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MEPE's Diverse Effects on Mineralization

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Abstract

Matrix extracellular phosphoglycoprotein (MEPE) is an inhibitor of mineralization in situ and in cell cultures where altered expression is associated with oncogenic osteomalacia and hypophosphatemic rickets. The purpose of this study was to determine whether the intact protein or the peptide(s) originating from this protein was responsible for the inhibition. The ability of the intact protein and the acidic, serine- and aspartate-rich MEPE-associated motif (ASARM) peptide to promote or inhibit de novo hydroxyapatite formation and growth of hydroxyapatite seed crystals, in both phosphorylated and dephosphorylated forms, was assessed at room temperature in a dynamic gel diffusion system at 3.5 and 5 days. The most effective nucleator concentration was also examined when associated with fibrillar type I collagen. The phosphorylated intact protein was an effective promoter of mineralization in the gelatin gel diffusion system, while the ASARM peptide was an effective inhibitor. When dephosphorylated both the intact protein and the ASARM peptide had no effect on mineralization. Associated with collagen fibrils, some of the effect of the intact protein was lost. This study demonstrates the importance of posttranslational modification for the site-specific activity of MEPE and its ASARM peptide.

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The authors have stated that they have no conflicts of interest.

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Keywords

Matrix extracellular phosphoglycoprotein; Mineralization; Posttranslational modification; ASARM peptide

Matrix extracellular phosphoglycoprotein (MEPE or OF45) was first cloned as a candidate substrate for PHEX (phosphaturic hormone with endopeptidase activity on the X chromosome) [1]. MEPE is a member of the SIBLING (small integrin binding ligand *N*-glycosylated) family of extracellular matrix proteins and, similar to the other SIBLING proteins, is believed to be multifunctional, playing roles in cell signaling, mineral homeostasis, and mineralization. In situ MEPE exists as smaller peptides, including the C-terminal ASARM (acidic, serine-, and aspartic acid-rich MEPE motif) peptide, which is analogous to the C terminus of the salivary protein statherin [3]. In bone, MEPE is mainly expressed by osteocytes [4].

Based on studies in mice in which MEPE was ablated [5] leading to enhancement of bone formation, MEPE is believed to inhibit mineralization. This was confirmed in cell culture experiments [6,7] as well as in cell-free solution studies [8]. In the cell-free studies, it was the ASARM peptide, a substrate and ligand for PHEX, that provided the inhibition [6,8,9,10]. Similar to the other SIBLING proteins, we hypothesized that posttranslational modification of MEPE would alter its effects on mineralization and that the intact protein would have a distinct effect compared to the peptides. Thus, we tested the intact protein and the ASARM peptide in the dynamic gelatin gel system [11] to validate this hypothesis. We also investigated whether phosphorylation and dephosphorylation would alter the effects of these proteins and peptides on the mineralization process.

Materials and Methods

Preparation of MEPE and MEPE Fragments

MEPE protein was generated by expression in insect cells using the sf9 system, as previously described [6]. ASARM peptides (phosphorylated and without phosphate) were synthesized and purchased from Neo-MPS (now called PolyPeptide Laboratories, San Diego, CA). Dephosphorylation of the intact protein in Tris buffer was performed with alkaline phosphatase-agarose beads (Sigma, St. Louis, MO) by incubating overnight at room temperature.

The Gel System

Hydroxyapatite formation and growth were monitored in the dynamic collagen gel hydroxyapatite growth system [10]. In this double diffusion system, Ca^{2+} and HPO_4^{2-} (Pi) ions circulate at room temperature and diffuse into opposite ends of a 6-cm-long 10% gelatin gel (Bloom 275; Fisher Chemicals, Fair Lawn, NJ). The pH of the 10% gelatin solution, prepared in tris-(hydroxymethyl)amino-methane (Tris) buffer by stirring at 50°C, was adjusted to 7.40 with 0.1 N NaOH. At a site 3.4 cm from the Pi entrance point, the gel included a 100-μL band containing the protein/peptide to be tested. This band consisted of a specified concentration of MEPE or ASARM peptide, in 0.15 M pH Tris. The rest of the 100-μL band was made of 20% gelatin so that the final concentration in the band was 10%. The experimental gels contained a range of 0–50 μg/mL protein or peptide. Once this gel had hardened (~30 min in a cold room), it was covered without any interface with the 10% gelatin. This provided a “sandwich” of blank gel, with the protein in the experimental tubes and without the protein in the control tubes. All experiments were performed at ambient temperatures.

For the de novo formation and growth studies, Ca and Pi accumulation in the precipitant band containing Tris buffer was compared to experimental gels with ASARM or MEPE at 3.5 and

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5.0 days. Identical "single-diffusion" gels, into which only Ca or Pi diffused, served as additional controls to correct for ion accretion due only to diffusion or to bonding of the ion to the protein or peptide. Control tubes and experimental tubes were run parallel to each other. Calcium and phosphate solutions were circulated at a continuous rate under nitrogen pressure, in separate loops at opposite ends of the gel. The calcium and phosphate solutions were maintained at 2 L, providing an "infinite reservoir" relative to the 3 mL contents of the 18 gels used for each experiment. The system was not thermostated; however, the ambient temperature around the gels was monitored, and experiments were not done if the temperature was outside the range of 20–25°C. Physiological temperatures could not be used since the gelatin began to melt at 35°C. After 3.5 days, a thin opaque band (precipitant band) formed perpendicular to the direction of the flow and either the precipitant band was cut out for mineral analysis (X-ray diffraction) or the entire gel was cut into slices (0.30 mL vol) and the Ca [12] and Pi [13] contents of each slice were determined following hydrolysis in 1 N HCl. Values in single-diffusion tubes were subtracted from those from double-diffusion tubes, as described previously [11].

For measurement of seeded growth, the protein or peptide was incubated overnight in 100 μL Tris with 0.5 mg hydroxyapatite, and the entire 100 μl was transferred to the position of the precipitant band. Controls containing 0.5 mg hydroxyapatite without protein or peptide were used. Analyses were performed, as above, at 5.0 days.

For studies of the interaction with fibrillar collagen, the most effective MEPE concentration (25 μg/mL) was mixed with fibrillar type I collagen (BD Biosciences, San Jose, CA), which was slowly neutralized with 0.1 N NaOH. MEPE was not added to the solution until the first signs of fibril formation were visible. After addition of MEPE, the pH was readjusted to 7.4. The control for this experiment contained only neutralized fibrillar collagen without MEPE. MEPE and the collagen fibrils or the collagen fibrils alone were incubated overnight at 37°C prior to suspension in the 100-μl band of the gelatin gel. The fibrillar collagen + MEPE was compared to fibrillar collagen alone after 5.0 days, as described above.

Statistical Analysis

To account for any time or temperature fluctuations during the course of analyses, all data were normalized to the controls run at the same time and expressed as experimental/control. Mean values for each set of concentrations were calculated for each experimental/control data set, and these were compared to 1.0 using the Dunnett test (GraphPad Instat2, La Jolla, CA). Data were excluded if the Ca/Pi ratio in the experimental or control tubes differed from 1.7 by >15%. In that case, additional experiments were added so that for some concentrations $n = 6$ and for others $n = 3$. Differences with $P < 0.05$ were considered significant. Where only single concentrations were tested, a one-sided *t*-test was used, assuming the samples represented a gaussian distribution of variables.

Results

The ASARM peptide inhibited both de novo hydroxyapatite formation and apatite-seeded growth in a dose-dependent fashion (Fig. 1a); this inhibition was lost when the peptide was dephosphorylated (Fig. 1b). The mineral formed in all cases was shown by X-ray diffraction to be a poorly crystalline hydroxyapatite (data not shown), with Ca/Pi concentrations in the experimental and control precipitant bands being 1.63 ± 0.20, and 1.56 ± 0.16, respectively.

In contrast, at low concentrations, the intact MEPE promoted hydroxyapatite formation and growth (Fig. 2a), although at higher concentrations it behaved like the ASARM peptide and was an inhibitor. The Ca/Pi concentrations in the experimental and control precipitant bands were 1.78 ± 0.27 and 1.64 ± 0.08, respectively. Similar to the ASARM peptides, once

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dephosphorylated there was no significant effect of the intact protein on mineralization (Fig. 2b).

When 25 μg/mL MEPE was combined with fibrillar collagen, to mimic what might occur in bone or dentin, much of its potential to promote hydroxyapatite formation was lost; however, the increased yield of mineral was still statistically significant. There was a difference between the yield with MEPE alone and MEPE + fibrillar collagen for Ca uptake, but phosphate uptake showed more scatter and did not differ significantly (Fig. 3).

Discussion

Our study demonstrated that, similar to the other SIBLING proteins, MEPE's mineralizing activity is a function of its state of phosphorylation and of the extent of cleavage. This is in agreement with other SIBLING proteins, osteopontin, dentin matrix protein 1, and bone sialoprotein (Table 1), which have different activities depending on their extent of cleavage and extent of phosphorylation, although in some cases the intact protein is the inhibitor and the cleaved protein is the promoter of mineralization.

It is interesting to note that recently MEPE with the ASARM peptide deleted was found to promote bone formation in culture and in mice [14]; similarly, a midterminal fragment of MEPE has been found to enhance cell binding [15] and pulp repair [16]. Thus, distinct components of MEPE appear to have different effects on mineralized tissue formation and signaling processes. It is also important to note that in dentin pulp cells and in osteoblasts MEPE expression decreases as the cells differentiate [17,18].

MEPE's interaction with collagen, at the concentration tested, slightly diminished its effect as a promoter of mineralization. This is in contrast to another SIBLING nucleator, bone sialoprotein (BSP), which has enhanced activity when bound to collagen [19]. Although this might be due to different methods of binding the protein to the collagen fibrils, it is likely that it reflects the relative importance of the interaction of BSP and MEPE with collagen.

Because MEPE was cloned as the substrate for PHEX [1], it is useful to compare the findings of the present study and the account of Addison et al. [8] for the hypophosphatemic phenotype in mice lacking PHEX. Cathepsin B is proposed to be a candidate protease for MEPE cleavage in bone [20]. A targeted "bone-specific" cathepsin B null mouse is required to resolve this hypothesis. However, one can explain the phenotype of the MEPE knockout and MEPE transgenics as well as the hypophosphatemic mouse based on the finding that the intact protein is a nucleator and the cleaved peptide an inhibitor. When MEPE is present, it and the other SIBLING proteins regulate initial mineral deposition in the osteoid and predentin of the mineralizing tissues. Their activities are regulated by phosphorylation, dephosphorylation, and, of course, cleavage. Once cleaved, the ASARM peptide is protected from binding to the mineral via its interaction with MEPE [8].

In hypophosphatemic rickets, when PHEX is absent, the ASARM peptides bind to the mineral and inhibit further mineralization. In the MEPE knockout the enhanced mineralization most likely results from the absence of the PHEX–ASARM interaction, while in the MEPE transgenic the increased release and accumulation of the ASARM peptide results in decreased mineralization [20].

While this study supports the hypothesis that posttranslational modifications of the SIBLING proteins determine their tissue-specific effects [21], it was limited in the following ways. First, we did not have sufficient intact protein to do assays at all concentrations, especially in the presence of fibrillar collagen. Second, we looked at only two time points (3.5 and 5.0 days), preventing discovery of what would have happened at shorter and longer times. Finally, this

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was a solution-based study, where the concentrations of the other SIBLING proteins did not vary, as they do in the knockout and transgenic. While results are thus easier to interpret, a caveat must be that in the animal the interactions of all these SIBLING proteins are linked in some way.

Acknowledgments

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References

1. Rowe PS, de Zoysa PA, Dong R, Wang HR, White KE, Econs MJ, Oudet CL. MEPE, a new gene expressed in bone marrow and tumors causing osteomalacia. Genomics 2000;67:54–68.10.1006/geno.2000.6235 [PubMed: 10945470]
2. Argiro L, Desbarats M, Glorieux FH, Ecarot B. Mepe, the gene encoding a tumor-secreted protein in oncogenic hypophosphatemic osteomalacia, is expressed in bone. Genomics 2001;74:342–351.10.1006/geno.2001.6553 [PubMed: 11414762]
3. Huq NL, Cross KJ, Ung M, Reynolds EC. A review of protein structure and gene organisation for proteins associated with mineralised tissue and calcium phosphate stabilisation encoded on human chromosome 4. Arch Oral Biol 2005;50:599–609.10.1016/j.archoralbio.2004.12.009 [PubMed: 15892946]
4. Nampei A, Hashimoto J, Hayashida K, Tsuboi H, Shi K, Tsuji I, Miyashita H, Yamada T, Matsukawa N, Matsumoto M, Morimoto S, Ogihara T, Ochi T, Yoshikawa H. Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone. J Bone Miner Metab 2004;22:176–184.10.1007/s00774-003-0468-9 [PubMed: 15108058]
5. Gowen LC, Petersen DN, Mansolf AL, Qi H, Stock JL, Tkalcevic GT, Simmons HA, Crawford DT, Chidsey-Frink KL, Ke HZ, McNeish JD, Brown TA. Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. J Biol Chem 2003;278:1998–2007.10.1074/jbc.M203250200 [PubMed: 12421822]
6. Martin A, David V, Laurence JS, Schwarz PM, Lafer EM, Hedge AM, Rowe PS. Degradation of MEPE, DMP1, and release of SIBLING ASARM-peptides (minhibins): ASARM-peptide(s) are directly responsible for defective mineralization in HYP. Endocrinology 2008;149:1757–1772.10.1210/en.2007-1205 [PubMed: 18162525]
7. Rowe PS, Kumagai Y, Gutierrez G, Garrett IR, Blacher R, Rosen D, Cundy J, Navvab S, Chen D, Drezner MK, Quarles LD, Mundy GR. MEPE has the properties of an osteoblastic phosphatonin and minhibin. Bone 2004;34:303–319.10.1016/j.bone.2003.10.005 [PubMed: 14962809]
8. Addison WN, Nakano Y, Loisel T, Crine P, McKee MD. MEPE-ASARM peptides control extracellular matrix mineralization by binding to hydroxyapatite: an inhibition regulated by PHEX cleavage of ASARM. J Bone Miner Res 2008;23:1638–1649.10.1359/jbmr.080601 [PubMed: 18597632]
9. Rowe PS, Garrett IR, Schwarz PM, Carnes DL, Lafer EM, Mundy GR, Gutierrez GE. Surface plasmon resonance (SPR) confirms that MEPE binds to PHEX via the MEPE-AS-ARM motif: a model for impaired mineralization in X-linked rickets (HYP). Bone 2005;36:33–46.10.1016/j.bone.2004.09.015 [PubMed: 15664000]
10. Guo R, Rowe PS, Liu S, Simpson LG, Xiao ZS, Quarles LD. Inhibition of MEPE cleavage by Phex. Biochem Biophys Res Commun 2002;297:38–45. [PubMed: 12220505]
11. Silverman L, Boskey AL. Diffusion systems for evaluation of biomineralization. Calcif Tissue Int 2004;75:494–501.10.1007/s00223-004-0019-y [PubMed: 15455184]
12. Willis JB. Determination of metals in blood serum by atomic absorption spectroscopy I. Calcium. Spectrochim Acta 1980;16:259–272.
13. Heinonen JK, Lahti RJ. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphates. Anal Biochem 1981;113:313–317. [PubMed: 6116463]

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14. Sprowson AP, McCaskie AW, Birch MA. ASARM-truncated MEPE and AC-100 enhance osteogenesis by promoting osteoprogenitor adhesion. J Orthop Res 2008;26:1256–1262.10.1002/jor.20606 [PubMed: 18383145]
15. Hayashibara T, Hiraga T, Yi B, Nomizu M, Kumagai Y, Nishimura R, Yoneda T. A synthetic peptide fragment of human MEPE stimulates new bone formation in vitro and in vivo. J Bone Miner Res 2004;19:455–462.10.1359/JBMR.0301263 [PubMed: 15040834]
16. Six N, Septier D, Chaussain-Miller C, Blacher R, DenBesten P, Goldberg M. Dentonin, a MEPE fragment, initiates pulp-healing response to injury. J Dent Res 2007;86:780–785. [PubMed: 17652210]
17. Liu H, Li W, Shi S, Habelitz S, Gao C, Denbesten P. MEPE is downregulated as dental pulp stem cells differentiate. Arch Oral Biol 2005;50:923–928.10.1016/j.archoralbio.2005.03.003 [PubMed: 16183369]
18. Siggelkow H, Schmidt E, Hennies B, Hüfner M. Evidence of downregulation of matrix extracellular phosphoglycoprotein during terminal differentiation in human osteoblasts. Bone 2004;35:570–576.10.1016/j.bone.2004.03.033 [PubMed: 15268910]
19. Baht GS, Hunter GK, Goldberg HA. Bone sialoprotein–collagen interaction promotes hydroxyapatite nucleation. Matrix Biol 2008;27:600–608.10.1016/j.matbio.2008.06.004 [PubMed: 18620053]
20. David V, Martin A, Hedge A-M, Rowe PSN. MEPE is a new bone renal hormone and vascularization modulator. Endocrinology 2009;150:4012–4023.10.1210/en.2009-0216 [PubMed: 19520780]
21. Qin C, Baba O, Butler WT. Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. Crit Rev Oral Biol Med 2004;15:126–136. [PubMed: 15187031]
22. Boskey AL, Maresca M, Ullrich W, Doty SB, Butler WT, Prince CW. Osteopontin–hydroxyapatite interactions in vitro: inhibition of hydroxyapatite formation and growth in a gelatin-gel. Bone Miner 1993;22:147–159. [PubMed: 8251766]
23. Gericke A, Qin C, Spevak L, Fujimoto Y, Butler WT, Sørensen ES, Boskey A. Importance of phosphorylation for osteopontin regulation of biomineralization. Calcif Tissue Int 2005;77:45–54.10.1007/s00223-004-1288-1 [PubMed: 16007483]
24. Tartaix PH, Doulaverakis M, George A, Fisher LW, Butler WT, Qin C, Salih E, Tan M, Fujimoto Y, Spevak L, Boskey AL. In vitro effects of dentin matrix protein-1 on hydroxyapatite formation provide insights into in vivo functions. J Biol Chem 2004;279:18115–18120.10.1074/jbc.M314114200 [PubMed: 14769788]
25. Gajjeraman S, Narayanan K, Hao J, Qin C, George A. Matrix macromolecules in hard tissues control the nucleation and hierarchical assembly of hydroxyapatite. J Biol Chem 2007;282:1193–1204.10.1074/jbc.M604732200 [PubMed: 17052984]
26. Boskey AL, Maresca M, Doty S, Sabsay B, Veis A. Concentration-dependent effects of dentin phosphophoryn in the regulation of in vitro hydroxyapatite formation and growth. Bone Miner 1990;11:55–65. [PubMed: 2176557]
27. He G, Ramachandran A, Dahl T, George S, Schultz D, Cookson D, Veis A, George A. Phosphorylation of phosphophoryn is crucial for its function as a mediator of biomineralization. J Biol Chem 2005;280:33109–33114.10.1074/jbc.M500159200 [PubMed: 16046405]
28. Boskey A, Spevak L, Tan M, Doty SB, Butler WT. Dentin sialoprotein (DSP) has limited effects on in vitro apatite formation and growth. Calcif Tissue Int 2000;67:472–478. [PubMed: 11289697]
29. Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. Proc Natl Acad Sci USA 1993;90:8562–8565. [PubMed: 8397409]

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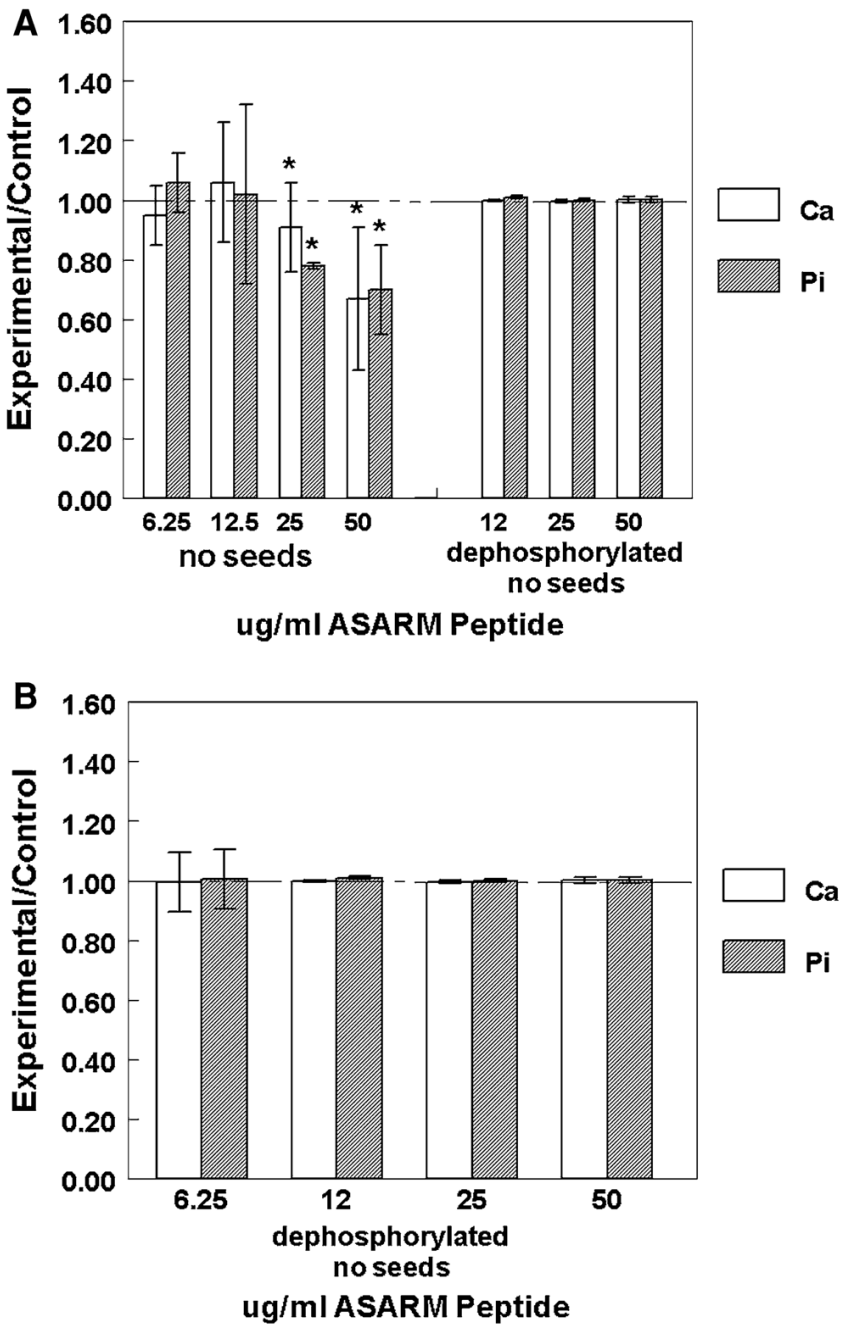

Fig. 1.

a Effect of the ASARM peptide on de novo formation (*left*) and seeded growth (*right*) of hydroxyapatite in the gelatin gel system at 5.0 days. **b** In the gelatin gel system, when dephosphorylated, the ASARM peptide has no detectable effect on seeded growth. Values (experimental/control) are mean (SD) for three to five determinations at each concentration and normalized to controls run at the same time. * $P < 0.05$ relative to control (*dotted line* = 1.00)

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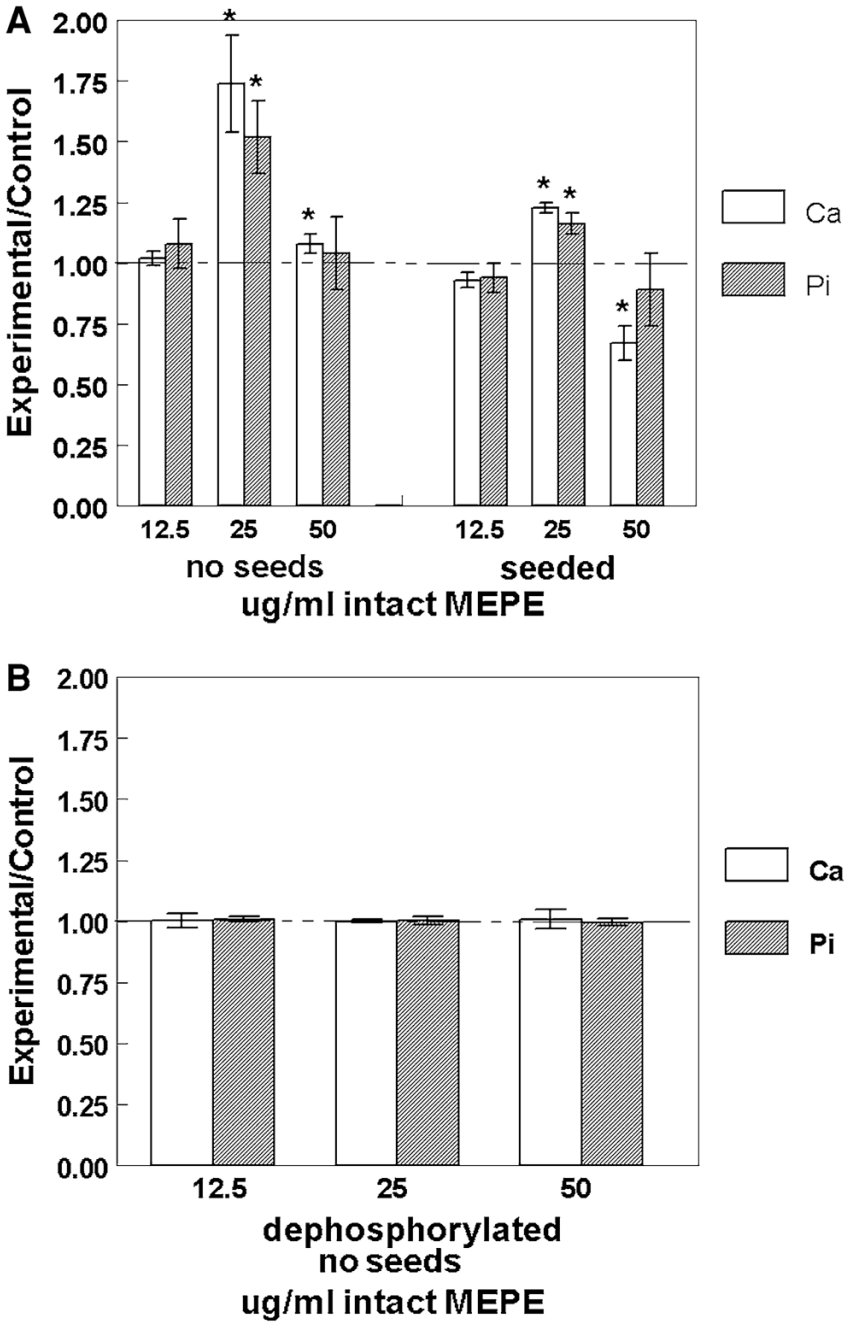

Fig. 2.

a There is a dose-dependent increase in calcium and phosphate accumulation in the gelatin gel system with increasing intact MEPE concentrations, with the observed maximum occurring at 25 μg/mL. With seeded growth, the 25 μg/mL concentration also stimulated mineral formation, but there was a statistically significant decrease at 50 μg/mL. **b** When dephosphorylated, intact MEPE has no effect on mineral accumulation. Values (experimental/control) are mean (SD) at 5 days for three to five determinations at each concentration and normalized to controls run at the same time. * $P < 0.05$ relative to control (*dotted line* = 1.00)

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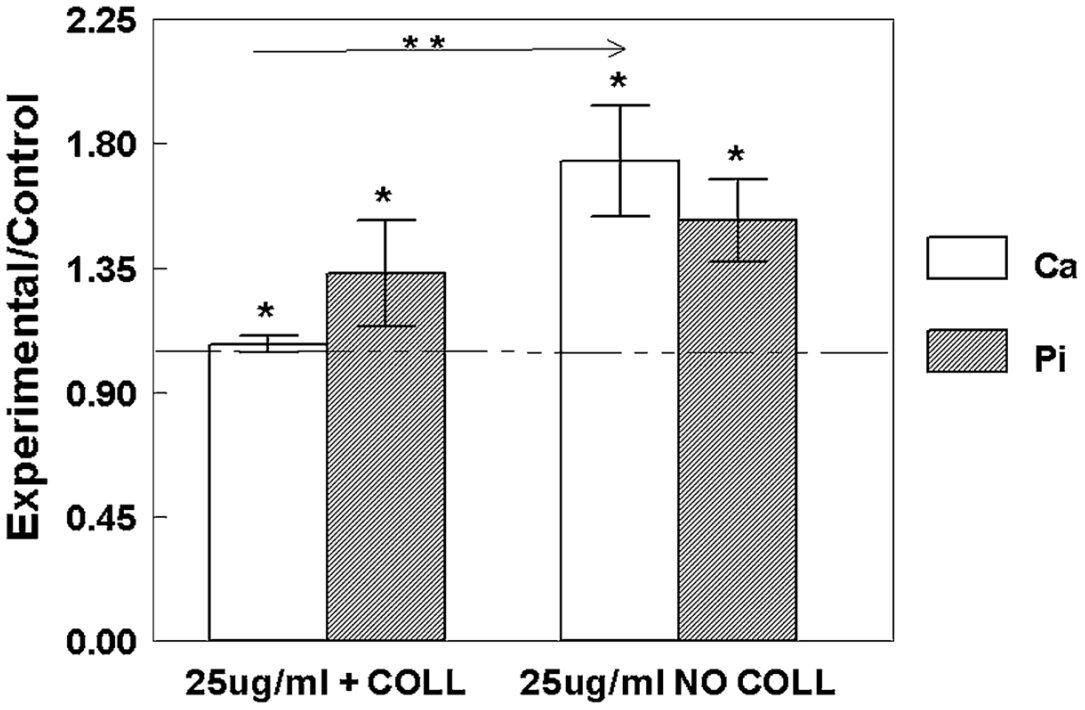

Fig. 3.

When collagen fibrils were coated with intact MEPE, there was a slight increase in the mineral yield relative to control (* $P < 0.05$), but the yield in terms of calcium uptake was significantly less (** $P < 0.05$) than that due to 25 μg/mL MEPE in the absence of fibrillar collagen. Values (experimental/control) are mean (SD) for three to five determinations at each concentration at day 5 and normalized to controls run at the same time. * $P < 0.05$ relative to control, ** $P < 0.05$ vs. group indicated by *arrow*

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Table 1

Effects of SIBLING proteins and their fragments and dephosphorylated (De-P) forms on hydroxyapatite formation based on solution data

Protein	Effect of intact protein	Effect of protein fragments
OPN		
Bone [22]	Inhibitor	?
Milk [23]	Promoter	?
DeP-OPN [22,23]	No effect	?
DMP1	Inhibitor [24] Nucleator [25]	Promoter
deP-DMP1 [24,25]	No effect	
DPP + DSP [26–28]	Promoter	Promoter
DeP DPP/DSP [27]	No effect	No effect
BSP [29]	Promoter	?
DeP-BSP	No effect	?
MEPE[a]	Promoter	Inhibitor
DeP-MEPE[a]	No effect	No effect

[a]This report