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Carboxyl-terminal Proteolytic Processing of Matrix Gla Protein*

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The present study was undertaken to determine the extent of COOH-terminal proteolytic processing in matrix Gla protein (MGP), a 10-kDa protein which contains 5 residues of the vitamin K-dependent Ca^{2+} binding amino acid, γ-carboxyglutamic acid (Gla). Two forms of MGP were isolated from demineralization and urea extracts of bovine cortical bone, one 79 residues in length with the COOH terminus Phe-Arg-Gln and the other 83 residues in length with the COOH terminus Phe-Arg-Gln-Arg-Arg-Gly-Ala. The 84-residue form of bovine MGP predicted from the message structure could not be detected in the bone extracellular matrix extracts, and it therefore seems probable that the lysine at position 84 was removed by the action of a carboxypeptidase B-like enzyme prior to secretion. A plausible sequence of proteolytic cleavages that could generate the 79-residue form of MGP would be a trypsin-like cleavage at Arg^{80}-Arg^{81} or Arg^{81}-Gly^{82} followed by carboxypeptidase B-like cleavage to remove COOH-terminal arginine(s). Since essentially equal amounts of the 79- and 83-residue forms of MGP were also detected in bovine articular cartilage and plasma, it seems likely that the COOH-terminal processing events identified in bone apply to many of the other tissues which synthesize this protein.

Only one form of MGP was detected in human bone extracts, a 77-residue protein that lacks the COOH-terminal residues Arg-Lys-Arg-Arg-Gly-Thr-Lys. This shortened version of human MGP is consistent with the proposed model for COOH-terminal processing, since the amino acid substitution in the COOH terminus of the human protein, Lys^{79} for Gln^{79}, would allow removal of the additional basic residues from the human MGP COOH terminus by the action of the carboxypeptidase B-like enzymic activity.

Recent studies have shown that MGP is strongly induced by retinoic acid in fibroblasts, chondrocytes, and osteoblasts, a response which suggests that MGP mediates an action of retinoic acid on an aspect of cell growth or differentiation. If this hypothesis is true, the present evidence for complex COOH-terminal processing events could provide a means to regulate the as yet unknown activity of MGP in the extracellular environment in a mechanism similar to the activation of hormones such as anaphlotoxins and kinins.

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Matrix Gla protein (MGP)[1] is a small, insoluble protein which contains 5 residues of the vitamin K-dependent Ca^{2+}-binding amino acid, γ-carboxyglutamic acid (Gla) (1, 2). MGP is found at high levels in the extracellular matrix of bone and cartilage (1–3) and is synthesized by all vertebrate tissues examined to date (4). The cDNA structures of bovine, rat, and human MGP predict a 19-residue transmembrane signal peptide and an 84-residue mature MGP (5–7). This mature protein differs in sequence from the 79-residue MGP structure determined by sequencing the protein isolated from bovine bone (2) in that it has an additional 5 residues at the COOH terminus, Arg-Arg-Gly-Ala-Lys. More recent studies have shown that the MGP in bovine cartilage and bone extracts can be resolved by SDS-gel electrophoresis into a closely spaced doublet which could indicate the presence of both the 79- and 84-residue forms of the protein in these tissues (3). The present studies were therefore undertaken in order to evaluate the extent of carboxyl-terminal proteolytic processing of MGP isolated from bone, and to determine the extent of COOH-terminal MGP processing in cartilage and plasma.

EXPERIMENTAL PROCEDURES

Materials—Calf bone was obtained from Vista Meat Packers, Vista, CA. Cyanogen bromide and trifluoroacetic acid were from Pierce Chemical Co., sequencer reagents were obtained from Applied Biosystems (Foster City, CA), electrophoresis grade chemicals were from Bio-Rad, and 9-fluorenylmethyl chloroformate was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Solvents for HPLC were from Fisher, and all other reagents were analytical reagent grade or better.

Cyanogen Bromide Digestion—Purified bovine or human MGP was dissolved in 70% (v/v) formic acid at a concentration of 1–4 mg/ml. Sufficient cyanogen bromide was added to achieve a final 50-fold molar excess of reagent to protein-bound methionine, the reaction vessel was flushed with N_2 and sealed, and the reaction mixture was stirred at room temperature for 16 h. To remove unreacted reagent, the digested protein was diluted 10-fold with water and dried, dissolved in the same volume of water, and redried. The digest was dissolved in 0.1% trifluoroacetic acid, and 20 μg of the digest was injected onto a Vydac C-4 reverse-phase column (4.5 × 250 mm) equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a gradient of 0–25% B (15 min), 25–50% (60 min), 50–100% B (10 min) where buffer B was 0.1% trifluoroacetic acid in 60% acetonitrile (v/v). Peptides were detected by their absorbance at 220 nm.

NH_2-terminal Sequence Analysis—Proteins and peptides were sequenced using an Applied Biosystems 470A sequenator equipped with a model 120 on-line HPLC using the 03RPTH program provided by the manufacturer (10). Data reduction was accomplished in a Perkin-Elmer Cetus 7500 computer equipped with Chrom 3 software.

Amino Acid Analysis—Proteins and peptides were hydolyzed in 6 N HCl for 24 h at 110 °C. Dried hydrolysates were derivatized with 9-fluorenylmethyl chloroformate (11, 12) and analyzed using a Perkin-Elmer Cetus Series 4 HPLC equipped with a Varian Amino Tag C_{18} column (4.6 mm × 15 cm) and a Kratos model FS970 fluorescence detector.

[1] The abbreviations used are: MGP, matrix Gla protein; Gla, E*, γ-carboxyglutamic acid; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; BGP, bone Gla protein.

Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed as described by Giulian *et al.* (13). One-mm thick slab gels were poured in which the separating gel was 21 cm in length. After the samples were loaded, the gel was run at 21 mA until the dye front was through the stacking gel, and the amperage was then increased to 28 mA until the dye front reached the bottom of the separating gel (~8 h); an aluminum plate was attached to one glass plate in order to help dissipate heat. Protein bands were visulaized by staining the gel for 1 h in 0.2% Coomassie Blue R-250 (Serva) in 10% 2-propanol and destaining overnight in 20% 2-propanol, 10% acetic acid. MGP bands were digested *in situ* with CNBr using the procedure of Jahnen *et al.* (14), and the resulting peptides were purified on a 2.1-mm × 25-cm Brownlee C-8 reverse-phase column. In a typical experiment, 13 μg of Sephadex C-25-purified MGP was loaded onto each of 13 lanes of a SDS-polyacrylamide gel. The two resulting bands were excised from each lane of the gel, and the pooled bands corresponding to each component were digested for 24 h in 1.3 ml of 70% formic acid (v/v) containing 8.45 mg of CNBr. The resulting peptides were separated as described above.

Immunoblotting Procedure—The procedures for the Western blot analysis of MGP and the antibody preparation used have been previously described (3). The 8 M urea extract of demineralized bovine bone and the 5 M guanidine HCl extract of bovine articular cartilage were dialyzed directly into SDS loading buffer. MGP was partially purified from bovine plasma by adsorption to an anti-bovine MGP antibody column and desorption with 5 M guanidine HCl; the guanidine eluate was dialyzed directly into SDS loading buffer. After electrophoresis, the proteins were electroblotted onto nitrocellulose, and Western blot analysis was performed (3).

Purification of Matrix Gla Protein—To prepare bone for protein extraction, the midshaft sections of tibias from 6-month-old calves were cut transversely into rings, freed of marrow and non-mineralized connective tissue, washed with acetone to remove lipid, and dried. Bone rings were subsequently frozen in liquid nitrogen and ground in a Wiley Mill equipped with a 2-mm filter; the resulting ground bone has the consistency of course sand. Ground bone was then washed with water at 4 °C and extracted overnight with a 10-fold excess (v/w) of 5 M guanidine HCl with vigorous mixing to remove cellular proteins and all other proteins not sequestered in the mineralized bone matrix. Ground bone was washed with distilled water to remove guanidine and then freeze-dried for noncollagenous protein purification.

In a typical purification, MGP was extracted from 300 g of ground calf bone by demineralization with 3 liters of 10% (v/v) formic acid for 2 h at 4 °C. It is critical at this step that the ground bone be suspended by vigorous stirring in order to ensure efficient demineralization. The extracted proteins were separated from the insoluble collagenous bone matrix by filtration through a GC50 borosilicate microfiber filter (Micro Filtration Systems, Dublin, CA) and then freed of dissolved bone mineral by one of two procedures. (*A*) For extracts of 300 g of bone or less, the entire 3-liter extract was further clarified by filtration through a 0.45-μm pore size cellulose nitrate filter and then applied to a 1.54 × 25-cm C_{18} HPLC column at a flow rate of 25 ml/min. Proteins were eluted from this column with a 1-h gradient from 0.1% trifluoroacetic acid in H_2O to 0.1% trifluoroacetic acid in 60% acetonitrile at a flow rate of 4 ml/min. The major component, which contains the extracted MGP as well as the related vitamin K-dependent protein bone Gla protein (BGP), was then dialyzed against distilled water at 4 °C to precipitate MGP. (*B*) For extracts of greater than 300 g of bone, the extract was adsorbed to Vydac C_{18} reverse-phase chromatography silica matrix (20–30 μm size, Separations Group, Hesperia, CA), by adding 1 volume of bulk silica matrix in 70% methanol to 10 volumes of the extract. The silica matrix was then washed several times with 500-ml volumes of 0.1% trifluoroacetic acid to remove dissolved bone mineral. Protein was eluted from the silica matrix with 250 ml of 0.1% trifluoroacetic acid in 50% acetonitrile, and the extracted proteins were dialyzed against distilled water at 4 °C to precipitate MGP. Although most of the extracted BGP remained in the supernatant after dialysis, some BGP coprecipitated with MGP at this stage. To remove BGP from the precipitated MGP, the precipitate after dialysis was dissolved in 8 M urea with 10 mM sodium acetate at pH 5.5 and applied to a 2 × 25-cm SP-Sephadex C-25 cation exchange column (Pharamacia LKB Biotechnology Inc.) previously equilibrated with the same buffer. The column was then washed with the same buffer until the absorbance of the effluent returned to base line. BGP does not bind to the column under these conditions and emerged in the wash step. MGP was eluted from the column with a linear 0–0.75 M NaCl gradient in 8 M urea with 10 mM sodium acetate, pH 5.5, and emerged at about 0.2 M NaCl. The fractions containing MGP were pooled and dialyzed against water to precipitate MGP, and the precipitate was either stored frozen or freeze dried.

About half of the MGP in bone remains with the collagenous bone matrix fraction after demineralization in 10% formic acid (8). To characterize the COOH-terminal structure of this matrix-associated MGP component, MGP was extracted from the demineralized bovine bone matrix with a 10-fold excess (v/v) of 8 M urea, 10 mM sodium acetate, pH 5.5, for 16 h at 4 °C with vigorous stirring. The extracted proteins were freed of collagenous residue by filtration through the GC-50 glass fiber filter and applied directly to a 2 × 25-cm SP-Sephadex C-25 column equilibrated with the same buffer. MGP was eluted from the column with a linear 0–0.75 M NaCl gradient in 8 M urea, 10 mM sodium acetate, pH 5.5; MGP again emerged at about 0.2 M NaCl.

Human MGP was purified from a low molecular weight fraction of an EDTA plus guanidine HCl extract of ~1 kg of human bone which was kindly provided by Dr. Larry Fisher (National Institute of Dental Research, Bethesda, MD). The bone was prepared and demineralized as described (9), the resulting extract was fractionated on tandem Sepharose CL-6B columns (2.6 × 190 cm) (9). The material corresponding to proteins between 5 and 18 kDa (see Fig. 2 of Ref. 9) was concentrated to 30 ml with a YM-5 membrane in an Amicon-stirred cell and sent to us by Dr. Fisher. We dialyzed this solution against 5 mM NH_4HCO_3 at 4 °C using molecular weight 3500 cutoff dialysis membrane to precipitate MGP. The precipitate was dissolved in 4 M urea, 10 mM sodium acetate, pH 4, and applied to a 2 × 25-cm SP-Sephadex C-25 cation exchange column equilibrated with the same buffer. MGP was eluted from the gel with a linear 0 to 0.75 M NaCl gradient in 4 M urea, 10 mM sodium acetate, pH 4.0. The fractions containing MGP were identified by their position on an SDS gel and their immunoreactivity, pooled, dialyzed against H_2O, dried, and stored frozen.

RESULTS

Purification of Matrix Gla Protein—In order to isolate sufficient purified MGP for structural study, we have developed an improved method for purification of the protein from bone. MGP was released from ground bone by 2 h of demineralization in 10% formic acid and freed of dissolved mineral by adsorption to a C_{18} reverse-phase matrix. The major protein component eluted from this matrix by an acetonitrile gradient or by washing with 50% acetonitrile in 0.1% trifluoroacetic acid is a mixture of MGP and BGP. This protein mixture was dialyzed against water to precipitate MGP and leave most of the BGP in solution. To remove that BGP which did coprecipitate with MGP, the precipitate was dissolved in 8 M urea and fractionated by gradient elution from a SP-Sephadex ion exchange column (Fig. 1). Dialysis of the MGP-containing fractions against water again precipitated MGP, and the precipitate was subsequently frozen or freeze-dried for storage.

About half of the total MGP extracted from bone emerges during a subsequent extraction of the demineralized collagenous bone matrix with denaturants such as 8 M urea or 6 M guanidine HCl (1, 8). We have also purified bovine MGP from an 8 M urea extract using gradient elution from the SP-Sephadex column and have found that the MGP in denaturant extracts elutes in the same position as the MGP in demineralization extracts (see "Experimental Procedures"). Human MGP was purified from an EDTA plus 6 M guanidine HCl extract of bone which had been first size fractionated by filtration over a column of Sepharose CL-6B (see "Experimental Procedures"). The size range corresponding to human MGP and BGP was again fractionated by gradient elution from the SP-Sephadex column.

Amino acid sequence analysis of the purified proteins revealed each to be homogenous. Bovine MGP purified from the demineralization and denaturant extracts gave a single NH_2-terminal sequence identical to that previously reported

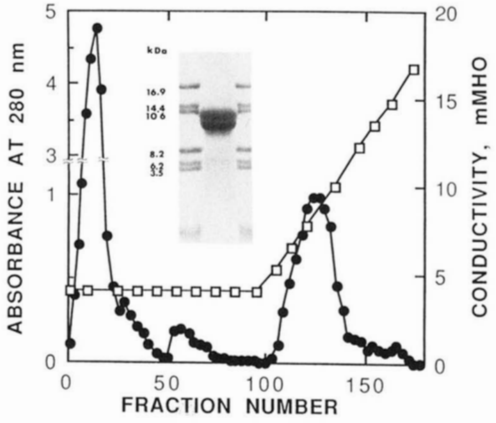

FIG. 1. **Purification of bovine MGP by SP-Sephadex C-25 cation exchange chromatography.** MGP was extracted from 800 g of bovine bone by demineralization and freed of dissolved mineral by adsorption to a C_{18} reverse-phase resin (see "Experimental Procedures"). Bound protein was then desorbed with 50% acetonitrile in 0.1% trifluoroacetic acid and dialyzed against water to precipitate MGP. This precipitate was dissolved in 25 ml of 10 mM sodium acetate, pH 5.5, with 8 M urea and loaded onto a 2 × 25-cm SP-Sephadex column equilibrated in the same buffer. A linear gradient from 0 to 0.75 M NaCl in 10 mM sodium acetate, pH 5.5, with 8 M urea (250 ml each condition) was started after 90 fractions were collected; fraction size was 5.5 ml. ●, absorbance at 280 nm; □, conductivity. *Inset*, SDS-polyacrylamide gel electrophoresis of purified bovine MGP. Twenty-five μg of purified MGP were electrophoresed on a 20% polyacrylamide gel and stained with Coomassie Blue (see "Experimental Procedures"). Sigma peptide molecular weight markers are on either side of MGP.

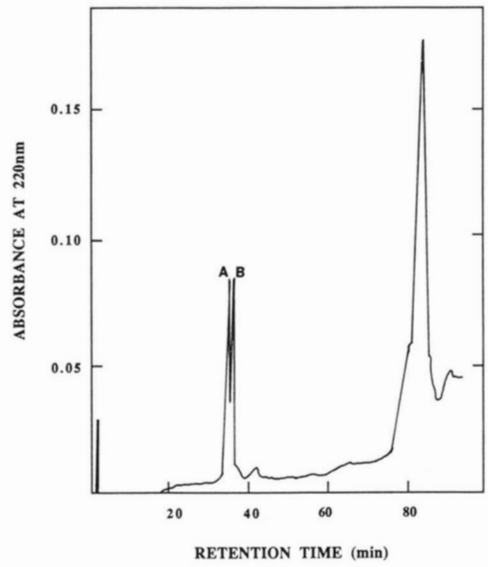

FIG. 2. **Separation by high performance liquid chromatography of the peptides generated by CNBr digestion of bovine MGP.** Separation of 20 μg of the cyanogen bromide digest of bovine MGP on a Vydac reverse-phase C-4 column (4.5 × 250 mm), equilibrated with 0.1% trifluoroacetic acid and run at a flow rate of 1.0 ml/min. Mobile phase: buffer A, 0.1% trifluoroacetic acid; buffer B, 0.1% trifluoroacetic acid in acetonitrile/H_2O (60, 40, v/v). Peptides were eluted with a linear gradient of 0–25% B (15 min), 25–50% B (60 min), and 50–100% B (10 min).

A. BOVINE MGP:

	66				70				75				80			
Sequence Predicted from cDNA:	V	Y	G	Y	N	A	A	Y	D	R	Y	F	R	Q	R	R	G	A	K
Sequence of CNBr Peak A:	V	Y	G	Y	N	A	A	Y	D	R	Y	F	R	Q	R	R	G	A	
Sequence of CNBr Peak B:	V	Y	G	Y	N	A	A	Y	D	R	Y	F	R	Q					

B. HUMAN MGP:

	66				70				75				80			
Sequence Predicted from cDNA:	V	Y	G	Y	N	A	A	Y	N	R	Y	F	R	K	R	R	G	T	K
CNBr Peptide:	V	Y	G	Y	N	A	A	Y	N	R	Y	F							

FIG. 3. **Structures of the COOH-terminal CNBr peptides of bovine and human MGP.** *Panel A*, the amino acid sequences of peptide A and peptide B (Fig. 2) were determined by NH_2-terminal sequencing. The COOH-terminal bovine MGP structure predicted from the cDNA sequence (6) is shown for comparison. *Panel B*, the amino acid sequence of the CNBr peptide which eluted at 44 min (Fig. 4) was determined by NH_2-terminal protein sequencing. The COOH-terminal human MGP structure predicted from the cDNA sequence (7) is shown for comparison.

for the protein by protein sequencing (2), while human MGP purified from the denaturant extract revealed a single NH_2-terminal sequence(Y-E*-S-H-E-S-M-E-S-Y-E-L-N-P-F-I-N-R-R-N-A-N-T-F-I-S-P-Q-Q) which is identical to that predicted from the human MGP cDNA and gene structures (6, 7).

Carboxyl-terminal Structure of Purified MGP—Previous investigations have shown that the COOH-terminal structure of the initial bovine MGP translation product has an additional 5 residues not found in the purified protein by amino acid sequencing, the residues Arg-Arg-Gly-Ala-Lys (2, 5, 6). In order to establish the COOH-terminal structure of bovine MGP purified by the above procedures, the protein was cleaved at the single methionine at position 65 with cyanogen bromide, and the resulting peptides were isolated by reverse-phase HPLC. As can be seen in Fig. 2, three components were obtained for MGP isolated from the demineralization extract. The component with the highest retention time, at 83 min, proved to have an NH_2-terminal sequence identical to the native protein and presumably corresponds to residues 1–65. The amino acid sequences of the peptides eluting at 34.5 and 36.5 min are shown in Fig. 3*A*. Peak A corresponds to residues 66–79 and presumably arises from cleavage of the 79-residue form of MGP previously described (2), and peak B corresponds to residues 66–83 and probably comes from cleavage of an 83-residue form of bovine MGP. The amino acid compositions of peaks A and B (data not shown) confirmed the amino acid sequence of each peptide and further showed that peak B indeed lacks the lysine residue that is at position 84 in the initial translation product.

The COOH-terminal structure of the bovine MGP component purified from the urea extract of demineralized bone matrix was also determined by the above procedures. The expected three peptides were again isolated from the CNBr digest, and amino acid sequencing and amino acid analysis of the components emerging at 34.5 and 36.5 min confirmed that they corresponded to residues 66–79 and 66–83 in the undigested protein. The ratio of the two COOH-terminal structural forms of bovine MGP in the urea extract was essentially identical to that in the formic acid extract (*cf.* Fig. 2).

The COOH-terminal structure of purified human MGP was also evaluated by cyanogen bromide cleavage at methionine 65 and isolation of the resulting peptides. As may be seen in Fig. 4, only one peptide was recovered in the region in which the carboxy-terminal bovine MGP peptides elute. The sequence of this peptide, shown in Fig. 3*B*, corresponds to residues 66–77 of human MGP and probably arises from cleavage of a 77-residue form of MGP. The sequence of this peptide was confirmed by its amino acid composition (data not shown).

Electrophoretic Analysis of Bovine MGP—As can be seen in the *inset* to Fig. 1, purified bovine MGP can be resolved into two components by electrophoresis using a procedure designed for high resolution separation of low molecular weight pro-

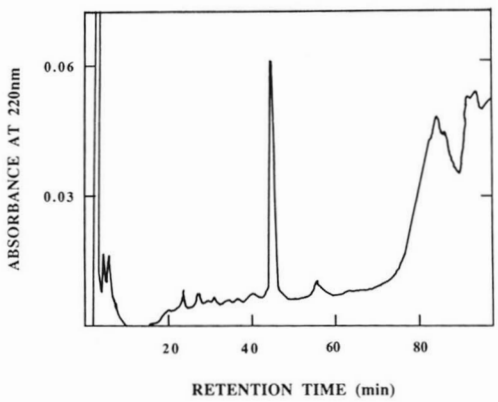

FIG. 4. **Separation by high performance liquid chromatography of the peptides generated by CNBr digestion of human MGP.** Separation of 20 μg of the cyanogen bromide digest of human MGP on a Vydac reverse-phase C-4 column (4.5 × 250 mm). Buffers and gradient are described in the Fig. 2 legend.

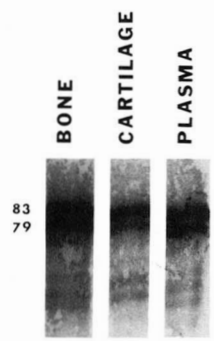

FIG. 5. **Western blot analysis of bovine MGP from bone, cartilage, and plasma.** *Lane 1*, 5 μg of the urea extract of bone. *Lane 2*, 25 μg of the guanidine HCl extract of articular cartilage. *Lane 3*, 3 μg of plasma MGP partially purified by adsorption to a rabbit anti-bovine MGP antibody column. After SDS-gel electrophoresis, proteins were transferred to nitrocellulose, probed with affinity purified rabbit antibody against bovine MGP, and detected using alkaline phosphatase-conjugated second antibody (see "Experimental Procedures"). The position of the 83- and 79-residue forms of MGP is indicated on the *left*.

teins (13). The difference in apparent molecular mass between these components, 450 daltons, is close to the molecular mass difference between the two MGP components, 458 daltons. When the smaller component was cleaved with cyanogen bromide *in situ* and the resulting peptides were extracted and purified, a single COOH-terminal peptide was obtained which coeluted with the authentic 66–79-COOH-terminal MGP peptide. The amino acid sequence of this peptide confirmed that it indeed corresponds to residues 66–79 of native MGP. The larger component was also cleaved with cyanogen bromide. The single COOH-terminal peptide that was obtained coeluted with the 66–83-COOH-terminal MGP peptide (see Fig. 2), and the amino acid sequence analysis confirmed that it in fact corresponds to residues 66–83 of native MGP.

The gel electrophoretic procedure for resolving the 79- and 83-residue forms of MGP was used in a Western blot analysis to investigate the nature of the MGP found in different bovine tissues. Cartilage and bone were chosen for this study because these are the only two tissues which are known to accumulate significant levels of MGP in the extracellular matrix, and each has, in fact, a comparable level of MGP (1, 3). Plasma was also selected because we have detected 0.5 to 1 μg/ml levels of MGP in rat, bovine, and human plasma by radioimmunoassay and have partially purified bovine plasma MGP by antibody affinity chromatography. As can be seen in Fig. 5, the bovine MGP in bone, cartilage, and plasma are each a mixture of the 79- and 83-residue forms of the protein. Since each of these samples was prepared for Western blot analysis by procedures which employed denaturants at every possible step, it seems likely that the two forms of MGP detected in plasma, cartilage, and bone indeed reflect the actual species of protein present in the tissues rather than a consistent degree of COOH-terminal proteolysis which occurs during each of the three sample preparations. It was not possible to repeat this experiment with extracts of human bone, cartilage, and plasma since we do not presently have antiserum suitable for detection of human MGP on a Western blot.

DISCUSSION

One of the objectives of the present investigation was to develop a simple and rapid method for the isolation of MGP from bone. The previous method for MGP purification from bone involved many steps (1) and, in our experience, yielded a product which still had contaminating proteins present, such as BGP and albumin. The simplest starting material for MGP purification by the present procedure is the demineralization extract of ground calf bone. Provided that acid demineralization is for 2 h or less, the proteins extracted at this step are predominantly less than 20 kDa in molecular mass. The major contaminating protein in this acid demineralization extract is BGP, the most abundant non-collagenous protein in bovine bone. Because BGP is exceptionally water soluble while MGP is not, dialysis against water precipitates MGP while leaving most of the BGP in solution. By adjusting the pH of the buffer used to equilibrate the cation exchange column, it proved possible to bind MGP to SP-Sephadex and not BGP. The bound MGP could be subsequently eluted with a linear salt gradient.

The other major objective of the present study was to resolve the discrepancy between the COOH-terminal structure of bovine MGP determined by protein sequencing and the 5-residue longer COOH-terminal structure predicted from the structure of the MGP message. Two forms of MGP were found in all bone extracts tested, one a 79-residue form of the protein identical to the structure previously determined by protein sequencing and the other an 83-residue protein with the additional COOH-terminal residues Arg-Arg-Gly-Ala. We could find no evidence for the 84-residue form of MGP in any bone extract examined, and therefore conclude that the COOH-terminal lysine predicted at residue 84 from the MGP mRNA structure must either be removed prior to secretion or be removed rapidly and completely after secretion.

The proteolytic cleavages which we believe generate the MGP structures found in bone begin with the removal of lysine 84 by the action of a carboxypeptidase B-like enzyme. If this step is intracellular, as we expect, the protease catalyzing this step is probably carboxypeptidase H, a peptidase which has been shown to specifically remove basic residues from the COOH-terminal of proteins prior to secretion (15).

The second proteolytic cleavage is probably catalyzed by a trypsin-like enzyme and cleaves either the Arg^{80}-Arg^{81} or the Arg^{81}-Gly^{82} bond. Since both the 79- and 83-residue forms of MGP are found in the extracellular matrix of bone, we believe that this trypsin-like enzymic cleavage is extracellular. A number of trypsin-like enzymes have been identified in the extracellular environment, including plasmin, kallikreins, and thrombin, each of which is activated by independent regulatory cascades. We suspect that one or more of these trypsin-like enzymes is able to cleave the COOH terminus of MGP and that COOH-terminal MGP cleavage is therefore a regulated event.

The final step in the postulated pathway for MGP proteolysis is the removal of Arg^{80} (and possibly Arg^{81}) from the COOH terminus of bovine MGP and the removal of these residues plus the additional Lys^{79} and Arg^{78} from the human protein. These steps are probably catalyzed by the ubiquitous extracellular carboxypeptidase B-like enzyme, carboxypeptidase N (16). Since this enzyme is not known to occur in an inactive zymogen form, it seems likely that this step takes place rapidly following the trypsin-like cleavage and that it is not regulated. It should be noted that extracellular proteases have been identified that cleave at the amino side of basic residues and could generate the 79-residue form of bovine MGP and the 77-residue form of human MGP directly, without the need for subsequent carboxypeptidase B-like enzymatic cleavages (17). However, this seems unlikely because the cleavage would need to occur at the Gln^{79}-Arg^{80} bond in bovine MGP and at the Phe^{77}-Arg^{78} bond in the human protein.

It seems reasonable to speculate that the COOH-terminal proteolytic processing of MGP regulates the as yet unknown activity of this protein. Assuming that the above model for proteolytic processing is correct, one can envision three possibilities. The trypsin-like cleavage could inactivate and the subsequent carboxypeptidase B-like cleavage is superfluous. Alternatively, the trypsin-like cleavage could activate, and the subsequent carboxypeptidase B-like cleavage(s) could either inactivate or modify the activity of the protein. Finally, the full activation of MGP could require both the trypsin-like and carboxypeptidase B-like cleavages. Examples of biological activities regulated by COOH-terminal proteolytic processing include the activation of kinins and anaphylatoxins by trypsin-like enzymes, such as kallikrein and convertase, and the subsequent inactivation or altered activity of these peptide hormones by the action of carboxypeptidase N (16). Such two-stage activation/inactivation systems provide a mechanism for tightly coupling the level of a biological response to the rate at which the regulated enzymatic step generates the active peptide.

COOH-terminal proteolytic processing could regulate a possible signaling function of MGP. We have recently demonstrated that MGP synthesis is strongly induced by the hormonally active metabolite of vitamin A, retinoic acid, in all cells tested, including normal human bone cells, fibroblasts, and articular cartilage chondrocytes (18). These observations strongly suggest that the increased secretion of MGP in response to retinoic acid mediates some aspect of the known potent activities of retinoic acid in differentiation and development, and further suggest that MGP could be a locally acting paracrine or autocrine factor which is important for growth in the wide variety of tissues that synthesize the protein. Should this hypothesis prove to be correct, the apparently complex COOH-terminal processing events in the metabolism of MGP demonstrated here could provide the additional level of extracellular regulation necessary to ensure that the activity of MGP is tightly constrained in space and time.

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REFERENCES

1. Price, P. A., Urist, M. R., and Otawara, Y. (1983) *Biochem. Biophys. Res. Commun.* **117,** 765–771
2. Price, P. A., and Williamson, M. K. (1985) *J. Biol. Chem.* **260,** 14971–14975
3. Hale, J. E., Fraser, J. D., and Price, P. A. (1988) *J. Biol. Chem.* **263,** 5820–5824
4. Fraser, J. D., and Price, P. A. (1988) *J. Biol. Chem.* **263:**11033–11036
5. Price, P. A., Fraser, J. D., and Metz-Virca, G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8335–8339
6. Kiefer, M. C., Bauer, D. M., Young, D., Hermsen, K. M., Masiarz, F. R., and Barr, P. J. (1988) *Nucleic Acids Res.* **16,** 5213
7. Cancela, L., Hsieh, C.-L., Francke, U., and Price, P. A. (1990) *J. Biol. Chem.* **265,** 15040–15048
8. Otawara, Y., and Price, P. A. (1986) *J. Biol. Chem.* **261,** 10828–10832
9. Fisher, L. W., Hawkins, G. R., Tuross, N., and Termine, J. D. (1987) *J. Biol. Chem.* **262,** 9702–9708
10. Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1981) *J. Biol. Chem.* **256,** 7990–7997
11. Einarsson, S., Josefsson, B., and Lagerkirst, S. (1983) *J. Chromatogr.* **282,** 609–618
12. Cunico, R., Mayer, A. G., Wehr, C. T., and Sheehan, T. (1986) *Biochromatography* **1,** 6
13. Giulian, G. G., Moss, R. L., and Greaser, M. (1983) *Anal. Biochem.* **129,** 277–287
14. Jahnen, W., Ward, L. D., Reid, G. E., Moritz, R. L., and Simpson, R. J. (1990) *Biochem. Biophys. Res. Commun.* **166,** 139–145
15. Fricker, L. D. (1985) *Trends Neurosci.* **8,** 210–214
16. Skidgel, R. A. (1988) *Trends Pharmacol. Sci.* **9,** 299–304
17. Gluschankof, P., Gomez, S., Morel, A., and Cohen, P. (1987) *J. Biol. Chem.* **262,** 9615–9620
18. Cancela, M. L., and Price, P. A. (1991) *Endocrinology*, in press