Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins

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E xtracellular matrix mineralization (ECMM) is a physiologic process in the skeleton and in teeth and a pathologic one in other organs. The molecular mechanisms controlling ECMM are poorly understood. Inactivation of Matrix gla protein (Mgp) revealed that MGP is an inhibitor of ECMM. The fact that MGP is present in the general circulation raises the question of whether ECMM is regulated locally and/or systemically. Here, we show that restoration of Mgp expression in arteries rescues the arterial mineralization phenotype of Mgp−/− mice, whereas its expression in osteoblasts prevents bone mineralization. In contrast, raising the serum level of MGP does not affect mineralization of any ECM. In vivo mutagenesis experiments show that the anti-ECMM function of MGP requires four amino acids which are γ-carboxylated (gla residues). Surprisingly, another gla protein specific to bone and teeth (osteocalcin) does not display the anti-ECMM function of MGP. These results indicate that ECMM is regulated locally in animals and uncover a striking disparity of function between proteins sharing identical structural motifs.

Introduction

ECM mineralization (ECMM) is a physiologic process in bone, teeth, and hypertrophic cartilage, whereas in other locations it must be inhibited. To date, few proteins acting as inhibitors of ECMM have been identified through mouse and human genetic studies. They include: Ank, a transmembrane protein controlling extracellular export of pyrophosphate, a small molecule that itself inhibits ECMM; NPPS, an ectoenzyme also generating pyrophosphate extracellularly; matrix gla protein (MGP), a mineral-binding protein of the ECM; and fetuin, a circulating protein that accumulates in bone ECM (Jahnen-Dechent et al., 1997; Luo et al., 1997; Okawa et al., 1998; Nakamura et al., 1999; Hagmann, 2000; Ho et al., 2000; Nurnberg et al., 2001; Schafer et al., 2003). Understanding at the molecular level how each of these proteins inhibits ECMM is a prerequisite to better understanding how ectopic ECMM develops, such as that observed in atherosclerosis or in osteoarthritis. Elucidation of the mechanisms behind protein inhibition of ECMM may lead eventually to the identification of novel therapeutic strategies for the treatment of these diseases.

With the long-term goal of understanding how ECMM is prevented in some tissues, whereas favored in others, our laboratory has embarked on a detailed study of the functions and mechanisms of action of proteins containing gla (or γ-carboxylated glutamic acid) residues (Pudota et al., 2000; Bandyopadhyay et al., 2002). This posttranslational modification confers to proteins a high affinity for hydroxyapatite crystals, the major mineral crystal present in mineralized ECMs (Romberg et al., 1986; Roy and Nishimoto, 2002; Hoang et al., 2003). We focused our work on two gla residue-containing proteins, namely MGP and bone gla protein (BGP or osteocalcin), the latter being a protein long thought to be involved in bone ECMM (Price et al., 1976, 1983; Celeste et al., 1986). Mgp is expressed in vascular smooth muscle cells (VSMCs) and in chondrocytes but not in osteoblasts, whereas Osteocalcin is expressed in osteoblasts and odontoblasts only (Ducy and Karsenty, 1995; Luo et al., 1995). In addition, both MGP and osteocalcin are circulating proteins (Lian et al., 1987; Ismail et al., 1988; Price et al., 2003). Consistent with the pattern of Mpg expression, MGP-deficient mice develop abnormal ECMM in their arteries and growth plate cartilage establishing that MGP is an inhibitor of ECMM in the vicinity of the cells expressing it (Luo et al., 1997). In contrast, osteocalcin-deficient mice did not have any detectable defect of bone ECMM indicating that osteocalcin is not required for bone mineralization (Ducy et al., 1996). This latter experiment did not address however, whether osteocalcin, like MGP, could inhibit ECMM.

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Abbreviations used in this paper: ECMM, ECM mineralization; MGP, matrix gla protein; VSMC, vascular smooth muscle cell; WT, wild-type.
not systemically. They also demonstrate that osteocalcin does not carry out the anti-ECMM function of MGP in vivo.

Results and discussion

Generation of transgenic mice

To study the roles of MGP and osteocalcin during ECMM, we generated several mouse models expressing, in a cell-specific manner, wild-type (WT) or mutated proteins (Fig. 1, A–E). We used a SM22α promoter fragment to drive their expression specifically in VSMCs, whereas liver-specific expression was achieved by using the promoter of the apolipoprotein E (ApoE) gene and its liver-specific enhancer (Simonet et al., 1993; Solway et al., 1995). To achieve osteoblast-specific expression, we used the 2.3-kb promoter fragment of the α1(I) collagen gene (Rossert et al., 1995). In each case, we obtained at least two different lines expressing the transgene of interest and we verified cell- or tissue-specific expression for each transgene (Fig. 1, A–E). SM22α-Mgp, ApoE-Mgp, SM22α-Osteocalcin, and α1(I)Col-Osteocalcin transgens had no metabolic or histological abnormalities. In particular, they had normally mineralized bone and no sign of ectopic ECMM (Table I and not depicted). These various transgenic mice were then used for subsequent experiments presented below.

Rescue of arterial but not of cartilage phenotype in Mgp−/−; SM22α-Mgp mice

SM22α-Mgp mice had no phenotypic abnormalities and serum PTH, phosphate and calcium levels were normal. To test if this transgene could rescue the arterial phenotype of Mgp−/− mice, we intercrossed SM22α-Mgp mice with Mgp+/− mice to obtain Mgp−/−; SM22α-Mgp mice. WT, Mgp−/− and Mgp−/−; SM22α-Mgp mice were analyzed at 4 wk old, the age at which most Mgp−/− mice die due to vascular rupture and hemorrhaging, by Alizarin red staining of skeletal preparations and histological analyses (Luo et al., 1997). Alizarin red staining of skeletal preparations failed to detect any abnormal mineralization of the aorta or of any arteries in Mgp−/−; SM22α-Mgp mice at that age or at 6 mo old (Fig. 2 A and not depicted). Accordingly, histological examination using von Kossa staining for mineral deposits failed to detect any ECMM in the arteries of Mgp−/−; SM22α-Mgp mice at 4 or 24 wk old (Fig. 2 B and not depicted). In contrast, mineralization of cartilage, an avascular tissue, was not prevented in Mgp−/−; SM22α-Mgp mice (Fig. 2 D). Thus, reintroducing MGP in VSMCs could rescue only the arterial phenotype of MGP-deficient mice.

Table I. Serum phosphate (Pi), calcium (Ca), and parathyroid hormone (PTH) concentrations in the transgenic mice

| Transgenic mice            | [Pi] mg/dL (σm) | [Ca]total mg/dL (σm) | [PTH] pg/mL (σm) |
|----------------------------|----------------|----------------------|-----------------|
| SM22α-Mgp                  | 7.91 (0.38)    | 9.58 (0.23)          | 34.76 (1.38)    |
| ApoE-Mgp                   | 7.05 (0.29)    | 10.20 (0.20)         | 29.67 (5.24)    |
| α1(I)Col-Mgp               | 7.39 (0.06)    | 9.91 (0.08)          | 38.11 (7.56)    |
| SM22α-Osteocalcin          | 7.35 (0.25)    | 10.00 (0.09)         | 25.95 (0.54)    |
| α1(I)Col-Osteocalcin       | 8.01 (0.19)    | 10.37 (0.15)         | 31.28 (4.47)    |
| WT                         | 7.89 (0.92)    | 10.37 (0.06)         | 29.02 (5.30)    |

SEM (σm) values are presented in the parentheses.
Systemic presence of MGP does not rescue the phenotype of MGP−/− mice

Because MGP is found in the general circulation where it forms a complex with small mineral nuclei and other circulating proteins (Price et al., 2003), the question arises as to its mode of action. Is MGP acting locally in the vicinity of the cells synthesizing it and/or is it using blood transport as its mode of action? Is MGP acting locally in the vicinity of the cells synthesizing it and/or is it using blood transport as its mode of action? Is MGP acting locally in the vicinity of the cells synthesizing it and/or is it using blood transport as its mode of action?

To that end, we studied bone mineralization at 4 wk old in WT, ApoE-Mgp, and in α1(I)Col-Mgp mice that express Mgp only in osteoblasts (Fig. 1 B). Skeletal preparations of 10-d-old skulls showed no mineralization defect in ApoE-Mgp mice, whereas a severe decrease in intramembranous bone mineralization was observed in α1(I)Col-Mgp mice (Fig. 3 E). Likewise, histological analysis of vertebrae of ApoE-Mgp mice failed to show any increase in unmineralized bone, whereas histological analysis of vertebrae of α1(I)Col-Mgp mice showed a marked, i.e., 8–12-fold, increase of unmineralized bone tissue (Fig. 3 F). When together, the analyses of Mgp−/−; SM22α-Mgp, ApoE-Mgp, and α1(I)Col-Mgp mice establish that, in animals fed a normal diet, MGP inhibits ECMM locally and not systemically.

MGP and osteocalcin do not share an antimineralization function despite structural similarities

The osteoidosis, i.e., increase in unmineralized bone ECM, observed in the α1(I)Col-Mgp mice provided us with an in vivo model to test the function of the gla residues within MGP and other proteins. In this context, we generated transgenic mice that produced two distinct mutated forms of MGP in osteoblasts. In MGP mutant1 (MGPm1), three of the four glutamic acid residues present in the mouse protein were replaced by aspartic acid residues. In MGP mutant2 (MGPm2) all four glutamic acid residues of the four glutamic acid residues present in the mouse protein were replaced by aspartic acid residues. Histological analysis performed in 4-wk-old mice failed to detect any evidence of osteoidosis in the α1(I)Col-Mgp mice, whereas osteoidosis was considerably milder in α1(I)Col-Mgp m1 mice than in α1(I)Col-Mgp (Fig. 4 C). These results establish that the gla residues are required for MGP antimineralization function.
The demonstration of the functional importance of gla residues in MGP leads to the question of whether other gla-containing proteins are also inhibitors of ECMM. Osteocalcin is the most abundant gla protein synthesized in the skeleton, yet its deletion in mice failed to show impaired ECMM (Ducy et al., 1996). However, because this experiment could not assess whether osteocalcin, like MGP, could act as an inhibitor of ECMM, we subsequently performed gain of function and ectopic expression experiments. SM22α-Osteocalcin mice did not show any metabolic or histological abnormalities (Table I and not depicted) despite a six- to eightfold increase in serum osteocalcin indicating efficient transcription/translation of the transgene (Fig. 5A). SM22α-Osteocalcin mice were then intercrossed with Mgp−/− mice to obtain Mgp−/−; SM22α-Osteocalcin mice. Surprisingly, these latter mutant mice did not survive past 2 mo old and possessed the phenotype of Mgp−/− mice (unpublished data). Indeed, Alizarin red staining of skeletal preparations and histological analysis showed that at 1 mo old the aorta of Mgp−/−; SM22α-Osteocalcin was fully mineralized (Fig. 5, B and C). This result indicates that unlike MGP and despite the presence of three gla residues, osteocalcin cannot inhibit ECMM in arteries. Alternatively, it could mean that this function of osteocalcin could not be incurred outside bone, its physiological site of expression. To test this possibility, we generated transgenic mice overexpressing Osteocalcin in osteoblasts under the control of the α1(I) collagen promoter. Again, unlike what we observed in α1(I)Col-MGP transgenic mice, α1(I)Col-Osteocalcin mice had normally mineralized bone. In particular, they had a normal osteoid volume relative to total bone volume (Fig. 5D).

In summary, using genetic and in vivo bioassay models of physiologic (bone) and pathologic (arterial) ECMM, we...
to an aspartic acid encoding codon by PCR mutagenesis. The mutated am-\pilo{p}licon was digested by CiaI and PpuMI to replace the CiaI and PpuMI di-

gested fragment of a\1(I)Col-Mgpm1. pApoE-Mgp was generated by inserting
\the Mgp ORF at a Hpal site of p\l\7 containing a 3-kb ApoE promoter
\fragment, the first intron, polyadenylation signal, and a liver-specific enhancer
(Simonet et al., 1993). To construct pSM22a-Osteocalcin, the complete
\Osteocalcin ORF was first subcloned in pBluScript (Stratagene). The
\SV40 polyadenylation signal was introduced at a 3' Xbal site and the
\3-kb SM22a promoter fragment was inserted at an upstream Xhol site.
p\1(I)Col-Osteocalcin was constructed by inserting the 2.3-kb a\1(I) col-
\lagen promoter fragment upstream to the Osteocalcin ORF and SV40 poly-
\adenylation signal.

Mice

Generation of Mgp\1/1 mices was previously described (Luo et al., 1997).
Transgenic founders were generated by pronuclear injection according to standard
techniques. All mice were maintained in a pathogen-free stan-
dard animal facility.

Genotyping and expression analysis

Genotypes were determined by PCR using isolated tail DNA. The following
sets of primers were used: for SM22a-Mgp transgene 5'-AAG-
\GAACGTTTTCAGGTCCTG-3' and 5'-CCGGGAAATGAGGAAGA-
\AGGG-3'; for a\1(I)Col-Mgp, a\1(I)Col-Mgpm1, and a\1(I)Col-Mgpm2 transgen-
\es 5'-CCAGTATGCTGAAAGATTACTACG-3' and 5'-CCG-
\GAAGATGAGGAAGAAGGG-3' and ApoE-Mgp transgene 5'-TTAGAG-
\GAATCACAGGGGAGGC-3' and 5'-GACCTGACAAACCGCCTAG-
\CTACATATACAAAGAGTGC-3'; for SM22a-Osteocalcin transgene 5'-
\AAGGAAAGGCTTCAAGGGTCCTG-3' and 5'-GGGATCTGGCCTGG-
\GACTGAGG-3' and for a\1(I)Col-Osteocalcin transgene 5'-CCAGTATG-
\CTGAAAGATTACTACG-3' and 5'-GGGATCTGGCCTGGGACT-
\GACTGAGG-3'. For analysis of transgene expression, RNA was isolated
\as previously described and analyzed by Northern blotting (Ausubel et al.,
\1996). Probes used were SV40 and Osteocalcin polyadenylation signals.

Primary osteoblast culture

WT osteoblasts were cultured in Alpha-MEM (Invitrogen) containing 10% WT
\or ApoE-Mgp serum and supplemented by 100 \mu\g/ml ascorbic acid
\(\Sigma\)-Aldrich). After formation of osteoblast nodules at 4 d, 5 mM \beta-
glycero phosphate (Sigma-Aldrich) was added to the culture medium and
\cells were grown for another 4 d. Fresh medium was added in every 48 h.
\von Kossa staining for mineral and alkaline phosphatase staining for osteo-
\blasts were performed at the end of the culture period.

Skeletal preparation

Thoracic aorta together with vertebrae were dissected, fixed overnight in
\100% ethanol, and stained in Alcian blue dye followed by Alizarin red s-
\olution as described previously (Luo et al., 1997).

Histology

Vertebr\e were fixed overnight in 4% PFA/PBS, embedded in methyl
\acrylate, sectioned (7 \mu\m), and stained by von Kossa and von Gieson.
\Unmineralized bone was measured using Osteomeasure software (Osteo-
\metrics Inc.). Aortas were fixed in 1% glutaraldehyde overnight, washed in
\0.1 M sodium cacodylate buffer, serially dehydrated in ethanol, and em-
\bedded in paraffin. 7-\mu\m sections were stained by von Kossa and counter-
\stained by Toluidine blue. Images were captured with a light micro-
\scope (model DMLB; Leica) using a SPOT CCD camera, acquired with
\SPOT software v2.1 (Diagnostic Instruments), and processed using Adobe
\Photoshop®.

Serum biochemistry

Serum calcium and phosphate were measured using commercially avail-
\able kits (Sigma-Aldrich). PTH was measured using an ELISA kit for immu-
\nological detection (Immunotopics). Dot blot analysis to detect serum MGP was
\performed using a polyclonal rabbit serum raised against a COOH-termi-
\nal MGP peptide (ERYAMVYGYNAAYNRYFRQRRGAKY). A commercially
\available antibody antibody conjugated with HRP was used as a second-
\ary antibody. HRP activity was detected by using standard protocols and
\signal intensity on an imaging film was measured by NIH software for den-
\sitometric analysis. Serum osteocalcin level was measured using an osteo-
\calcin RIA kit (Biomedical Technologies Inc.).

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\useful suggestions.

Materials and methods

DNA constructs

DNA constructs for VSMCs and osteoblast-specific expressions of Mgp
\transgenes were generated using a plasmid (p\5\5\4) containing Mgp ORF
\with a downstream SV40 polyadenylation signal. To generate pSM22a-
\Mgp, a 3-kb SM22a promoter fragment was inserted at a Xhol site up-
\stream to the Mgp ORF in p\5\5\4. Similarly, to generate p\1(I)Col-Mgp, a
\3.3-kb a\1(I) collagen promoter fragment was inserted upstream to the Mgp
\ORF. p\1(I)Col-Mgpm1 with three g\l\-encoding codons mutated to Asp
\encoding codons was generated by PCR mutagenesis. The mutated am-
\pilo{p}licons were digested by NcoI to replace the NcoI fragment of Mgp ORF
\in p\5\5\4 and was followed by the insertion of a 2.3-kb a\1(I) collagen pro-
\moter at an upstream Xhol site. To generate p\1(I)Col-Mgpm2, the re-
\maining g\l\ encoding codon in p\1(I)Col-Mgpm1 construct was mutated

demonstrate that inhibition of ECM by proteins is ex-
tected locally and not systemically. They also uncover great
\functional disparity between proteins sharing identical func-
tional motifs thought to be pivotal in controlling ECM.

Indeed, our mutagenesis experiments establish that the g\l\ residues are required for MGP inhibition of ECM func-
tion, yet g\l\ residue-containing osteocalcin cannot inhibit
\ECM. This observation again underscores the importance
\of in vivo tests for protein function even when their struc-
ture might suggest an obvious function. The observation that
\MGP can inhibit ECM in arteries and bone suggests that
\the function of inhibitors of ECM does not overly
\depend on the composition of a given ECM but rather de-
\pends on the expression of these inhibitors. The similarity
\of function of MGP in bone and in preventing arterial ECM
\implies also that if one can elucidate the molecular bases for
\the spatial restriction of ECM to bone then this informa-
tion could possibly be used to prevent ectopic mineralization
\in diseases of arteries and joints.

Figure 5. Osteocalcin does not share antimineralization function of MGP.
(A) Radioimmuno-assay shows a six- to eightfold increase of osteocalcin serum level in SM22a-Osteocalcin mice in comparison to their WT littermates. The error bars represent SDs of five independent measurements. Skeletal preparation of the thoracic aorta (B) and von Kossa staining of aorta sections from a 4-wk-old Mgp\1/1 mice. (C) mouse show mineral deposition as seen in Mgp\1/1 mice. (D) von Kossa and von Gieson staining of a vertebra section show normal bone mineralization in 4-wk-old a\1(I)Col-
\Osteocalcin mice.

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