Matrix GLA Protein, a Regulatory Protein for Bone Morphogenetic Protein-2*

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Matrix GLA protein (MGP) has been identified as a calciumification inhibitor in cartilage and vasculature. Part of this effect may be attributed to its influence on osteoinductive activity of bone morphogenetic protein-2 (BMP-2). To detect binding between MGP and BMP-2, we performed immunoprecipitation using MGP and BMP-2 tagged with FLAG and c-Myc. The results showed coprecipitation of BMP-2 with MGP. To quantify the effect of MGP on BMP-2 activity, we assayed for alkaline phosphatase activity and showed a dose-dependent effect. Low levels of MGP relative to BMP-2 (<1-fold excess) resulted in mild enhancement of osteoinduction, whereas intermediate levels (1–15-fold excess) resulted in strong inhibition. High levels of MGP (>15-fold excess), however, resulted in pronounced enhancement of the osteoinductive effect of BMP-2. Cross-linking studies showed that inhibitory levels of MGP abolished BMP-2 receptor binding. Immunoblotting showed a corresponding decrease in activation of Smad1, part of the BMP signaling system. Enhancing levels of MGP resulted in increased Smad1 activation. To determine the cellular localization of BMP-2 in the presence of MGP, binding assays were performed on whole cells and cells-synthesized matrix. Inhibitory levels of MGP yielded increased matrix binding of BMP-2, suggesting that MGP inhibits BMP-2 in part via matrix association. These results suggest that MGP is a BMP-2 regulatory protein.

MGP† is a small matrix protein that was initially isolated from bone and characterized by Price and Williamson (1). MGP deficiency in mice results in premature calcification in bone, calcification of normally noncalcifying cartilage, such as the trachea, and severe vascular calcification leading to premature death (2). Thus, MGP functions as a calcification inhibitor; however, its molecular mechanism is incompletely understood.

MGP appears to play a role in cell differentiation. In the artery wall of the MGP knockout mouse, medial smooth muscle cells are replaced by chondrocyte-like cells undergoing endochondral ossification, and in the growth plate of growing bones, hypertrophic chondrocytes are lacking (2). Further support for an effect of MGP on cell differentiation comes from the work of Yagami et al. (3), who show that overexpression of MGP in developing limbs delays chondrocyte maturation and blocks endochondral ossification. In addition, MGP inactivation triggers mineralization in cultured hypertrophic chondrocytes but not in immature chondrocytes. This is consistent with recent data from Newman et al. (4) demonstrating that overexpression of MGP in hypertrophic chondrocytes reduces mineralization. These authors also show that MGP expression is biphasic and stage-specific during chondrocyte differentiation and that MGP has an effect on chondrocyte viability. Increased expression of MGP induces apoptosis in maturing chondrocytes, whereas decreased expression induces apoptosis in proliferative and hypertrophic chondrocytes.

Previous studies from our laboratory using the multipotent cell line C3H10T1/2 cells demonstrate that MGP inhibits differentiation induced by BMP-2 (5). BMP-2 belongs to the TGF-β superfamily of growth factors, and it is a potent inducer of bone and cartilage (6). When initially purified from bone, it was observed to be strongly associated with MGP, and strong denaturants were required to separate the two proteins (7, 8). More recently, Wallin et al. (9), have shown binding between MGP and BMP-2 using ligand blotting. If such a binding also occurs in cellular systems or in vivo, it may at least partly explain MGP’s function as a calcification inhibitor.

In the present study, we further explore the relationship between MGP and BMP-2. We show that the effect of MGP on BMP-2 differentiation is dose-dependent. In addition, we show that MGP is able to bind BMP-2 and promote an association between BMP-2 and matrix components. Interaction between MGP and BMP-2 interferes with binding of BMP-2 to its receptor and activation of Smad1, part of the BMP signaling system.

MATERIALS AND METHODS

Cell Culture—M2-10B4 (M2) mouse marrow stromal cells were obtained from American Tissue Culture Collection and cultured in RPMI 1640 (Irvine Scientific). The cells were split at 70–80% of confluency. HEK293 human embryonic kidney cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Irvine Scientific). Both media were supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories), penicillin (100 units/ml), streptomycin (100 units/ml), sodium pyruvate (1 mM), and L-glutamine (2 mM).

Transfections were performed using SuperFect™ or Polyfect™ (Qiagen), after optimization of the ratio of DNA to transfection agent as per manufacturer’s instructions.

Conditioned Media—Conditioned media containing BMP-2, Myc-tagged BMP-2, MGP, or FLAG-tagged MGP were prepared from HEK293 cells transfected with 16 μg of the respective plasmid per 100-mm culture dish as previously described (5). 5 or 10 ml of conditioned medium was collected and filtered after 48 h of incubation starting after transfection. Control medium was collected from HEK293 cells transfected with empty vector.

Vector Construction—To construct the Myc-tagged BMP-2 vector, a Myc tag was introduced upstream of the first cysteine of the mature

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‡ The abbreviations used are: MGP, matrix GLA protein; BMP-2, bone morphogenetic protein-2; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid.
N-terminal domain of BMP-2. Four oligonucleotides were synthesized, which together created a dsDNA fragment that included (in 5’ to 3’ order): two random nucleotides, an EcoRI site, the signal sequence of MGP (corresponding to MKSLILLAILAALAVVTLC, Ref. 10), the c-Myc tag (corresponding to EQKISLISEEL), 11 nucleotides corresponding to nucleotide sequence between nucleotide 1208 (BMP-2 sequence, Ref. 11) and the 3’ insertion point for the BMP-2 cDNA in that vector. The 150- and 300-bp fragments were subsequently inserted into pCDNA3.1(+)/Neo (Invitrogen). The construct was confirmed by restriction enzyme analysis and DNA sequencing of the complete coding sequence.

**Immunoblotting**—For detection of BMP-2 and Myc-BMP-2 by immunoblotting, either 30 µl of culture medium or protein extracted from immunoprecipitation beads were electrophoresed through 10% NuPAGE™ Bis-Tris (MES) gels. Proteins were transferred to a nitrocellulose filter using NuPAGE™ transfer buffer (Invitrogen). The blots were incubated with specific antibodies (1 µg/ml) to either BMP-2 (polyclonal antibody H-51, Santa Cruz Biotechnology) or the c-Myc tag (Santa Cruz Biotechnology). Specific antibody binding was visualized with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibody (Santa Cruz Biotechnology).

To estimate the concentration of MGP-FLAG, 0.5–4 ng of C-terminally FLAG-tagged bacterial alkaline phosphatase standard protein (Sigma) was electrophoresed and transferred as described above, in parallel with 1–2 µl aliquots of conditioned media containing MGP-FLAG. The blots were incubated with anti-FLAG M2 monoclonal antibody horseradish peroxidase-conjugate (Sigma), diluted 1:2,500, and specific antibody binding was visualized. Densitometry using NIH Image J, version 1.08i (public domain program, Internet address: rsb.info.nih.gov/nih-image) was performed to compare the amount of FLAG in the samples to that in the standards. The difference in calculated molecular weight between the two proteins, 49.4 kDa for the standard protein and 11.6 kDa for MGP-FLAG, was taken into account when estimating the concentration of MGP-FLAG in ng/ml.

**Alkaline Phosphatase Assay**—M2 cells were plated in triplicate in 96-well plates at 10,000 cells per well. 16 h later, 200 µl of conditioned medium containing the indicated combinations of BMP-2 and MGP, ascorbic acid (50 µg/ml), and β-glycerophosphate (3 mM) were applied to the cultures. The alkaline phosphatase activity was determined 48 h later. The cells were washed once with phosphate-buffered saline and lysed by freeze-thawing twice in 50 µl of 0.2% Nonidet P-40 with 1 mM MgCl2. Cell lysates were assayed for alkaline phosphatase activity by mixing 150 µl of alkaline phosphatase buffer (40 mg of Sigma 104 alkaline phosphatase substrate dissolved in 20 ml of Sigma 221 alkaline buffer solution diluted 1.2 with distilled water) per well and incubating for 2 h at 37 °C. The proteins were analyzed on 8% SDS-PAGE, and immunoblotting was performed using anti-phosphorylated Smad1(P) antibodies (Upstate Biotechnology) at a concentration of 1 µg/ml. Specific antibody binding was visualized with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibody (Santa Cruz Biotechnology).

**Immunoprecipitation of Myc-BMP-2 and MGP-FLAG**—Two aliquots of equal volume (~3 ml) of conditioned media containing MGP-FLAG and Myc-BMP-2, respectively, were mixed and incubated at room temperature for 30 min with gentle agitation. A 15% (v/v) gentle overnight pre-incubation with agarose-conjugated nonspecific rabbit antibodies, 1 µg of anti-FLAG antibody (polyclonal antibody F7425, Sigma), was added, and incubation continued overnight at 4 °C. The following day, 25 µl of protein G-agarose (Santa Cruz Biotechnology) was added, and incubation continued for 1–2 h at room temperature. The protein G-agarose was spun down and washed 12–15 times with DMEM with 25 mM HEPS, pH 7.5, and 2 mg/ml of bovine serum albumin (BSA), or six times with radioimmunoprecipitation buffer (RIPA) (phosphate-buffered saline with 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). The clearest results were obtained after extensive washing with DMEM; the number of washes was determined by the number of washes it took to remove all nonspecific binding to the agarose-conjugated nonspecific rabbit antibodies. Co-precipitated Myc-BMP-2 was detected by immunoblotting as described above.

**Affinity Labeling and Immunoprecipitation of BMP Receptors**—HEK293 cells were transfected with pCMV-BMPRI-HA (12), alone or in combination with pCMV5-BMPRI-His (12), or with pCMV5 empty vector. The vectors were graciously supplied by Dr. Joan Massague, Columbia University, New York.

Recombinant human BMP-2, obtained from R&D Systems, was radioactive labeled using IODOBEADS™ (Pierce). 3 µg of BMP-2 and 0.5 µCi of [125I]NaI were incubated with the IODOBEADS™ as per manufacturer’s instructions. Free [125I]NaI was separated from 125I-BMP-2 by dialysis against phosphate-buffered saline.

Whole cell binding and affinity labeling were performed using previous methods (11) (Table I). The BMP-2 protease inhibitor mixture (10 µl/ml Sigma, for mammalian cells) was added during the preincubation and binding procedures. Briefly, ~3 nm 125I-BMP-2 was preincubated for 30 min with or without 10-fold excess of MGP or MGP-FLAG in binding buffer (DMEM with 25 mM HEPS and 2 mg/ml of BSA) prior to binding to cell monolayers in 6-well plates for 3.5 h at 4 °C. After the incubation, the cells were washed three times in ice-cold binding buffer and twice in binding buffer without BSA. Cross-linking was performed using 0.26 mM dithiobisimidyl suberate in DMEM for 15 min at 4 °C. Cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 150 mM NaCl, 0.75% Triton X-100 and protease inhibitors mixture for 20 min at 4 °C. Extracts were clarified by centrifugation. After pre-clearance with agarose-conjugated nonspecific rabbit antibodies, 1 µg of anti-human BMP antibodies (HE908, Sigma) was added, and incubation continued overnight at 4 °C. The following day, 25 µl of protein G-agarose was added, and incubation continued for 1–2 h at room temperature. The protein G-agarose was washed three times with lysis buffer and three times with RIPA before the protein was extracted and analyzed with SDS-PAGE and autoradiography.

**Detection of Smad1 Activation**—M2 cells were plated the day before the experiment in 60-mm Petri dishes, at a density resulting in ~80–90% confluency on the day of the experiment. The cells were incubated with BMP-2 alone or in combination with excess MGP for 30 min at 37 °C. After incubation, the cells were scraped into 150 µl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 20 µl/ml of protease inhibitor mixture) and sonicated. The proteins were analyzed on 8% SDS-PAGE, and immunoblotting was performed using anti-phosphorylated Smad1(P) antibodies (Upstate Biotechnology) at a concentration of 1 µg/ml. Specific antibody binding was visualized with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibody.

**Preparation of Cell-synthesized Matrix (Dilute Alkaline Treatment)**—Cell-synthesized matrix was prepared as previously described (13). M2 cells were allowed to grow on tissue culture plastic for 3 days followed by detachment of the cell monolayer with 25 mM NH4OH. The monolayers were incubated with NH4OH for 10 min, followed by extensive washing with calcium and magnesium-free phosphate-buffered saline (CMF-PBS). The cells were inspected in the dishes to ensure complete cell removal. If cells remained after the initial incubation, the treatment was repeated.

**Whole Cell Binding Assay**—Assays for cellular binding of radioactively trace-labeled BMP-2 in the presence of increasing amounts of MGP were performed at 4 °C as previously described by Massagué (14) for identification of TGF-β receptors. M2 cells were plated in 12- or 24-well plates either 1 or 2 days prior to the assay, in both cases at a density resulting in 100% confluence 1 day after plating. ~1.2 nm radioactively trace-labeled BMP-2 per well was added in ice-cold binding buffer (DME with 25 mM HEPS, pH 7.5 and 2 mg/ml BSA), together with different amounts of conditioned media containing cold MGP or MGP-FLAG. The final concentration of MGP was between 0.05 and 18 nM (15-fold excess). Conditioned medium from sham-transfected cells were used as a control. The assay was incubated for 4 h at 4 °C and 120 rpm and was subsequently washed five times with ice-cold binding buffer. The receptor-bound BMP-2 was solubilized as described using a Triton X-100-containing buffer (1% (v/v) Triton X-100, 10% (v/v) glycerol, 25 mM HEPS, pH 7.5, 1 mg/ml BSA) for 40 min at 4 °C and 120 rpm. After removal of the Triton X-100-containing buffer, an equal amount of 5 nM guanidine hydrochloride in 0.1% Triton X-100, pH 8.0 was added. The plates were then incubated for 60 min at 37 °C and 120 rpm. 125I-BMP-2 was detected by gamma counting. At least three independent experiments for each time point were performed, each for MGP and MGP-FLAG. After cell removal with dilute alkaline treatment, assays for matrix binding were performed in the identical manner.

**Immunocytochemistry**—Cells were fixed in 4% paraformaldehyde for 30 min at room temperature. The fixed cells were performed as previously described (15). Rabbit polyclonal antibodies to MGP were graciously supplied by Dr. Reidar Wallin, Wake Forest University, NC.

**Statistics**—Data was analyzed for statistical significance by analysis of variance (ANOVA) with post-hoc Scheffe’s analysis, unless otherwise stated. The analyses were performed using StatView, version 4.51 (Abacus Concepts).
RESULTS

Tagged Proteins—We constructed a Myc-tagged BMP-2 (Myc-BMP-2), adding the Myc tag to the N terminus of the mature protein, similar to what previously has been described for BMP-4 (12). The Myc-BMP-2 was easily detected in conditioned medium by immunoblotting (Fig. 1) and retained 20–25% of the osteoinductive effect of native BMP-2 in M2 cells, as measured by alkaline phosphatase (data not shown). The FLAG-tagged form of human MGP (MGP-FLAG) used in the present study has been shown previously to have the same ratio. No difference was seen between the two methods. The concentration of MGP-FLAG in conditioned medium was estimated by using its ability to induce osteoblastic differentiation in M2 cells, as measured by alkaline phosphatase, an early osteoblastic marker.

Conditioned Media—To study the interaction between BMP-2 and MGP or MGP-FLAG, conditioned media were used as the source of the proteins. The conditioned media for the respective proteins were always prepared in parallel using equimolar amounts of plasmid. The transfection efficiency was monitored using β-galactosidase. The concentration of biologically active BMP-2 in conditioned medium was estimated by using its ability to induce osteoblastic differentiation in M2 cells, as measured by alkaline phosphatase, in comparison to purified BMP-2 standards (Fig. 1A). Immunoblotting was used to confirm the presence of BMP-2 in the conditioned medium (Fig. 1A).

The concentration of MGP-FLAG in conditioned medium was determined using a comparison with FLAG-tagged standard protein on immunoblots probed with anti-FLAG antibodies (Fig. 1B). Because there were no available MGP-antibodies suitable for immunoblotting, the biological activity of native MGP was compared with that of MGP-FLAG of known concentration in alkaline phosphatase assays using 10T1/2 cells (5) and M2 cells (see below). Known amounts of BMP-2 were combined with the same amounts of conditioned media for MGP-FLAG and native MGP, respectively. In all conditions, conditioned media containing MGP-FLAG and native MGP, respectively, that had been prepared in parallel showed similar activity. The results also showed that the concentration of MGP-FLAG corresponded to that of BMP-2 as well when conditioned media for the respective protein were prepared in parallel.

For experiments requiring a mix of BMP-2 and MGP (MGP-FLAG or MGP), medium containing both proteins were prepared either by mixing separately produced conditioned media at the appropriate ratio or by obtaining conditioned medium from cells co-transfected with the two vectors at the same ratio. No difference was seen between the two methods. In addition, we have taken into account that MGP has poor solubility in aqueous solutions, about 200 μg/ml (1), and adjusted transfections to keep BMP-2 and MGP concentrations to no more than 10 μg/ml.

Co-precipitation of Myc-BMP-2 and MGP-FLAG—To study whether MGP binds BMP-2, we performed immunoprecipitation studies. Conditioned media for BMP-2 and Myc-BMP-2 were mixed at a ratio of 1:1, a ratio where MGP significantly inhibited the effect of BMP. MGP-FLAG was immunoprecipitated using FLAG antibodies, and the immunoprecipitated proteins were analyzed by immunoblotting using anti-Myc antibodies. The results showed that Myc-BMP-2 co-precipitated with MGP-FLAG (Fig. 2). The results were confirmed for native BMP-2 using radioactively labeled BMP-2, which also co-precipitated with MGP-FLAG (data not shown).

Differential Effect of MGP on Osteoinduction of BMP-2 in M2 Cells—To quantify the effect of MGP on osteoinduction by BMP-2, we established a 96-well plate assay for alkaline phosphatase, an early osteoblastic marker, using M2 marrow stromal cells. Medium containing either BMP-2 (500 ng/ml) alone or in combination with MGP or MGP-FLAG at a molar ratio between 0.1 and 15 were prepared as described above. A differential effect on osteoinduction was seen when the ratio of MGP to BMP-2 was changed (Fig. 3). Molar ratios of MGP to BMP-2 of less than 1 consistently resulted in a small but reproducible increase in osteoinduction, whereas ratios between 1 and as much as 10–15 resulted in significantly decreased osteoinduction. For ratios between 10 and 15, a trend toward a second increase was seen.

Effect of MGP on Receptor Binding of BMP-2 and Smad1 Activation—To study whether MGP inhibits binding of BMP-2 to its receptor, we transiently transfected HEK 293 cells with hemagglutinin-tagged BMP receptor type IA (BMPRIA), alone or in combination with BMP receptor type II (BMPRII)
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Effect of MGP on Cellular Localization of BMP-2—To study the effect of MGP on cellular localization of BMP-2, we performed whole cell binding assays as described by Massague (14), using M2 cells. The binding assays were performed using radioactively trace-labeled BMP-2, with increasing amounts of cold MGP or MGP-FLAG or control medium. Cell membranes were solubilized with Triton X-100-containing buffer, while Triton X-100 insoluble cell and matrix components were solubilized with guanidine hydrochloride-containing buffer.

The results showed that the whole cell binding of BMP-2 increased significantly with increasing concentration of MGP, even though previous experiments had showed decreased osteoinduction with increasing MGP concentration. If the binding assay was performed within 24 h of plating of the cells, most of actively trace-labeled BMP-2 was subsequently added alone or in combination with 10-fold excess of MGP or MGP-FLAG. Cross-linking was performed followed by immunoprecipitation using anti-hemagglutinin antibodies, polyacrylamide gel electrophoresis, and autoradiography. The results showed that BMP-2 bound to the receptors, both after transfection with BMPRIA alone and after co-transfection with BMPRII (Fig. 4A, lanes 3 and 5). The binding decreased significantly when MGP was added to the medium (Fig. 4A, lanes 4 and 6).

We also tested whether MGP inhibited activation of Smad1, part of the intracellular signaling system for BMP-2 (16). We treated M2 cells with BMP-2 alone or in combination with 10-fold excess of MGP and studied the activation of Smad1 by immunoblotting, using antibodies recognizing the phosphorylated (activated) form of Smad1 (P-Smad1). The results showed that phosphorylation of Smad1 was inhibited by the addition of MGP to the medium (Fig. 4B) consistent with the decreased receptor binding. Together, these results suggest that MGP inhibits BMP-2 signaling by preventing receptor binding and subsequent Smad1 activation.

Effect of MGP on Osteoinductive Activity of BMP-2—To assess whether the results of the binding assays could be reproduced by cell-synthesized matrix alone, we removed the M2 cells after 3 days in culture using dilute alkali treatment and performed binding assays identical to those above. The results were similar to those in whole cells after 3 days of culture (Fig. 6A), with most of the labeled BMP-2 being detected in the Triton X-100-containing fraction (Fig. 5A). However, after 3 days of culture, most of the labeled BMP-2 was instead detected in the guanidine hydrochloride fraction (Fig. 5B).

The results suggest an increased association of BMP-2 to the extracellular matrix in the presence of MGP. Shortly after plating, the matrix is likely to remain immature and soluble in Triton X-100-containing buffers. With time, however, the matrix would be expected to mature and become less soluble in Triton X-100-containing buffers. Instead, it would require guanidine hydrochloride for solubilization. A large excess of MGP resulted in a trend for reduced binding of labeled BMP-2 (Fig. 5, A and B), possibly suggesting a limited number of sites for MGP-dependent association of BMP-2.

To assess whether the results of the binding assays could be reproduced by cell-synthesized matrix alone, we removed the M2 cells after 3 days in culture using dilute alkali treatment and performed binding assays identical to those above. The results were similar to those in whole cells after 3 days of culture (Fig. 6A), with most of the labeled BMP-2 being detected in the guanidine hydrochloride fraction. The BMP-2 content of the Triton X-100 fraction increased only slightly or not at all. Again, large excess resulted in reduced binding of labeled BMP-2 (Fig. 6A). For comparison, the binding assay was performed on plastic not previously used for cell culture. The results showed that labeled BMP-2 was found mostly in the guanidine hydrochloride-containing fraction, but decreased with increasing concentrations of MGP. This is consistent with previous observations that BMP-2 binds to plastic (14), a binding with which MGP may interfere. Taken together, these results support that there is an increased matrix association of BMP-2 in the presence of MGP.

The Effect of a Large Excess of MGP on Osteoinduction by
BMP-2—In the previous experiments, an excess of MGP to BMP-2, greater than 10–12-fold, resulted in both a slight increase in osteoinduction (Fig. 3) and a decrease in whole cell and matrix binding of labeled BMP-2 (Figs. 5 and 6). To further clarify the effect of an excess of MGP above 15-fold on osteoinduction of BMP-2 in M2 cells, we used the 96-well plate assay described above. We prepared media containing either BMP-2 (100 ng/ml) alone, or in combination with MGP or MGP-FLAG at an excess of 20-fold or more. Interestingly, the results showed that induction of alkaline phosphatase continued to increase to levels significantly above that for BMP-2 alone (Fig. 7A). In addition, immunoblotting showed an increase in phosphorylated (activated) Smad1, indicating an increased receptor activation (Fig. 7B). One possible explanation for this would be a large excess of MGP interfering with matrix association of BMP-2-MGP complexes. However, osteoinduction appears to be enhanced to levels beyond what would be expected with liberated BMP-2, possibly by a mechanism not yet identified.

**Immunocytochemistry for MGP in M2 Cells**—MGP is a component of the extracellular matrix. Previous studies have shown a possible association of MGP with RGD-containing proteins (17), and its low solubility in water-based buffers may be because of a self-aggregation of MGP monomers reminiscent of other proteins.
of that in organized bone matrix (1). Many cells in culture express MGP that has been suggested to aid in the attachment of cells to plastic (17). It is possible that exogenous MGP added to the cells would associate with endogenous MGP.

We used immunocytochemistry to visualize the amount of endogenous MGP in M2 cells after 1 or 3 days in culture. The results showed that within 24 h of plating MGP was primarily intracellular, and the nuclei were well visualized (Fig. 8A). However, after 3 days, MGP accumulated extracellularly and became part of a sheet of matrix covering the cells (Fig. 8B). This parallels the increase of BMP-2 associated with matrix components in the presence of exogenous MGP, seen after 3 days in culture.

**DISCUSSION**

MGP is a small matrix protein, initially isolated from bone and characterized by Price and Williamson (1). It is not known how MGP is bound or incorporated into the extracellular matrix; however, high levels of MGP in nonmineralized cartilage indicates that MGP does not require hydroxyapatite as an anchor (1, 18). Despite a low content of hydrophobic amino acids (1), MGP has low solubility in water-based buffers, possibly reflecting a specific aggregation of monomers reminiscent of the structure in organized bone or cartilage matrix.

This study provides evidence that MGP is a regulatory protein for BMP-2. MGP has a dose-dependent effect on BMP-2-induced osteoblastic differentiation in M2 cells. Low relative levels of MGP to BMP-2 (less than 1-fold) result in mild enhancement of BMP-2 activity. Intermediate levels (1–15-fold) result in inhibition, and high levels (above 15-fold) result in strong enhancement of BMP-2 activity.

Immunoprecipitation results demonstrating co-precipitation of BMP-2 with MGP suggest that the modulating effect involves direct binding of MGP to BMP-2. This is consistent with earlier studies that showed BMP-2 to be tightly associated with MGP during protein purification (7, 8) and to bind to membrane-fixed MGP in ligand-blotting experiments (9). It is not clear from our experiments whether BMP-2 can still exert an effect when bound to MGP. The promoting effect of low levels of MGP on BMP-2 activity may be similar to the facilitating effect of betaglycan on TGF-β receptor binding (20).

Binding of BMP-2 to its receptor, as well as Smad1 activation, is decreased in the presence of intermediate levels of MPG, consistent with the inhibitory effect on osteoinduction in M2 cells. However, overall binding of BMP-2 to whole cells or matrix is increased and inversely correlated with the inhibitory effect. This suggests that MGP promotes association of BMP-2 to the extracellular matrix.

When the levels of MGP exceeded 12–15-fold relative to...
BMP-2, there was a trend to increased osteoinduction in the alkaline phosphatase assay and to decreased binding of BMP-2 to whole cells and matrix. In the experiments designed to study large excesses of MGP (20–80-fold) relative to BMP-2, we found a strong enhancement of the osteoinductive effect of BMP-2 and a corresponding activation of Smad1. One possible explanation for this phenomenon may be saturation of association sites. However, the level of osteoinduction far exceeds the one by BMP-2 alone, suggesting an additional mechanism not yet identified.

The variable effect of MGP, depending on its ratio to BMP-2, may explain the apparently contradictory observations in vascular calcification. On one hand, MGP knockout mice develop profound vascular calcification (2), whereas on the other hand, calcifying atherosclerotic lesions show an increased expression of MGP (19).

In the MGP knockout mouse, decreased control of BMP-2 may explain the effects of MGP-deficiency in the artery wall and also in the growth plate. In the developing aorta, BMP-2 is strongly expressed in the primitive endothelium as SMC precursor cells are recruited to form the media (21). It is possible that MGP, which is expressed throughout the embryonic aorta, interferes with BMP-2 at a certain stage inhibiting osteogenic and chondrogenic differentiation of precursor cells. In the growth plate, both MGP and BMP-2 have distinct patterns of expression (22, 23). Alterations in this pattern may contribute to the lack of hypertrophic chondrocytes seen in MGP knockout mice (2). Both BMP-2 and MGP are expressed at increased levels in calcified atherosclerotic vessels (19, 24), BMP-2 as a possible promoter and MGP as a possible inhibitor of calcification. However, interactions between the two proteins may also be important in determining vascular cell differentiation and calcification.

Our results are consistent with the previous observations that both BMP-2 and MGP are present in bone matrix and are closely associated during protein purification (7, 8). It has also been suggested that BMP-2 is liberated from matrix in areas of bone fracture (25), supporting healing and new bone formation in the fracture area. Interestingly, osteoblasts in nonhealing fractures were positive for MGP expression, whereas osteoblasts in normally healing fractures were not (26). It is possible that MGP interferes with BMP-induced bone formation.

Several BMP-binding proteins have previously been identified, including Noggin (12), Chordin (27), Cerberus (28), Follistatin (29), and Twisted (30). The majority have been identified as BMP-4 antagonists and play crucial roles in the development of Xenopus and mice. In addition, type IIA procollagen containing the cysteine-rich NH₂-propeptide, deposited in extracellular matrix of preochondrogenic tissue, has been shown to bind BMP-2 and the related growth factor TGF-β1 (31). Matrix binding of BMP may be a relatively common way to limit the activity of BMP-2 to critical time periods in development or healing.

In summary, our results demonstrate that MGP may act as a regulator of BMP-2. Its effect is dose-dependent and appears to involve direct binding between the proteins as well as a matrix association.

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