gla-Ser-His-Glu-Ser-Met-Glu-Ser-Tyr-Glu-Val-Ser-Pro-Phe-Thr-Asn-Arg-Arg-Asn-Ala-Asn-Thr-Phe-Ile-Ser-Pro-Gln-Gln-Arg-Trp-His-Ala-. The following oligonucleotide probes were synthesized by the University of California at San Diego Oligonucleotide Synthesis Facility and end-labeled (11): 17A, d(GTYGTYGCNACCGTRCG); 17B, d(GTYGTYTCYACCGTRCG); 14C, d(GTRCTYTCRTACCT); and 14D, d(GTRCTYAGNTACCT). Probes 17A and B correspond to residues 28–33 in rat MGP, and probes 14A and B correspond to residues 4–8. Y refers to C and T, R to G and A, and N to all four bases, each at equimolar levels. Clone λMGP-6 was purified based on its hybridization with 17B and 14C. The hybridization of these probes rather than 17A and 14D is in agreement with the final cDNA sequence (Fig. 3).

RNA Blot Hybridization. RNA was isolated from rat calvaria and from ROS 17/2 cells treated for 6 days with 1,25-$(OH)_2D_3$ at 1 ng/ml (7) by the guanidinium isothiocyanate/CsCl method (8, 11). Poly(A)$^+$ RNA selection, deadenylylation of mRNA, and blot hybridization of electrophoretically fractionated RNA were carried out as described (8, 11, 12).

RESULTS

Isolation of cDNA Clones by Antibody Screening. The initial screening of 5×10^5 recombinants from our ROS 17/2 cDNA library with rat MGP antibodies yielded 3 positive plaques. After plaque purification, the clone containing the largest insert, designated λMGP-1, was excised with restriction endonuclease *Eco*RI, subcloned into pUC8, and sequenced. To better establish the sequence in the middle of this insert, MGP-1 was also cleaved with *Stu* I and the two fragments were subcloned into pUC8 and sequenced. The 495-base-pair insert of λMGP-1 contains one long open reading frame coding for a polypeptide that exactly corresponds to the 33 N-terminal residues of rat bone MGP, which had been determined by amino acid sequence analysis (see *Experimental Procedures*) and matches the amino acid sequence of MGP purified from bovine bone (2) in 66 out of 79 positions. The open reading frame extends to the 5′ end without revealing any ATG triplet that might act as a translational start site. In the 3′ direction, the open reading frame contains a TAA termination codon followed by 197 nucleotides.

Analysis of MGP mRNA. In order to estimate the size of the full-length MGP mRNA, RNAs from rat calvaria (Fig. 1, lane A) and ROS 17/2 cells treated for 6 days with 1,25-$(OH)_2D_3$ (lane B) were analyzed by gel electrophoresis followed by blot hybridization with ^{32}P-labeled MGP-1 DNA. The probe recognized a single broad band at 660–740 nucleotides in both RNA samples, indicating that the MGP cDNA we had cloned from ROS 17/2 was indeed representative of MGP message in normal bone but was not full-length. Analysis of deadenylylated ROS 17/2 RNA yielded a single discrete band at 570–580 nucleotides (Fig. 1, lane C). No hybridization was detected to RNA from ROS 17/2 cells that had not been treated with 1,25-$(OH)_2D_3$ (data not shown), a finding consistent with the absolute dependence of MGP synthesis in these cells on treatment with 1,25-$(OH)_2D_3$ for at least 48 hr (7).

Nucleotide Sequence of Complete MGP cDNA. Since none of the original 3 clones contained a full-length cDNA insert, ^{32}P-labeled MGP-1 DNA was used to identify additional MGP clones in the library by plaque hybridization. This screening yielded 11 positive clones, none of which proved longer than MGP-1. Most inserts were subcloned into pUC8 and sequenced; one of these, designated λMGP10, is shown in Fig. 2. Independent screening of 3×10^5 recombinants from our ROS 17/2 cDNA library with two oligonucleotide probes

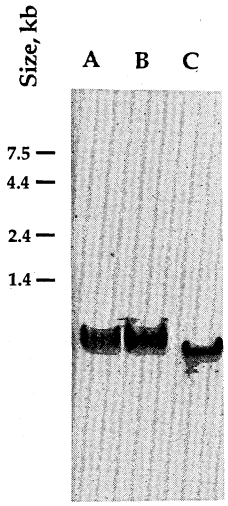

FIG. 1. Blot hybridization analysis of MGP mRNA. All mRNA samples were purified by a single round of oligo(dT)-cellulose chromatography. Lanes: A, 10 μg of rat calvarial mRNA; B, 2 μg of mRNA from ROS 17/2 cells treated for 6 days with 1,25-$(OH)_2D_3$ at 1 ng/ml; C, 2 μg of deadenylylated ROS 17/2 mRNA [same 1,25-$(OH)_2D_3$ treatment as lane B]. A 96-hr autoradiographic exposure was used for all lanes. Positions and sizes [in kilobases (kb)] of RNA standards are indicated.

based on the N-terminal sequence of rat MGP identified 1 plaque positive for both probes; this plaque also hybridized with ^{32}P-labeled MGP-1. The insert from this clone, designated λMGP-6, was excised with *Eco*RI, subcloned into pUC8, and sequenced. The cDNA inserts whose sequences were used to obtain the cDNA structure of MGP are diagrammed in Fig. 2. The sequences of all other clones examined agreed with this structure.

Overlapping sequences were consolidated into the 521-nucleotide sequence shown in Fig. 3. The first ATG triplet (nucleotide positions 1–3) in the longest open reading frame has been designated the translational start site because the sequence surrounding this triplet matches the proposed consensus sequence for initiation of eukaryotic translation at 6 of 7 bases (13). The C-terminal region of the 103-residue-predicted polypeptide (corresponding to nucleotide positions 58–309) has 83% sequence identity with the amino acid

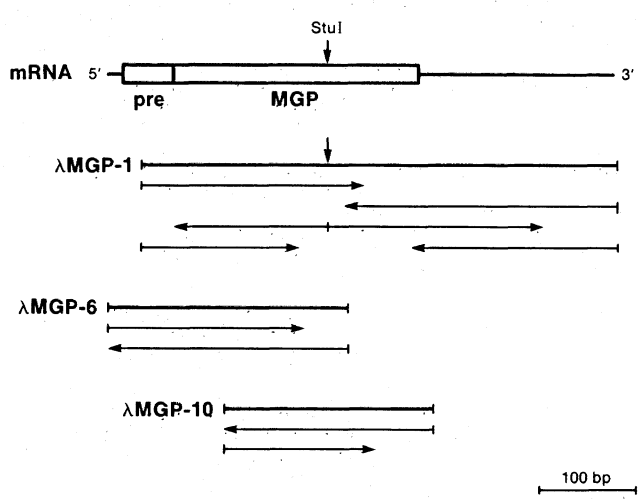

FIG. 2. Strategy for determining the nucleotide sequence of MGP cDNA. Inserts of the λgt11 cDNA clones that were sequenced are depicted by heavy lines. Horizontal arrows show the direction and extent of sequencing. Vertical arrows indicate *Stu* I restriction site. bp, Base pairs.

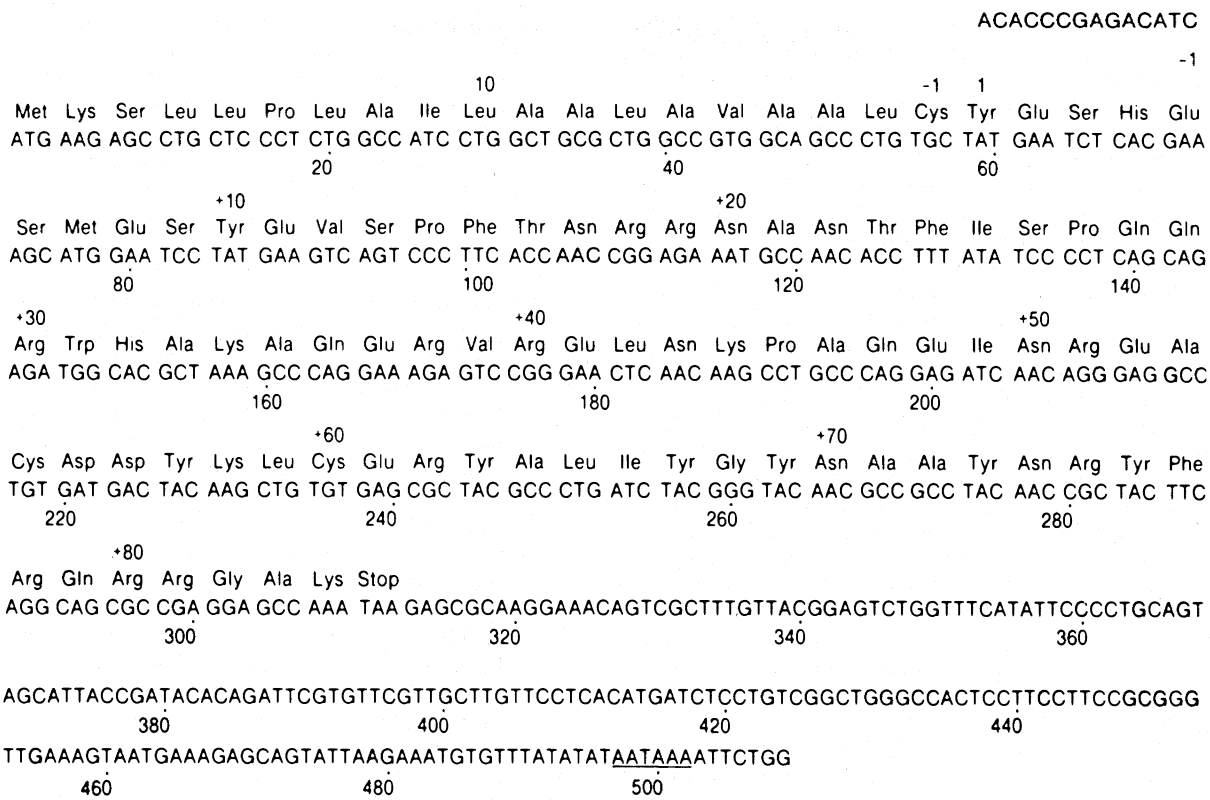

FIG. 3. Complete nucleotide sequence of MGP cDNA and sequence of its predicted polypeptide. The nucleotide sequence is numbered below each line, with position 1 corresponding to the start of the protein-coding region. The amino acid sequence is numbered above each line, with position 1 corresponding to the N-terminal residue of the protein isolated from bone. The polyadenylylation signal is underlined.

sequence of bovine MGP (2) and, in the first 33 amino acid residues of this region, is identical to the N-terminal sequence of MGP purified from rat bone. The coding region is terminated by a single TAA triplet (positions 310–312). An AATAAA polyadenylylation signal (14) is found beginning at position 497.

DISCUSSION

Processing of the Predicted Polypeptide. An unusual feature of the 103-residue primary translation product predicted from the MGP cDNA is the absence of a propeptide. To our knowledge, MGP is the only known example of a vitamin K-dependent protein that lacks a propeptide. The first ATG triplet in MGP is followed by codons for lysine, serine, and a stretch of 16 hydrophobic residues that presumably form the transmembrane core of a signal peptide (15). Signal-peptide cleavage is predicted to occur after the cysteine residue at position −1 (16), and tyrosine at position +1 is the first residue in the N-terminal sequence of mature rat and bovine MGP (Fig. 4).

The primary translation product predicts an 84-residue rat MGP that has an additional 5 amino acids at the C terminus that are not present in the MGP previously isolated from bovine bone and sequenced (Fig. 4). The first two residues of this C-terminal pentapeptide are Arg-Arg, a dibasic sequence that is often a site of proteolytic cleavage in the processing of proteins (17). Although it is not known whether proteolytic cleavage to remove the C-terminal pentapeptide precedes secretion from the cell, a closely spaced doublet has been noted in the protein blot analysis of MGP in guanidine extracts of bone and cartilage from rat and calf (3). If this doublet represents the 84-residue protein predicted from the

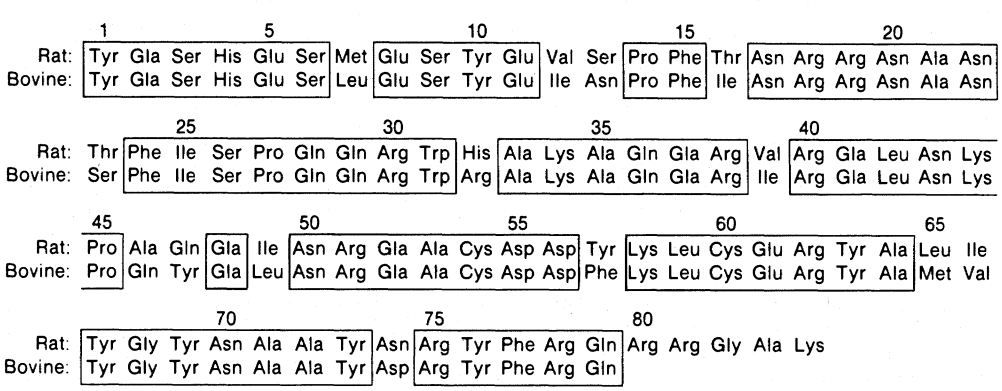

FIG. 4. Comparison of bovine and rat MGP sequences. The bovine MGP sequence was determined by direct amino acid sequence analysis; the position of each γ-carboxyglutamic (Gla) residue in this sequence is therefore known (2). The rat MGP sequence was deduced from the cDNA sequence and the position of the 5 Gla residues in rat MGP is assumed to be the same as in the bovine protein.

cDNA and the 79-residue protein sequenced from bone, the two proteins must be present at comparable levels in the extracellular matrix and proteolytic processing probably occurs after secretion. It will clearly be of great interest to know the function of proteolytic processing at the C terminus of MGP and, in particular, whether the extraordinary water-insolubility of the 79-residue MGP isolated from bone, an unusual feature of the protein given its high percentage of hydrophilic amino acids and small size (1–4), is also a property of the 84-residue protein.

Mechanisms by Which Substrates Are Recognized by the γ-Carboxylase. Previous work showed that the propeptide of BGP is homologous to the propeptide of other vitamin K-dependent proteins (8), and it was postulated that the homologous propeptide domain is a recognition site for the γ-carboxylase (8, 18). Although MGP has no propeptide, there is a region of the mature protein, residues +15 to +30 in Fig. 4, that is homologous to the propeptide of other vitamin K-dependent proteins. The absence of a propeptide in MGP demonstrates that the γ-carboxylation and secretion of a vitamin K-dependent protein need not be linked to the presence of a propeptide or to its proteolytic removal.

If residues 15–30 in MGP correspond to the propeptide component of the binding site for the γ-carboxylase, MGP is the first example of a vitamin K-dependent protein in which a glutamic residue on the N-terminal side of this γ-carboxylase binding site is known to be γ-carboxylated. Previous N-terminal sequence analysis of bovine MGP revealed approximately 80% γ-carboxyglutamic acid and 20% glutamic acid at position 2 (2). The other three glutamic residues on the N-terminal side of this γ-carboxylase binding site were not γ-carboxylated. In the course of the present studies, we carried out N-terminal sequence analysis of rat MGP. The results support the presence of partial γ-carboxylation at position 2 and no γ-carboxylation at positions 5, 8, and 11. Based on extrapolated repetitive yields in the N-terminal sequence of rat MGP, position 2 is 20% glutamic acid and 80% γ-carboxyglutamic acid. The consistent finding of a γ-carboxyglutamic residue on the N-terminal side of the γ-carboxylase binding site demonstrates that glutamic residues on either side of the binding site have access to the active site of the γ-carboxylase. It follows from these observations that the γ-carboxylase binding site in as-yet-uncharacterized vitamin K-dependent proteins can be anywhere in the mature protein and that γ-carboxylation of glutamic residues can occur to either side of the propeptide component of the binding site.

A previously unrecognized invariant structure in the γ-carboxyglutamic acid-containing region of vitamin K-dependent vertebrate proteins is the sequence Glu-Xaa-Xaa-Xaa-Glu-Xaa-Cys (EXXXEXC, positions 17–23, Fig. 5). The separation between the first glutamic residue in this invariant structure (at +17) and the previously recognized invariant arginine in the homologous propeptide domain (at −1) is 15–17 residues in all cases except mouse BGP, where the separation is 12 residues. If the sequences of the homologous propeptide domain and the γ-carboxyglutamic acid-containing region of known vitamin K-dependent vertebrate proteins are compared (−16 to +30, Fig. 5), the two vitamin K-dependent bone proteins, BGP and MGP, are each as closely related to coagulation proteins as to each other. There are 2 additional residues that are invariant in MGP and in all coagulation proteins but not in BGP (alanine at −10 and glutamic acid at +30), and 1 additional residue that is invariant in BGP and in all coagulation proteins but not in MGP (leucine at +6). There are, for comparison, only 2 residues that are invariant in BGP and MGP but are not found in coagulation proteins (arginine at +20 and cysteine at +29). These sequence relationships are consistent with the hypothesis that the propeptide and γ-carboxyglutamic acid domain units of known vitamin K-dependent proteins evolved from a common ancestor by gene duplication and subsequent divergent evolution and indicate that the two bone proteins

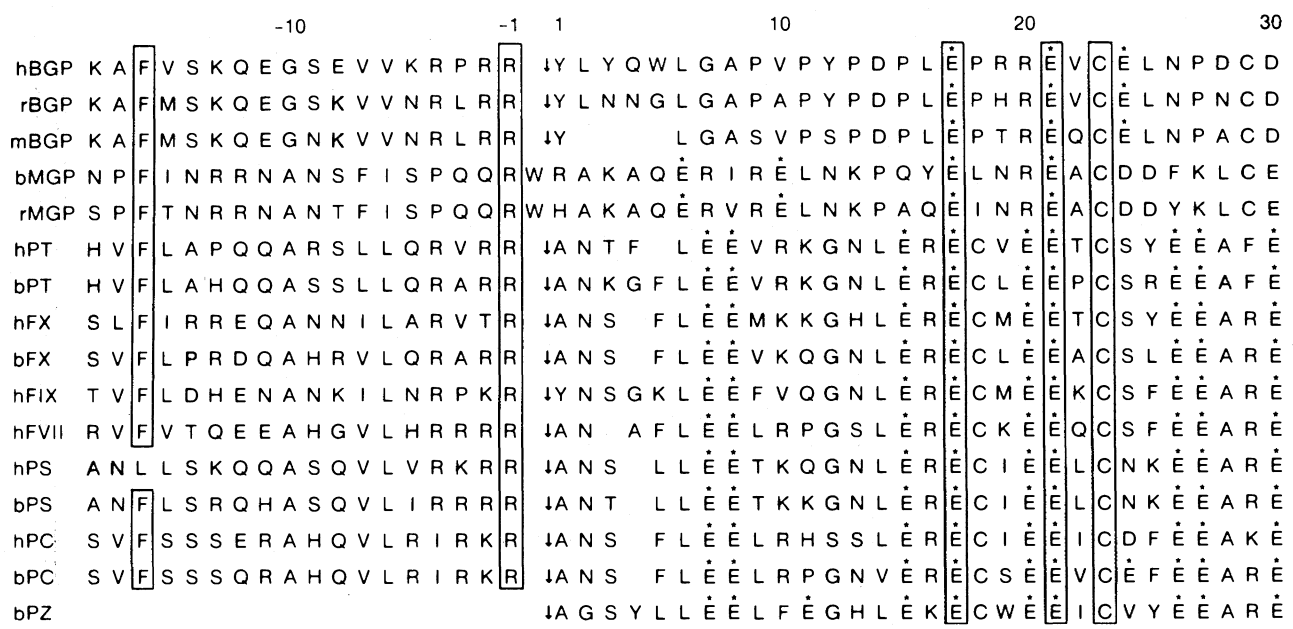

FIG. 5. Conserved amino acids in the propeptide and γ-carboxyglutamic acid-containing domains of known vertebrate vitamin K-dependent proteins. Amino acid sequence positions are numbered so that 1 corresponds to the first residue of mature BGP. All other sequences have been aligned to give maximum homology. Arrows preceding residue 1 indicate the site of propeptide cleavage; the absence of an arrow in the MGP structure is due to the absence of a propeptide in this protein. Sequences are given in standard one-letter symbols [Ė represents γ-carboxyglutamic acid (Gla)]. Sequences (from top to bottom): hBGP, human BGP (19); rBGP, rat BGP (8); mBGP, mouse BGP (19); bMGP, bovine MGP (2); rMGP, rat MGP (Fig. 3); hPT, human prothrombin (20); bPT, bovine prothrombin (21); hFX, human factor X (22); bFX, bovine factor X (23); hFIX, human factor IX (24); hFVII, human factor VII (25); hPS, human protein S (26); bPS, bovine protein S (27); hPC, human protein C (28); bPC, bovine protein C (29); bPZ, bovine protein Z (30).

probably diverged from this common ancestor at about the same time as coagulation proteins.

There is evidence that the homologous propeptide and invariant EXXXEXC structures of vitamin K-dependent proteins both play a role in substrate recognition by the γ-carboxylase. Site-directed mutagenesis studies (31) showed that two conserved amino acids in the propeptide region of factor IX (phenylalanine at −16 and alanine at −10; Fig. 5) are indeed critical to recognition by the γ-carboxylase. Although the importance of the EXXXEXC unit has not yet been tested by site-directed mutagenesis, there is indirect evidence to support a role for this unit in γ-carboxylase binding to substrate. A variety of peptides have been synthesized that correspond to the sequences of vitamin K-dependent proteins in γ-carboxyglutamic acid-containing regions but that lack the invariant EXXXEXC unit (32). None of these peptide substrates proved to have K_m values lower than a few millimolar (32–34). In contrast, both intact BGP and the peptide corresponding to residues 13–29 in prothrombin proved to be excellent γ-carboxylase substrates (33, 34) after γ-carboxyglutamic residues were converted to glutamic residues by decarboxylation (35). For both decarboxylated polypeptides, K_m values are 2–3 orders of magnitude lower than for the best known synthetic peptide substrate, Phe-Leu-Glu-Glu-Leu (33, 34). Since both decarboxylated BGP and decarboxylated prothrombin-(13–29)-peptide lack the homologous propeptide domain yet retain the EXXXEXC invariant structure, the excellent substrate activities of these decarboxylated polypeptides is strong evidence for a role of the EXXXEXC structure in γ-carboxylase binding to substrate.

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