The Identification of Matrix Gla Protein in Cartilage*

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The vitamin K-dependent bone protein matrix γ-carboxyglutamic acid (Gla) protein (MGP) has been identified by radioimmunoassay in the guanidine extract of rat cartilage. MGP was present in all cartilages tested at levels comparable to the MGP level in bone. Western blot analysis indicated that the molecular weight of cartilage MGP is the same as bone MGP, and Northern blot analysis revealed that MGP mRNA from cartilage is the same size as the MGP mRNA from bone. The structurally related vitamin K-dependent protein bone Gla protein could not be detected in cartilage by radioimmunoassay or by Northern blot analysis. The discovery that MGP is synthesized by growth plate cartilage could provide an explanation for the excessive growth plate mineralization disorder seen in rats treated with the vitamin K antagonist warfarin and the punctate mineralization of the growth plate seen in infants whose mothers received warfarin in the first trimester of pregnancy (the fetal warfarin syndrome). Both disorders appear to be caused by the inactivation of a vitamin K-dependent mineralization inhibitor in cartilage, an inhibitor which we suggest is MGP.

Bone contains two small vitamin K-dependent proteins, matrix Gla1 protein (MGP) and bone Gla protein (BGP). As isolated from bovine bone, MGP is a 79-residue protein with 5 residues of the Ca2+ binding amino acid Gla, and BGP is a 49-residue protein with 3 Gla residues (1, 2). There is sufficient sequence identity between the two proteins to conclude that BGP and the COOH-terminal portion of MGP arose from a common ancestor by gene duplication (1). The level of sequence identity is quite low (20% identity), however, and in most respects the proteins are remarkably different. MGP is exceptionally insoluble in water, which is unusual given its small size and high percentage of hydrophilic amino acids, while BGP is quite water-soluble. Adult levels of MGP are found in the recently calcified matrix of newborn rat bone, while BGP levels in newborn rat bone are only 5% of adult levels (3, 4). Finally, a major fraction of MGP is anchored to the organic matrix of bone while nearly all of the BGP in bone is bound to the mineral phase (3, 4).

The present studies were undertaken to evaluate the possible presence of MGP and BGP in growth plate cartilage, the proximal (tibial) growth plates were dissected from control rats and, because the width of the growth plate is increased by vitamin D deficiency (7), rachitic rats. To induce rickets, eight weanling Holtzman Co. Sprague-Dawley rats were purchased from Simonsen. Rachitogenic diet 2 (catalog no. 904652) and 125I (as NaI, 16 mCi/µg) were obtained from ICN. Enzyme-grade guanidine HCl and the RNA size markers were purchased from BRL (Bethesda Research Laboratories). Electrophoresis reagents and the transblot apparatus were obtained from Bio-Rad. Prestained low molecular weight protein standards were purchased from Diversified Biotech. The ProteoBlo™ immunoscreening system was obtained from Promega Biotech. 5′-[α-32P]dCTP (3 Ci/µmol) was purchased from Amersham Corp. Nytran and nitrocellulose paper were obtained from Schleicher and Schuell. Kodak XAR-5 film was bought from Eastman Kodak. Cronex Lighting Plus screens were obtained from Du Font. Unless otherwise stated, all other chemicals were of analytical reagent grade or better.

**EXPERIMENTAL PROCEDURES**

**Materials**—Low vitamin D rats were purchased from Holtzman Co. Sprague-Dawley rats were purchased from Simonsen. Rachitogenic diet 2 (catalog no. 904652) and 125I (as NaI, 16 mCi/µg) were obtained from ICN. Enzyme-grade guanidine HCl and the RNA size markers were purchased from BRL (Bethesda Research Laboratories). Electrophoresis reagents and the transblot apparatus were obtained from Bio-Rad. Prestained low molecular weight protein standards were purchased from Diversified Biotech. The ProteoBlo™ immunoscreening system was obtained from Promega Biotech. 5′-[α-32P]dCTP (3 Ci/µmol) was purchased from Amersham Corp. Nytran and nitrocellulose paper were obtained from Schleicher and Schuell. Kodak XAR-5 film was bought from Eastman Kodak. Cronex Lighting Plus screens were obtained from Du Font. Unless otherwise stated, all other chemicals were of analytical reagent grade or better.

**Extraction of MGP and BGP from Tissues**—To evaluate the possible presence of MGP and BGP in growth plate cartilage, the proximal (tibial) growth plates were dissected from control rats and, because the width of the growth plate is increased by vitamin D deficiency (7), rachitic rats. To induce rickets, eight weanling Holtzman low vitamin D rats were fed a rachitogenic diet and housed under lights with a low uv content. Six control weanling Holtzman low vitamin D rats were housed in the same room and provided with normal rat chow. After 4 weeks on the diets, all rachitic and control rats were exsanguinated while under ether anesthesia, and left tibias were excised and cleaned of adhering connective tissue. Subsequent dissections were carried out with the aid of a dissecting microscope. Fascia were first cut away from the surface of the proximal end of the tibia to expose the edge of the growth plate, which could be visually distinguished by its translucent and columnar appearance compared to the opaque appearance of the epiphysis and metaphysis. The growth plate tissue was also softer than the adjacent epiphysis and metaphysis, which had some grtiness due to calcification. The epiphysis was separated from the growth plate by cutting with a scalpel at a depth of approximately 1 mm around the exterior surface of the junction between the epiphysis and the growth plate. When the entire circumference had been scored, a second 1-mm deep cut was made around the circumference of the junction, and this process was repeated until the epiphysis was removed from the growth plate. The growth plate cartilage was separated from the metaphysis by again sequentially scoring around the circumference of the junction between the growth plate and the metaphysis until the growth plate...
was separated from the metaphysis. After dissection, traces of blood were scraped off with a scalpel to yield a soft translucent cartilaginous tissue which is termed growth plate in Table I. These growth plate specimens included the lower hypertrophic zone and must, therefore, have contained a small amount of calcified cartilage in the form of calcified longitudinal septa (8). The widths of the growth plates dissected from the epiphyses of control and rachitic rats were comparable to the 0.5- and 2-mm widths measured from radiographs of the right tibiae from the respective groups of animals. To determine bone levels of BGP and MGP, the left tibiae of three control rats were further dissected into serial sections of 2-mm width beginning at the metaphysis. The first 2-mm section is termed proximal metaphysis in Table I, and the three 2-mm sections from the tibial midshaft are termed cortical bone. Marrow was removed from all bone samples by suction prior to biochemical analyses.

Additional cartilage, connective, and soft tissues were obtained from two 2-month-old Sprague-Dawley rats. Cartilage tissues were removed and cleaned of adhering connective tissue and perichondrium with the aid of a dissecting microscope. The different cartilages had a translucent to white appearance and were free of contaminating tissue by visual inspection under a dissecting microscope.

All tissue samples were lyophilized and weighed. In order to remove mineral-associated noncollagenous protein, both bone growth plate, and costal cartilage samples were first extracted with a 20-fold excess (volume/weight) of 10% (v/v) formic acid for 16 h with continuous end-over-end turning at 4 °C. The supernatant was removed for biochemical analyses, and the insoluble collageen matrix was washed with a 20-fold excess (volume/weight) of distilled water to remove excess acid. These insoluble residues and the dried tissues of all other tissues were then extracted with a 20-fold excess (volume/weight) of 5 M guanidine HCl (0.1 M Tris, pH 8.0) for 16 h with continuous end-over-end turning at 20 °C.

Biochemical Analysis—MGP and BGP levels in extracts were determined by radioimmunoassay as previously described (3, 9). The extract volumes assayed were less than or equal to 5 μL of 5 M guanidine HCl or 0.5% of 10 M formic acid. These volumes have previously been shown not to affect the ability of MGP or BGP to interact with their respective antibodies (3). Phosphate levels in formic acid extracts were determined as described (3, 10).

Immunoblotting Procedure—Guanidine-extracted proteins from cartilage samples were dialyzed against 8 M urea, and MGP purified from rat bone was dissolved in 8 M urea. Forty-microliter aliquots containing 100 or 200 ng of MGP were lyophilized and then taken up in SDS sample buffer to a volume of 40 μL. The proteins were separated on 17.5 or 15% SDS-polyacrylamide slab gels (11) along with prestained molecular weight standards. Following electrophoresis, proteins were electroblotted onto nitrocellulose (12) and subsequently visualized with the aid of a dissecting microscope. The different cartilages were ground to a powder while adhering connective tissue, perichondrium, and periosteum, rinsed in phosphate-buffered saline, and immersed in liquid nitrogen. Samples of all other tissues were then extracted with a 20-fold excess (volume/weight) of 5 M guanidine HCl followed by 5 M formic acid extracts were determined as described (3, 10).

RESULTS

Detection of MGP in Cartilage Extracts—One objective of the present investigations was to evaluate the possibility that either of the two vitamin K-dependent bone matrix proteins, BGP or MGP, is also present in the growth plate and, if so, could be a candidate for the putative vitamin K-dependent protein which prevents growth plate mineralization. As can be seen in Table I, MGP could be detected in growth plate cartilage of control animals at levels significantly higher than in bone. MGP could also be detected in the growth plate cartilage of rachitic rats, a tissue which is larger and easier to dissect without contamination with bone. The lower level of MGP in the rachitic growth plate than in the control could indicate the vitamin D regulates MGP in growth plate cartilage, since 1,25(OH)2D3 has been shown to stimulate MGP synthesis by 10-fold in the osteoblastic cell line UMR 106-01 (13). No BGP could be detected in the growth plate cartilage of control or rachitic rats. In agreement with earlier observations (3), BGP levels are approximately 10-fold lower in metaphyseal bone than in the cortical bone at the midshaft.

There is a high level of acid-extractable phosphate in the growth plate cartilage of control rats (Table I). Since there is no detectable BGP in growth plate cartilage from control rats, most of the extractable phosphate in the growth plate could not be due to contamination with adjacent metaphyseal or epiphyseal bone. A similarly high level of phosphate has been reported previously for the growth plate cartilage dissected from the rat (21). Other studies have shown that phosphate accounts for about 200 μg/mg dry weight in the hypertrophic zone of the growth plate in pigs and fetal calves (22, 23), a region which occupies at least 50% of the growth plate cartilage in rats of this age (8).

To see if the presence of MGP is unique to growth plate cartilage or is a general property of many tissues, several additional tissues were extracted with guanidine HCl and assayed for MGP. As can be seen in Table II, high MGP levels are found in all cartilages examined and in Swarm chondrosarcoma, a transplanted cartilaginous rat tumor that has been widely used as a model for the study of cartilage proteoglycans. No MGP could be detected in tendon, skin, liver, or skeletal muscle, and no BGP could be detected in any tissue but bone.

Characterization of Cartilage MGP—The immunodiffusion curves for cartilage and bone MGP are parallel (Fig. 1), which indicates that the cartilage and bone proteins are identical with respect to the epitopes recognized by the antiserum used for radioimmunoassay. As shown in Fig. 2, the apparent molecular weight of MGP in cartilage is comparable to that for MGP purified from bone. The calculated molecular mass for both proteins is 14 kDa, in good agreement with the 14 kDa previously determined by SDS gel electrophoresis for MGP purified from bone (13). Both the cartilage and bone

| Tissue                | MGP (μg/mg tissue) | BGP (μg/mg tissue) | Phosphate (μg/mg tissue) |
|-----------------------|--------------------|--------------------|--------------------------|
| Control growth plate  | 0.66 ± 0.12        | <0.02              | 116.0 ± 20.0             |
| Rachitic growth plate | 0.22 ± 0.09        | 9.2 ± 5.5          |                          |
| Control epiphysis     | 0.51 ± 0.12        | 1.38 ± 0.11        | 298.0 ± 11.0             |
| Control metaphysis    | 0.33 ± 0.15        | 0.34 ± 0.05        | 222.0 ± 51.0             |
| Control cortical bone | 0.41 ± 0.13        | 2.13 ± 0.24        | 368.0 ± 20.5             |

The left tibiae of control and rachitic rats were dissected into the indicated tissues, and each tissue was dried, weighed, and extracted with 10% formic acid followed by 5 M guanidine HCl. The level of MGP and BGP in both extracts was determined by radioimmunoassay, and the values were then added to give the total MGP and BGP level for the indicated tissue from a given animal. The phosphate level in the formic acid extract was determined colorimetrically (see "Experimental Procedures" for details). The values are the average ± S.D. of the indicated number of animals whose tissues were analyzed.
TABLE II
MGP and BGP levels in the extracts of rat cartilages, bone, and soft tissues

Tibial and epiphyseal bone, and costal cartilage were dried, weighed, and extracted with 10% (v/v) formic acid. The insoluble residue of these tissues and the dried samples of all other tissues were then extracted with 5 M guanidine HCl. MGP and BGP levels in all extracts were determined by radioimmunoassay (see "Experimental Procedures" for details). The data are the average for tissues analyzed from two animals; for bone and costal cartilage the values represent the combined level of protein in the formic acid and guanidine HCl extracts.

| Tissue                        | MGP (µg/mg tissue) | BGP (µg/mg tissue) |
|-------------------------------|--------------------|--------------------|
| Cartilages                    |                    |                    |
| Costal                        | 0.99               | <0.02              |
| Tracheal                      | 0.80               | <0.02              |
| Vertebral disc                | 0.33               | <0.02              |
| Xiphoi process                | 0.32               | <0.02              |
| Nasal septum                  | 0.19               | <0.02              |
| Auricular                     | 0.13               | <0.02              |
| Chondrosarcoma                | 0.77               | <0.02              |
| Bone                          |                    |                    |
| Tibia (diaphysis and metapysis) | 0.41              | 2.19               |
| Epiphysis                     | 0.57               | 1.56               |
| Connective tissue             |                    |                    |
| Achilles tendon               | <0.01              | <0.02              |
| Tail tendon                   | <0.01              | <0.02              |
| Tail skin                     | <0.01              | <0.02              |
| Soft tissue                   |                    |                    |
| Liver                         | <0.01              | <0.02              |
| Skeletal muscle               | <0.01              | <0.02              |

Fig. 1. Radioimmunoassay of MGP from rat bone and its cross-reactivity with MGP from growth plate and nasal septal cartilage. The relative fraction of 125I-labeled rat MGP bound to antibody (B/B0) at increasing amounts of purified rat bone MGP (◆) and at increasing volumes of 5 M guanidine HCl extracts from normal rat growth plate cartilage (□), nasal septal cartilage (○), and rachitic growth plate cartilage (△).

MGP bands can often be resolved into a closely spaced doublet (Fig. 2B). This doublet has been noted in all preparations of MGP purified from calf and rat bone. The relative amounts of the higher and lower molecular weight components of the doublet are constant for a given preparation. For all preparations of MGP from nasal septal cartilage and growth plate cartilage the larger component is the more abundant, while the opposite is the case for MGP from bone.

Detection of MGP mRNA in Cartilage—In order to determine whether the MGP found in cartilage is actually synthesized in this tissue, RNA was extracted from nasal septal cartilage, Swarm chondrosarcoma, and calvaria and subjected to Northern blot analysis. As seen in Fig. 3, there is strong hybridization signal for MGP in the total RNA from nasal septal cartilage and chondrosarcoma while the signal for MGP in the total RNA from calvaria is barely detectable. Hybridization to the total RNA from calvaria could be seen more easily in longer exposures (data not shown) and in the poly(A+)-purified RNA from calvaria (Fig. 3). The MGP mRNA detected in all three tissues is identical in size to the 700-base message previously reported for bone (13). No BGP hybridization could be detected in the total RNA from nasal septal cartilage or Swarm chondrosarcoma even at long exposures, while the BGP signal was strong in the total RNA from calvaria (Fig. 3).

DISCUSSION

We have demonstrated the synthesis and accumulation of the vitamin K-dependent protein matrix Gla protein in cartilage. To our knowledge this represents the first Gla-containing protein to be identified in cartilage. By all criteria examined, the MGP in cartilage is essentially identical to MGP in bone. Radioimmunoassay immunodilution curves for cartilage and bone MGP are parallel indicating antigenic identity. The apparent molecular weight of MGP from cartilage is comparable to that of MGP from bone. Finally, MGP mRNA in cartilage is identical in size to the MGP mRNA in bone. The presence of high MGP levels in all unmineralized cartilages examined indicates that MGP is associated with the organic matrix of cartilage and does not require a Gla-dependent association with hydroxyapatite to anchor in a matrix. The organic matrix association of the major fraction of MGP
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**Fig. 3. Northern blot analysis of MGP and BGP mRNA levels in cartilage and calvaria.** RNA was extracted from rat chondrosarcoma, nasal septal cartilage, and calvaria. 10 μg of total RNA from chondrosarcoma, nasal septal cartilage, and calvaria and 10 μg of poly(dT)-cellulose-selected calvarial RNA were electrophoresed on a 1.4% formaldehyde agarose gel and blotted onto Nytran. The blot was then hybridized to 32P-labeled MGP cDNA (MGP). Following autoradiography, the probe was boiled off and the blot was hybridized to 32P-labeled BGP cDNA (BGP) and autoradiographed again.

Bone is supported by the observation that only 20–50% of bone MGP is extracted by demineralization, while the rest remains associated with the bone matrix (3).

Although the level of MGP in cartilage is approximately the same as the level in bone, the level of MGP mRNA in cartilage is far higher than in bone. If the higher level of MGP mRNA in cartilage does, as we believe, reflect a higher rate of synthesis in this tissue, the higher ratio of synthesis to tissue levels of the protein in cartilage could either mean that most MGP synthesized in cartilage is not deposited in the tissue or that the turnover rate of MGP in cartilage is more rapid than it is in bone. The latter possibility is supported by the observation that the half-life of proteoglycans in cartilage may be as short as 84 h (24), while typical bone matrix proteins such as BGP turn over only at the far slower rate at which bone is resorbed and reformed (25).

This is the first report that MGP migrates as a closely spaced doublet on SDS gel electrophoresis. In previous studies the presence of this doublet must have been obscured by a higher protein load and a lower percentage of acrylamide in the gel, since we now know that all preparations of MGP purified from bone can be resolved into a doublet in the system employed in this study. One explanation for the doublet could be proteolytic processing at the COOH-terminus of MGP. The cDNA structure of rat MGP predicts an 84-residue secreted protein which has an additional 5 COOH-terminal residues (-Arg-Arg-Gly-Ala-Lys) not seen in the 79-residue bovine MGP structure determined by amino acid sequencing of the purified protein (1, 19). The doublet seen on SDS gel electrophoresis may, therefore, reflect partial proteolytic conversion of the 84- to the 79-residue form of MGP. This hypothesis is consistent with the fact that the lower molecular weight form of MGP is the major component in MGP purified from bone, since direct sequence analysis of MGP isolated from bone did reveal a 79-residue protein. Further work will clearly be needed to determine whether the doublet observed on electrophoresis is due to proteolytic cleavage at the COOH-terminus of MGP and to establish the relationship between molecular weight differences and MGP function.

Several lines of evidence support the hypothesis that MGP is a mineralization inhibitor whose function in cartilage is to prevent abnormal mineralization. Rats treated from birth to 8 months of age with warfarin plus vitamin K, a protocol which ultimately reduces bone levels of BGP by 50-fold but does not affect blood coagulation times (25, 26), have only one major abnormality, the excessive mineralization of growth plate cartilage with eventual fusion of the growth plate (5). A similar disorder characterized by punctate mineralization of growth plate cartilage has also been described in infants born to mothers who received warfarin during the first trimester of pregnancy (27). Both disorders could be explained by the hypothesis that the mineralization of growth plate cartilage is normally regulated by the action of a vitamin K-dependent mineralization inhibitor. Because the present studies demonstrate that MGP is made in cartilage while BGP is not, MGP is the better candidate for the mineralization inhibitor whose abnormal synthesis leads to cartilage mineralization in a warfarin-treated animal (5). Preliminary evidence indicates that MGP is indeed an inhibitor of hydroxyapatite formation in vitro and that this property is lost when the Gla residues are thermally decarboxylated to Glu.3 We are presently examining the effect of warfarin on unmineralized cartilages in order to clarify the function of matrix Gla protein in these tissues.

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