A marked dissociation has been observed between the timed accumulation in calcified tissues of two related vitamin K-dependent proteins, bone Gla protein (BGP) and the recently discovered matrix Gla protein (MGP). In long bone diaphyses, total levels of MGP were essentially equivalent in newborn, juvenile, and adult rats. In agreement with previous studies, BGP levels were only 5% of adult levels in newborn rat bones and increased to 90% of adult levels by 19 days of age. Similar results were obtained from the analysis of the longitudinal distribution of MGP and BGP in 14-day-old rat tibia, a bone in which new mineral is added rapidly at both growth plates. Again, MGP was essentially at the same level in the regions nearest the growth plates as in the midshaft while BGP levels were 10-fold lower in the regions nearest the growth plates. These differences in the timed accumulation of MGP and BGP in calcifying tissues indicate that MGP could function earlier in bone formation than does BGP.

To further characterize the MGP antigen in bone, extracts from newborn and adult rat bones were chromatographed by gel filtration over Sephacryl S-200. All of the antigen extracted by formic acid and most of the antigen subsequently extracted by guanidine HCl emerged at the position expected for the 79-residue MGP. There was a significant difference in the fraction of total MGP which was extracted by guanidine HCl in newborn (50%) and adult (20%) bone. The radioimmunoassay for rat MGP which was developed for these studies employs rabbit antibody directed against calf MGP and rat MGP for standards and radioiodinated tracer. This assay has a sensitivity of 0.1 ng and does not detect rat or calf BGP.

The quantitative accumulation of non-collagenous proteins during the calcification of bone can provide important insights into the role of these proteins in bone formation. In pursuit of our interests in the structure and function of vitamin K-dependent bone proteins, we have previously examined the appearance of bone Gla protein (BGP) in calcifying rat and human bone (1, 2). In both species, BGP accumulates in calcifying tissues 1-2 weeks after mineral deposition. This observation, which was subsequently confirmed for calcifying chicken bone (3), suggests that BGP functions at a later stage of bone formation than initial mineralization.

We have recently isolated a second Gla-containing protein from bone, matrix Gla protein (MGP) (4). MGP is a water insoluble 79-residue protein which contains 4.8 Gla residues (5), while BGP is a water soluble 49-residue protein which contains 3 Gla residues (6, 7). MGP is associated with the organic matrix of demineralized bone and is present in bovine bone at a level of 0.4 mg of MGP/g (4). In contrast, BGP is extracted quantitatively during demineralization and is present in bovine bone at the higher level of 2 mg of BGP/g. Although there is no immunological cross-reactivity between MGP and BGP (4), there is sufficient sequence homology between the two proteins to conclude that they arose from a common ancestor by gene duplication and subsequent divergent evolution (5).

The sequence homology between BGP and MGP suggests that there may be a common aspect to their function. Since homology is greatest in the region of the Gla residues, we have previously suggested that sequence homology reflects structural features which are important to hydroxyapatite binding (5). These include the postulated bidentate association of the malonyl side chain of Gla residues with surface Ca$^{2+}$ in hydroxyapatite, and hydrogen bond complexes between the guanidino side chain of arginine and surface phosphate in hydroxyapatite. For BGP, the interaction between Gla residues and Ca$^{2+}$ in hydroxyapatite appears to be essential for accumulation in bone (6, 9). The late accumulation of BGP in calcifying bone has therefore been attributed to the maturation of the initial mineral phase to fully crystalline hydroxyapatite (1, 2), a transition whose timing (10) correlates well with the delayed accumulation of BGP in bone.}

**Developmental Appearance of Matrix Gla Protein during Calcification in the Rat**

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The quantitative accumulation of non-collagenous proteins during the calcification of bone can provide important insights into the role of these proteins in bone formation. In pursuit of our interests in the structure and function of vitamin K-dependent bone proteins, we have previously examined the appearance of bone Gla protein (BGP) in calcifying rat and human bone (1, 2). In both species, BGP accumulates in calcifying tissues 1-2 weeks after mineral deposition. This observation, which was subsequently confirmed for calcifying chicken bone (3), suggests that BGP functions at a later stage of bone formation than initial mineralization.

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**MATERIALS AND METHODS**

Preparation of MGP—MGP was extracted and purified from demineralized calf and rat cortical bone as described previously (4). The molecular weight of purified rat MGP was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amino acid composition of purified rat MGP was determined by amino acid analysis of protein samples which had been hydrolyzed in vacuo in 6 N HCl at 110 °C for 24 h. The Gla and Glu levels in samples were determined by amino acid analysis of alkaline hydrolysates as described (11). The NH$_2$-terminal amino acid sequence of rat MGP was determined by automatic Edman degradation using an Applied Biosystems Model 470A Gas Phase Protein Sequencer employing the standard "NoVac" program supplied by the manufacturer (12). Phenylthiobenzydantoin derivatives were methylated with 1 M methanolic HCl and identified on an IBM cyano column (13). The high performance liquid chromatography system employed consisted of a Perkin-
Preparation of Radiolabeled MGP—Two micrograms of purified calf or rat MGP were labeled with 1 μCi of \(^{35}\text{S}\) (4 × 10^6 cpm/mole of I, Amer sham Corp.) by the chloramine-T method (14). The labeled MGP was separated from unincorporated \(^{35}\text{S}\) by gel filtration on a Sephadex G-25 column equilibrated with assay diluent (0.14 M NaCl, 0.01 M phosphate, 25 mM EDTA, 0.1% gelatin, 0.1% Tween 20, pH 7.4). Over 80% of labeled calf or rat MGP bound to an excess (1:160 dilution) of antibody raised against purified calf MGP.

Preparation of Antibodies—Three rabbits were immunized by monthly injections at multiple intradermal sites with 0.1-0.5 mg of purified calf MGP adsorbed to polyvinylpyrrolidone (15) and emulsified with either complete (initial challenge) or incomplete Freund's adjuvant. Serum samples were withdrawn at regular intervals and tested for the titer of antibody to rat and calf MGP and tested for the presence of radioimmunoassay. The antiserum that bound 20% of the iodinated rat MGP at the highest dilution was used for all radioimmunoassays reported here.

Radioimmunoassay—The assay for rat MGP contained (in order of addition): 0.1 ml of assay diluent; either a known amount of unlabelled rat MGP, 5 μl or less of bone formic acid extract, or 5 μl or less of bone residue guanidine HCl extract in 0.1 ml of assay diluent; 0.4 μl of antiserum (final 1:2500 dilution) and 2.1 μl of normal rabbit serum in 0.1 ml of assay diluent; and 30,000 cpm of \(^{125}\text{I}\)-labeled rat MGP in 0.1 ml of assay diluent. All components except \(^{125}\text{I}\)-labeled MGP were combined and incubated for 24 h at 4 °C followed by addition of \(^{125}\text{I}\)-labeled MGP and a second 24-h incubation at 4 °C. Assays were terminated by precipitation of rabbit antibody with the addition of 1.9 units of goat antiserum to rabbit γ-globulin (Cappel-Lederle-Behring) in 0.1 ml of assay diluent. After 2 h at 4 °C, reaction mixtures were centrifuged to sediment \(^{125}\text{I}\)-labeled MGP bound to rabbit antibody and the supernatant was discarded. Background counts/min in precipitate divided by total counts/min in assay; Bo is the value of B when no unlabeled MGP is present. The Bo values for all standards and unknowns are the average of three independent measurements. BGP levels in formic acid and guanidine HCl extracts of bone were determined as described (1, 2).

Table I

| Rat MGP | Calf MGP |
|---------|---------|
| Asp     | 11.3    | 12.7   |
| Thr     | 2.9     | 0      |
| Ser     | 6.8     | 6.3    |
| Glu     | 9.2     | 11.7   |
| Pro     | 5.0     | 3.8    |
| Gln     | 2.7     | 1.3    |
| Ala     | 10.7    | 8.9    |
| Cys     | 1.2     | 2.5    |
| Val     | 4.0     | 1.3    |
| Met     | 1.4     | 1.3    |
| Ile     | 3.8     | 5.1    |
| Leu     | 3.9     | 5.1    |
| Tyr     | 9.5     | 10.1   |
| Phe     | 4.1     | 6.1    |
| His     | 3.2     | 1.5    |
| Lys     | 4.1     | 3.8    |
| Arg     | 11.0    | 12.7   |
| Trp     | ND*     | 1.3    |
| Glu     | 5.1     | 6.1    |

*ND, not determined.
Developmental Appearance of MGP

![Diagram](Image)

**FIG. 1.** Radioimmunoassay of rat MGP and its cross-reactivity with bovine MGP and with formic acid and guanidine HCl extracts of rat bone. Relative fraction of $^{125}$I-labeled rat MGP bound to antibody ($B/B_0$) at increasing levels of rat MGP (○), calf MGP (●), formic acid extract of rat bone (△), and guanidine HCl extract of rat bone (□).

**FIG. 2.** Developmental changes in BGP and MGP levels in rat long bones. Total BGP (○) and MGP (●) levels were determined by radioimmunoassay of protein solubilized from 10 mg of bone after demineralization in 0.2 ml of 10% formic acid for 16 h at 4°C and subsequently solubilized in 0.2 ml of guanidine HCl for 16 h at room temperature; each point is the average of 2 separate 10-mg extractions of the pooled long bones from five rats.

BGP, mg/g bone slice, ○, µg of BGP/bone slice, 0, µg of MGP/bone slice, ○, µg of MGP/bone slice.

The MGP in each extract is identical antigenically to MGP purified from rat bone.

**Developmental Changes in Total MGP and BGP Levels**—The developmental changes in the levels of MGP and BGP in rat long bones are shown in Fig. 2. In bones from newborn and juvenile rats, approximately equal amounts of MGP emerged in the formic acid demineralization and the subsequent guanidine HCl extraction steps. Total MGP levels in newborn rats were about 0.5 mg/g of dry bone and did not change significantly over the first 19 days of postnatal life or in the adult rat. In marked contrast, the same extracts of newborn rat bone revealed a total BGP content of only 0.15 mg/g of dry bone. Total BGP levels increased to 3 mg/g by 19 days of age, and to 3.7 mg/g in the adult rat. The same pattern, delayed developmental appearance for BGP and constant MGP levels from birth through adulthood, was obtained on analysis of calvarial extracts (data not shown). It should be noted that mineral could first be detected 2–3 days before birth, and newborn rat bone consequently represents a tissue in which most mineral has been deposited quite recently.

**Distribution of MGP and BGP in Tibias from 2-Week-old Rats**—Another system for correlating the appearance of MGP with calcification is provided by the longitudinal distribution of MGP in tibias from juvenile rats. Since this bone increases rapidly in length by the formation of new bone at the growth plate, the segments of bone closest to the growth plate have the highest proportion of newly deposited mineral and the lowest level of BGP (2). As can be seen in Fig. 3, total MGP levels are actually somewhat higher in the proximal and distal growth plate regions of the tibia than in the midshaft region. Since the dry weights of the two segments closest to the proximal (1 and 2) and distal (6 and 7) growth plates weighed an average of 25% more than the midshaft segments, the total MGP content/weight of segment was essentially constant. Analysis of BGP levels in the same extracts confirmed the earlier report (2) that BGP levels are lowest in the growth plate region and highest in the midshaft. The longitudinal distribution of MGP in juvenile rat bones therefore further supports the concept that MGP appears earlier in calcification than does BGP.

**Size Distribution of MGP in Bone Extracts**—As noted above, essentially equivalent amounts of MGP were present in the formic acid and guanidine HCl extracts of juvenile rat bones. Both antigens appear to be immunologically identical to purified MGP from rat bone (Fig. 1). To further examine the nature of MGP in the two extracts, we established the size distribution of each by filtration over Sephacryl S-200. As seen in Figs. 4a and 5a, most of the antigenic material in the formic acid and guanidine HCl extracts of newborn rat bone emerges in the position expected for purified MGP. A small amount of MGP antigen in the guanidine HCl extract (<10%) emerges in the excluded volume. This fraction of apparently higher molecular weight MGP was not characterized further.

The size distribution of MGP antigen in the formic acid demineralization extract and the subsequent guanidine HCl extract of adult rat bone, shown in Figs. 4b and 5b, reveal a qualitatively similar picture to the size distribution of MGP.
Developmental Appearance of MGP

FIG. 4. Sephacryl S-200 filtration of the formic acid demineralization extract from newborn and adult rat bone. The formic acid extracts from 100 mg of newborn and adult rat bone were applied to a 0.9 × 150-cm column of Sephacryl S-200. Buffer, 5 M guanidine HCl, 0.1 M Tris (pH 8); 4 °C. ○, absorbance at 220 nm. ●, µg/ml of MGP as determined by radioimmunoassay. The arrow denotes the elution position of MGP and BGP purified from rat bone.

antigen in newborn rat bone. The major differences are that quantitatively less of the total MGP in adult bone emerges in the guanidine HCl extract, and the percentage of apparently higher molecular weight material in the guanidine HCl extract of adult bone is somewhat higher.

DISCUSSION

The present results demonstrate that MGP levels are already high at the earliest stages of bone formation examined. Due to the inherent limitations of this system and the sensitivity of our radioimmunoassay, we cannot presently determine whether the appearance of MGP in bone precedes mineralization or is coincident with the onset of mineralization. This important question will require careful immunohistological analysis of the earliest stages of calcification.

The dramatic difference between the developmental appearance of MGP and BGP could reflect either the delayed expression of the BGP gene or accumulation of MGP in bone matrix by a mechanism different from that for BGP. Although the question of the timing of BGP gene expression has not yet been completely resolved, the available evidence indicates that some expression of the BGP gene occurs quite early in development. For example, serum BGP levels are high in newborn rats (1) and BGP can be demonstrated in osteoblasts of 2-day-old rat bone (16) and in odontoblasts of developing rat dentine (17). It is not presently known, however, whether the relative rates of BGP and MGP gene expression are developmentally regulated. The major evidence which supports differential gene regulation is the observation that

FIG. 5. Sephacryl S-200 filtration of the guanidine HCl extracts from newborn and adult rat bone. The residue remaining after demineralization of 100 mg of newborn and adult rat bone (Fig. 4) was subsequently extracted with 5 M guanidine HCl as described under "Experimental Procedures." Columns and buffer are described in the legend to Fig. 4. ○, A220; ●, µg/ml of MGP as determined by radioimmunoassay. The arrow denotes the elution positions of MGP and BGP purified from rat bone.
clonal cells derived from the same osteosarcoma produce BGP and MGP, but not both.\(^2\) Clear resolution of the timing of MGP and BGP gene expression will require measurement of BGP and MGP message levels in the bone of developing rats.

There is also evidence which indicates that the dramatic difference in the timing of the accumulation of MGP and BGP in bone could reflect differing modes of association with bone matrix. Several observations indicate that BGP is anchored to bone exclusively by its binding to hydroxyapatite. Nearly all BGP emerges from bone during demineralization with EDTA or with formic acid. In addition, the abnormal, non-γ-carboxylated BGP synthesized in a vitamin K-deficient rat neither binds to hydroxyapatite nor accumulates significantly in bone (8, 9). Because BGP appears to anchor to bone through its affinity for the mineral phase, it has been suggested that the delayed appearance of BGP reflects the well-established maturation of bone mineral during this phase of rat development (10). We have postulated that MGP binds both to the mineral and the organic components of bone (5). Evidence for mineral binding is based on the substantial sequence homology between the Gla-containing regions of MGP and BGP, a region of BGP known to be critical for mineral binding (8), and on the observation that over half of the total MGP in bone is extracted during demineralization. Support for binding to the organic bone matrix is based on the observation that much of the MGP in bone can only be extracted by treatment of demineralized bone matrix with denaturants. In addition, treatment with the vitamin K-antagonist warfarin from birth to 8 months of age reduces BGP levels to 2% of normal (9) while, in preliminary analyses, it reduces MGP levels to only 26% of normal. If the γ-carboxyglutamic acid residues in MGP and BGP are equally critical for hydroxyapatite binding, the failure of vitamin K deficiency to lower MGP to levels comparable to BGP can be taken as evidence for a mechanism of association between MGP and bone matrix which is independent of bone mineral.

The high levels of MGP in newborn rat bone provide a partial explanation for the observation that γ-carboxyglutamic acid levels become high in calcifying bone long before BGP levels do (1, 3, 18). One particularly striking example of this dissociation is the demonstration that alkaline hydrolyzates of newborn rat bone contain 37% of adult levels of γ-carboxyglutamic acid while demineralization extracts contain only 3% of adult levels of BGP (1). Like MGP, the Gla-containing component in newborn rat bone is not detected by the radioimmunoassay for BGP and is substantially associated with denatured bone matrix (1). While we cannot at present rule out the possible presence of other vitamin K-dependent proteins which appear early in calcification, quantitative calculations indicate that at least half of the Gla in newborn rat bone must be MGP.

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