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MEPE has the properties of an osteoblastic phosphatonin and minihibin

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Abstract

Matrix extracellular phosphoglycoprotein (MEPE) is expressed exclusively in osteoblasts, osteocytes and odontoblasts with markedly elevated expression found in X-linked hypophosphatemic rickets (Hyp) osteoblasts and in oncogenic hypophosphatemic osteomalacia (OHO) tumors. Because these syndromes are associated with abnormalities in mineralization and renal phosphate excretion, we examined the effects of insect-expressed full-length human-MEPE (Hu-MEPE) on serum and urinary phosphate in vivo, ³³PO₄ uptake in renal proximal tubule cultures and mineralization of osteoblast cultures. Dose-dependent hypophosphatemia and hyperphosphaturia occurred in mice following intraperitoneal (IP) administration of Hu-MEPE (up to 400 µg kg⁻¹ 31 h⁻¹), similar to mice given the phosphaturic hormone PTH (80 µg kg⁻¹ 31 h⁻¹). Also the fractional excretion of phosphate (FEP) was stimulated by MEPE [65.0% (*P* < 0.001)] and PTH groups [53.3% (*P* < 0.001)] relative to the vehicle group [28.7% (SEM 3.97)]. In addition, Hu-MEPE significantly inhibited ³³PO₄ uptake in primary human proximal tubule renal cells (RPTEC) and a human renal cell line (Hu-CL8) in vitro (*V*_{max} 53.4% inhibition; *K*_m 27.4 ng/ml, and *V*_{max} 9.1% inhibition; *K*_m 23.8 ng/ml, respectively). Moreover, Hu-MEPE dose dependently (50–800 ng/ml) inhibited BMP2-mediated mineralization of a murine osteoblast cell line (2T3) in vitro. Inhibition of mineralization was localized to a small (2 kDa) cathepsin B released carboxy-terminal MEPE peptide (protease-resistant) containing the acidic serine–aspartate-rich motif (ASARM peptide). We conclude that MEPE promotes renal phosphate excretion and modulates mineralization.

Keywords

MEPE; Renal phosphate excretion; Mineralization

Introduction

Matrix extracellular phosphoglycoprotein (MEPE) was first cloned from a tumor resected from a patient with oncogenic hypophosphatemia (OHO). This was achieved by expression screening of an OHO tumor cDNA library with polyclonal antibodies that neutralized OHO tumor secreted phosphate uptake-inhibiting factor(s) [71]. MEPE is exclusively expressed in osteoblasts, osteocytes and odontoblasts and markedly up-regulated in murine X-linked hypophosphatemic rickets (Hyp) osteoblasts and OHO tumors [2,3,15,26,29,45,62,71]. The primary defect in HYP is due to mutations and functional inactivation of the gene product PHEX resulting in defective calcification of bone, renal phosphate wasting (hypophosphatemia) and abnormal vitamin D metabolism [33,72,74]. The mechanisms responsible for these abnormalities are not known. However, extensive studies in Hyp mice indicate that the defect in mineralization and renal function is mediated by circulating factor(s) derived from Phex defective osteoblasts. These investigations include parabiosis experiments [48,49], Hyp/normal kidney cross-transplantations [57], Hyp/normal osteoblast intra-muscular cross-transplantation and mineralization studies [18,20–22,102], Hyp osteoblast conditioned media phosphate-uptake and mineralization studies [58,59,101], PHEX antisense studies in normal osteoblasts [80], demonstration of intrinsic abnormalities in the Hyp osteoblast [13,30,50,67,69] and direct analysis of mineralization inhibition and phosphate-uptake inhibition activity of Hyp serum [37]. Thus, in Hyp, the evidence strongly implicates a circulating humoral factor or factors [phosphatonin(s)] that are secreted by the Hyp osteoblast and impact on mineralization and renal phosphate handling.

Recent discoveries have confirmed that autosomal dominant hypophosphatemic rickets (ADHR) is caused by activating mutations in FGF23 [4,82,97,98]. The specific ADHR mutations increase the stability of mutated FGF23 and the uncleaved mutated molecule is phosphaturic and elicits osteomalacia/rickets [4,77,81,98]. Wild-type FGF23 is also expressed in OHO tumors and the tumor over-expression of the wild-type FGF23 molecule is thought to swamp proteolytic mechanisms that normally inactivate full-length FGF23 [11,15,81]. Evidence suggests, however, that other factors may also play a role in this complex bone-renal pathway. The mineralization and phosphate-inhibiting modalities of conditioned media derived from Hyp osteoblasts and normal osteoblast cell lines stably transfected with PHEX [17,51,59,80,101], the confirmation of intrinsic Hyp osteoblast mineralization defects [18,20–23,67–69,102,103], in osteoblasts/bone indicates that there may be osteoblastic phosphatonins (OB-PTN) and extra-osseous phosphatonins (EO-PTN) molecules and pathways. Both OB-PTN and EO-PTN pathways may be contiguous, overlapping or separate.

One of the candidate OB-PTNs is MEPE (also called OF45 in the rat) [62,71]. MEPE is a secreted RGD-matrix phosphoglycoprotein up-regulated in OHO tumors and Hyp osteoblasts. Moreover, MEPE is exclusively expressed in osteoblasts, osteocytes and odontoblasts [2,3,15,26,29,62,71,79,81]. MEPE also belongs to the short integrin-binding ligand interacting glycoprotein (SIBLING) family that all map to 4q21.1 [24,71]. These proteins include MEPE, dentin sialophosphoprotein (DSPP), osteopontin (SPP1), dentin-matrix-protein-1 (DMP-1) and bone sialophosphoprotein (BSP) and have many features in common [24,71]. We originally observed the occurrence of a highly conserved acidic serine-aspartate-rich MEPE-associated motif (ASARM motif) in MEPE, DSPP, osteopontin and DMP-1 (Fig. 1) and suggested that it could play a role in modulating mineralization [71]. MEPE expression is markedly up-regulated in osteoblasts and bone from Hyp mice and is suppressed by PHEX and 1,25-dihydroxy vitamin-D₃ [2,3,29]. Moreover, recent evidence indicates that MEPE is a substrate or forms a complex with PHEX [12,29]. Recently we observed that PHEX inhibits the cathepsin B cleavage of MEPE through the C-

terminal domain of PHEX [29]. The observed dramatic increase in MEPE (a secreted protein) as a consequence of loss of PHEX (a cell surface protein) in HYP could potentially result in the generation of large quantities of protease-resistant ASARM peptide (Fig. 2) originally identified by us [71]. Moreover, this peptide has been implicated in inhibiting calcification of the urinary tract [32,96] and the motif is also present in salivary statherin and is responsible for maintaining the phosphate/calcium supersaturated mineralization solution dynamics of saliva [5,43,64,78]. Statherin may also function in the transport of calcium and phosphate during secretion in the salivary glands [5,64,78].

This investigation demonstrates that the MEPE ASARM peptide is also a potent inhibitor of osteoblast (murine 2T3 cells) BMP2-mediated mineralization in vitro. We would propose that PHEX may modulate mineralization by preventing cleavage and release of the small (<3 kDa) protease-resistant ASARM peptide and may also interact with other matrix proteins. Our data also show for the first time unequivocal hypophosphatemia, hyperphosphaturia and changes in 1,25-dihydroxy vitamin-D₃ metabolism following bolus intraperitoneal (IP) administration of recombinant human-MEPE (Hu-MEPE). Hu-MEPE also inhibits Na-dependent phosphate uptake in vitro in primary human proximal tubule epithelial cells and a human renal cell line Hu-CL8. Thus, we conclude that MEPE is a good OB-PTN candidate and the MEPE ASARM peptide inhibitory effects on mineralization are likely modulated by a novel PHEX-MEPE interaction. Of major interest in this regard is the recent finding that MEPE (OF45) knock-out mice have accelerated mineralization and increased bone formation [26] and loss of function mutations in the cathepsin C gene are responsible for the dental disease and ectopic intracranial calcification in PLS [88].

Materials and methods

Recombinant Hu-MEPE

Insect *Spodoptera frugiperda* cells were infected with Baculovirus containing the human MEPE gene at a multiplicity of 1.0. Cells were grown in a 10-l bioreactor for 48 h and conditioned medium was concentrated (5-fold) and used as the starting material for purification. The purification scheme employed cation exchange chromatography as the capture step followed by affinity chromatography and size exclusion chromatography for final polishing and buffer exchange. The final product integrity was verified by N-terminal sequence analysis and Western blot analyses using antibodies to the N terminus, mid-region and C terminus. Purified protein contained N-terminal amino acid residues APTFQ confirming cleavage of predicted nascent-MEPE signal peptide by the *S. frugiperda* insect cells. Fig. 3 shows SDS-polyacrylamide gel electrophoresis (PAGE) (Coomassie stain) and Western blot of representative material used in these studies.

MEPE ASARM peptide and polyclonal antisera

The C-terminal MEPE ASARM peptide (CFSSRRRDDSSSESDSGSSSESDDGD) with NH₂-terminal cysteine was used as an experimental peptide and also to raise polyclonal antisera (Fig. 1). The following peptide was used as a control in the mineralization experiments (CGSGYTDLQERGDNDISPFSGDGQPF). Synthetic MEPE peptides corresponding to regions in the N terminus (APTFQPQTEKTKQSC) mid-region (TDLQERGDNDISPFSGDGQ) and C-terminal region (GRQPHSNRRFSSRRRDDSS) were also used to raise polyclonal antisera in rabbits. Antibodies to these peptides were affinity purified using the respective peptides and titred for optimal use in Western blot detection of MEPE. All peptides are oriented amino-terminal to carboxy-terminal.

Measurement of $^{33}\text{PO}_4$ uptake and ^{14}C - α -methyl D -glucose uptake

A novel technique, scintillation proximity assay (SPA), was used to measure $^{33}\text{PO}_4$ uptake in vitro. Human renal proximal tubule primary cells (RPTEC) were purchased from Clonetics-Biowhittaker (cat. no.: CC2553) and were cultured and subcultured using the manufacturer's recommended protocols, methods, buffers and media (cat. nos.: CC3190, CC5034). Also a human renal cell line (Hu-CL8) was cultured as previously described [73]. For $^{33}\text{PO}_4$ uptake measurement, cells were grown in REBM media supplemented with 5% fetal calf serum (REBM-FCS) to 80% confluence (Clonetics-Biowhittaker cat. nos.: CC2553, CC3190 and CC5034) and seeded into 96-well (3000 cells/well) cytostar-T scintillating-microplates (Amersham-Pharmacia Biotech cat. no: RPNQ 0162). Cells were then cultured in 200 μl of REBM-FCS media for 24 h. Medium was then replaced with serum-free media (REBM) and the cells incubated for a further 3–4 h with Hu-MEPE, parathyroid hormone (PTH) (1–34) or media control, respectively, in REBM media. The concentrations of PTH (1–34) and Hu-MEPE were 1–500 ng/ml and each experimental dose was repeated eight times for statistical analysis. Following incubation, medium was removed and replaced with either phosphate uptake buffer (PUB) consisting of 150 mM NaCl/1 mM CaCl_2 /1.8 mM MgSO_4 /10 mM HEPES pH 7.0/0.1 mM phosphate buffer pH 7.0 and 3 $\mu\text{Ci ml}^{-1}$ ^{33}P as orthophosphoric acid (18 MBq/ml or 5775.7 Gbq/mg stock solution Perkin Elmer cat. no.: NEZ080). To determine the Na-dependent phosphate co-transporter activity, a separate set of control cells was incubated with the same buffer but with choline chloride (150 mM) substituted for NaCl (CHUB buffer). After 10 min at 37°C, the reaction was terminated by removing the PUB and CHUB buffers and replacing with STOP buffer (CHUB buffer without radioisotope). The cytostar scintillation plates were then directly assayed for counts (CPM) using a Hewlett-Packard Multiplate Top-counter calibrated for ^{33}P SPA assay. Due to the unique design of the 96-well cytostar plates, radioactive decay in solution is not detected by the counter. In contrast, radioisotope attached to or taken into the cells initiates release of plastic-scintillant photons that are detected by the top-counter photo-multiplier tube. Thus, no washes are required and direct CPM can be counted in living cells. Following CPM determination, the buffers were removed from the living cells and fresh media supplemented with cell-titre-96 (Promega-Biotech cat no: G5421) cell-number detection media. Cells were then further incubated at 37°C for 4 h. Conversion of a tetrazolium salt to a colored formazan product by living cells was then measured at 490 nm using a 96-well multi-well spectrophotometer with KC-4 software (absorbance at 490 nm is directly proportional to the number of living cells). Na-dependent ^{33}P uptake was then calculated by subtracting background counts and the choline chloride non-Na-dependent ^{33}P uptake and results expressed as $^{33}\text{PO}_4$ fmol 10^4 cells $^{-1}$ 6 min $^{-1}$. The measurement of Na-dependent α -methyl D -glucose uptake was accomplished using a previously described method of ^{14}C incorporation [73]. The culture of cells and incubation with Hu-MEPE, PTH and controls were identical to the experiment for determination of phosphate uptake in RPTEC and Hu-CL8 cells.

Intraperitoneal administration of Hu-MEPE and PTH (1–34) into mice

Male ICR-Swiss mice were used as recipients for intraperitoneal administration (100 μl) of 0.1% phosphate-buffered saline/0.1% bovine serum albumin (vehicle) and experimental conditions (Hu-MEPE and PTH 1–34) dissolved in 100 μl of vehicle. Two separate (repeat) experiments were conducted consisting of four groups with six and seven animals per group. The control group was administered with 100 μl of vehicle (Group 1). Group 2 consisted of animals administered with 0.8 μg /injection of parathyroid hormone (PTH 1–34). Group 3 animals were injected with 0.4 μg /injection of Hu-MEPE and Group 4 with 4 μg /injection of Hu-MEPE. On day 1, three bolus injections were given at 0, 3 and 6 h, respectively. Serum samples were then collected via retro-orbital bleed 1 h after the 6-h bolus administration. The next day or 30 h after the first bolus, a fourth bolus (same amount) was

given and after 1 h, the animals were sacrificed and serum prepared after cardiac exsanguination. The total amounts of PTH and MEPE administered over the 30 h were $80 \mu\text{g kg}^{-1} 30 \text{ h}^{-1}$ PTH 1–34, $40 \mu\text{g kg}^{-1} 30 \text{ h}^{-1}$ Hu-MEPE (MEPE40) and $400 \mu\text{g kg}^{-1} 30 \text{ h}^{-1}$ Hu-MEPE (MEPE400). Each group was placed in a separate metabolic cage and urine was collected for the 6-h period. After the first 6-h urine collection, mice were replaced in thoroughly cleaned metabolic cages for the remaining 24 h. The second 24 h urine was collected immediately after the animals were sacrificed. The urine volumes for the second and first collections were measured. Collecting urine samples from large numbers of small mice over the time frame of the experiment was not feasible. Therefore, urine samples from each group ($n = 7$) were pooled and volumes measured via a specially designed collecting duct in the metabolic cage. To ensure reproducibility, the experiment was repeated twice (the same changes occurred). To calculate the fractional excretion of phosphate (FEP), the following equation was used: $100 - (100 \times (1 - (A/B)))$, where $A = (\text{urine phosphorus in mg/dl}) \times (\text{serum creatinine in mg/dl})$ and $B = (\text{urine creatinine in mg/dl}) \times (\text{serum phosphorus in mg/dl})$. To calculate renal phosphorus clearance (RPC) in ml/min, the following computation was used: $(\text{urine phosphorus in mg/dl}) \times (\text{urinary volume in ml}) / ((\text{serum phosphorus in mg/dl}) \times (\text{time of collection in min}))$. The phosphate excretion index (PEI) was calculated using the same method described by Shimada et al. [81] using the following formula: $((\text{urinary concentration of phosphorus in mg/dl}) / (\text{serum concentration of phosphorus in mg/dl})) / (\text{total urinary creatinine in mg})$.

Serum and urine assays

Serum and urine assays for phosphate, calcium, chloride, potassium, creatinine, glucose and alkaline phosphatase were accomplished using 96-well format KC4-spectrophotometer multi-plate kinetic/end-point analyses using routine methods and diagnostic kits; (Sigma-Diagnostics). Serum 1,25-dihydroxy vitamin-D₃ levels were estimated using the method of Reinhardt et al. [66]. Analysis of data for fractional excretion of phosphate (FEP) was carried out following the same method described previously by Shimada et al. [82] for FGF23 bolus administration studies and by Nesbitt et al. [57] for renal cross-transplantation experiments in Hyp mice.

Mineralized bone matrix formation assay

A mouse osteoblast cell line (2T3 cells) was monitored using a mineralized matrix formation assay as described by Bhargava et al. and cultured as described previously [7,14,105]. Mineralization of the murine osteoblastic 2T3 cell line has been extensively studied using physical techniques, electron microscopy and histological techniques. This cell line (2T3) undergoes exogenous BMP2-induced mineralized matrix indistinguishable from normal bone-mineral hydroxyapatite in vitro [7,14,105]. The cells were plated in 24-well culture plates at a density of 2×10^4 cells/well and cultured with minimal essential medium (MEM) supplemented with 10% FCS. When the cells reached confluency (day 0), the medium was changed to MEM containing 5% FCS, 100 $\mu\text{g/ml}$ ascorbic acid and 5 mM β -glycerol phosphate with or without 100 ng/ml of BMP-2. The effects of Hu-MEPE, MEPE ASARM peptide and control peptide at different doses (see Results section) in the absence and presence of BMP2 were measured using the von Kossa stain for mineralization. The medium was changed every other day and fresh reagents were added. Von Kossa stain of mineralized bone matrix was performed as follows. The cell cultures were washed with PBS twice, fixed in phosphate-buffered formalin for 10 min, and then washed with water, and serially dehydrated in 70%, 95%, and 100% ethanol, twice each and air-dried. The plates were rehydrated from 100% to 95% to 80% ethanol to water before staining. The water was removed, a 2% silver nitrate solution was added, and the plates were exposed to sunlight for 20 min, after which the plates were rinsed with water. Five percent sodium thiosulfate was added for 3 min and the plates were then rinsed with water. The modified van Gieson stain

was then used as a counterstain after the von Kossa stain. The unmineralized collagen matrix can be recognized by the yellow/red van Gieson stain. The acid fuchsin solution (5 parts of 1% acid fuchsin, 95 parts of picric acid, and 0.25 part of 12 M HCl) was added for 5 min. The plates were washed with water, and then $2 \times 95\%$ ethanol and $2 \times 100\%$ ethanol, and dried for image analysis. The area of von Kossa-stained matrix was quantitated as previously described [14]. Briefly matrix was quantified by automated image analysis using a video analysis program (Jandel Scientific, San Rafael, CA) and also a Bio-Rad quantity 1 image analysis capture program. A video screen camera (CCD/RGB; Sony Corp., Park Ridge, NJ) linked to a microscope (model BH2; Olympus Corp., Precision Instruments Division, Lake Success, NY) equipped with metal-lurgical lens was used to image plates.

SDS-polyacrylamide electrophoresis and Western analysis

Proteins were separated and visualized using 4–12% SDS-PAGE Novex gel gradient and Coomassie blue staining as described previously [71]. Polyclonal antisera raised in rabbits were used to screen SDS-PAGE separated proteins electro-transferred and immobilized onto PVDF membranes (Western blotting) using methods also previously described [71].

Statistical methods

Differences were assessed statistically by the use of Newman–Keuls or Bonferroni (as indicated) multiple comparison equations after one-way analysis of variance (ANOVA, non-parametric). A *P* value of less than 0.05 was considered significant. The standard error of the mean (SEM) was used as a representative measure of how far the sample mean differed from the true population mean.

Results

Measurement of $^{33}\text{PO}_4$ uptake and ^{14}C - α -methyl D -glucose uptake (SPA)

Dose-dependent inhibition of Na^+ -dependent phosphate co-transport occurred in both primary human renal proximal tubule epithelial cells (RPTEC) and a human renal proximal tubule cell line (Hu-CL8) with PTH (1–34) and Hu-MEPE (Figs. 4A–C). Although the K_m for Hu-MEPE was similar with RPTEC (27.4 ng/ml) and Hu-CL8 cells (23.8 ng/ml), the $V_{\max}$ of percentage inhibition differed significantly. Primary RPTEC cells exhibited a $V_{\max}$ of 53.4% inhibition and Hu-CL8 cells a lowered $V_{\max}$ of 9.1% inhibition (Figs. 4A and B). Fig. 4C graphically (histogram) illustrates the dose-dependent inhibitory effects of Hu-MEPE and PTH (1–34) on RPTEC cells. No significant differences were noted in the Na -dependent uptake of ^{14}C - α -methyl D -glucose uptake between controls and experimentals (data not shown).

Intraperitoneal administration of Hu-MEPE and PTH (1–34) into mice

There were no significant differences in serum phosphate between vehicle, PTH and MEPE animals after 7 h. However, a marked and significant reduction in serum phosphate relative to vehicle (11.94 mg/dl; SEM 0.9) occurred in the PTH (8.32 mg/dl; SEM 0.48; $P < 0.01$) animals and the high dose MEPE400 (8.23 mg/dl; SEM 0.334; $P < 0.01$) group after 31 h (1 h after final bolus administration). A drop in serum phosphate in the low dose MEPE40 animals (10.6 mg/dl; SEM 0.65) was also observed relative to vehicle after 31 h but this was not statistically significant (Fig. 5A). Urine phosphate increased dose dependently relative to vehicle (31.8 mg/dl; SEM 0.23) in PTH (61.6 mg/dl; SEM 1.0), MEPE40 (41.0 mg/dl; SEM 0.61) and MEPE400 (56.1 mg/dl; SEM 0.82) groups after 7 h dramatically, and reproducibly (Table 1). Also consistent with the observed hypophosphatemia in PTH and MEPE mice, the 31-h second urine phosphate excretion was also markedly and reproducibly elevated in PTH mice (238.3 mg/dl; SEM 2.88) and MEPE400 (297.4 mg/dl; SEM 7.43) mice relative to

vehicle (175.9 mg/dl; SEM 4.39) (Table 1). In contrast to the 7 h collections, the 31-h low-dose MEPE40 urine was not significantly different to the 31 h vehicle. This was not surprising as the smaller change elicited by the low Hu-MEPE dose was probably diluted by the overnight collection of urine (24 h since last low-dose Hu-MEPE bolus). The dose-dependent MEPE and PTH hyperphosphaturia was reproducible and occurred after three bolus injections of Hu-MEPE and PTH at 0, 3 and 6 h and was also markedly evident in the second overnight urine collection. Thus, the administration of Hu-MEPE and PTH resulted in hypophosphatemia and hyperphosphaturia.

Although the hypophosphatemia was manifest in PTH and Hu-MEPE groups after 31 h but not after 7 h, hyperphosphaturia was apparent dose dependently after 7 h and also after 31 h. Moreover, measurement of serum/urine creatinine, phosphate, urine volume and calculation of the fractional excretion of phosphate (FEP) together with renal phosphorus clearance (RPC) confirmed a dramatic and dose-dependent loss of phosphate after 7 h and also 31 h (Table 1 and Figs. 5B and C). This loss was underlined by a dramatic and reproducible increase in FEP (after 7 h) relative to vehicle (7.8%; SEM 0.94), in PTH (27.4%; SEM 3.38, $P < 0.001$) and MEPE400 (14.43%; SEM 0.99, $P < 0.01$) animals (Fig. 5B). Also the dramatic increase in FEP relative to vehicle (28.7%; SEM 3.97) was still evident in the PTH (53.3%; SEM 4.51, $P < 0.01$) and MEPE400 (65.0%; SEM 6.47, $P < 0.001$) after 31 h (Fig. 5C). The low dose MEPE40 group was not significantly different in FEP to the vehicle confirming a dose-dependent Hu-MEPE phosphaturic effect. The levels of serum calcium were significantly elevated in the PTH animals after 7 h (1 h after third bolus administration). The low Hu-MEPE dose and vehicle dose were not significantly different. There were no differences in serum calcium between groups after 31 h (single bolus second day administration). Although urine calcium was not significantly different to vehicle in all groups after 7 h, the PTH animals were significantly hypocalciuric after 31 h compared to Hu-MEPE and vehicle (Table 1). Serum values for a range of parameters including K, Cl, Na, glucose, protein and albumin were not significantly different between groups (Table 1).

As expected, the serum levels of 1,25-dihydroxy vita-min-D₃ in the PTH 1–34 animals (61.2 pg/ml) were reproducibly elevated after 31 h relative to vehicle (25.2 pg/ml). A dose-dependent increase of 1,25-dihydroxy vitamin-D₃ also occurred reproducibly with Hu-MEPE with values of 31.5 and 45.05 pg/ml for MEPE40 and MEPE400, respectively (Table 1). Insufficient serum was obtained via retro-orbital bleed to assay the 7-h time point. Serum alkaline phosphatase was not significantly different between vehicle and other groups after 7 h. In contrast, a highly significant and reproducible suppression of serum alkaline phosphatase was observed after 31 h between vehicle (65.06 U/l SEM 4.4) and MEPE40 (45.63 U/l; SEM 4.52, $P < 0.05$), MEPE400 (46.31 U/l; SEM 2.8, $P < 0.01$) and PTH (46.3 U/l; SEM 3.56, $P < 0.01$) groups (Table 1).

Mineralized bone matrix formation assay and affects of Hu-MEPE and MEPE ASARM peptide

BMP2 (100 ng/ml) induced mineralization of 2T3 osteoblasts as assayed using the von Kossa stain after 12 days. No von Kossa-positive staining was apparent in the control cells without BMP2 up to 31 days. The results for 26 days shown in Figs. 6 and 7 show quantification of nodule formation after days 13 and 20, respectively. Addition of Hu-MEPE at concentrations of 10, 100, 500 and 800 ng/ml in the presence of BMP2 (100 ng/ml) dose dependently and reproducibly inhibited mineralization of 2T3 osteoblasts (Figs. 6A and B and 7A and B). Doses above 100 ng/ml were very effective inhibitors. MEPE in the absence of BMP2 had no effect on mineralization and cells were identical to controls, confirming an inhibitory role for MEPE or derived MEPE peptide(s). Addition of the MEPE ASARM peptide (DDSSSESSDSGSSSESDDGD) also dose dependently inhibits BMP2-mediated von Kossa staining and bone mineral nodule formation in 2T3 cells (Figs. 6A and C). A control

peptide failed to elicit any changes in mineralization (positive or negative) (see Fig. 6A). Quantification of the inhibitory effects of Hu-MEPE is shown in Fig. 7 for days 13 and 20.

Discussion

The primary defect in HYP is a mutated Zn-metalloendopeptidase (PHEX) and MEPE is dramatically up-regulated [2,3,29,33,74]. There is overwhelming evidence implicating the osteoblast as the cell intrinsically defective [13,18,20–22,30,37,50,58,59,67,69,80,101,102] and the HYP osteoblast directly secretes factors (phosphatonins) that impact adversely on renal phosphate uptake and mineralization in vivo and in vitro. FGF23 activating mutations are responsible for the changes in phosphate, vitamin D metabolism and mineralization in ADHR [4,82,97,98] and overexpression of wild-type FGF23 in some but not all OHO tumors is directly or indirectly responsible for the changes in some tumor-induced osteomalacias [15,81,97,99]. Also, a number of groups have reported lack of FGF23 expression in bone/osteoblasts [3,27,29,41,98,104] and others using more sensitive techniques have detected low levels of FGF23 mRNA in bone tissue but not in normal murine osteoblasts [40]. This suggests a complex pathway involving extra-osteoblastic phosphatonins (EO-PTN) and osteoblastic phosphatonins (OB-PTN) in Hyp. In this regard, MEPE and FGF23 expression is tightly correlated in Hyp and the full-length molecules do not appear to be PHEX proteolytic substrates [40]. PHEX is primarily expressed in osteoblasts and odontoblasts and the coordinated maturational expression of PHEX with mineralization supports a role in mineralization and/or processing of phosphatonins [16,28,50,75,86]. MEPE is exclusively expressed in osteoblasts and osteocytes in rodents [2,3,26,29,62,71]. In man, MEPE expression also occurs in one other tissue, brain [2,26,29,62,71]. MEPE expression occurs in all OHO tumors screened and is notably absent in non-phosphaturic tumors [15,71,79, 81]. The exclusive osteoblast expression profile of MEPE, its temporal expression with PHEX, its marked up-regulation in HYP, its presence as a secreted protein in serum and matrix, the coordinate down-regulation of MEPE expression by PHEX and 1,25-dihydroxy vitamin-D₃, and the overexpression and secretion by OHO tumors indicated to us that MEPE is a good candidate for an OB-PTN [2,3,15,26,29,50,71,79,81].

To date, no definitive experiments have been reported that describe the effects of MEPE on phosphate or mineralization in vivo or in vitro. A very recent report of the phenotype of MEPE null-mutant mice, however, showed conclusively that lack of MEPE increases bone formation and mineralization and the authors concluded that MEPE plays a role in bone homeostasis by impacting negatively on mineralization and/or bone formation. To determine the possible functional role of MEPE as a candidate OB-PTN, we investigated the biological effects of recombinant (Hu-MEPE) in vivo and in vitro. Bolus administration of Hu-MEPE in vivo resulted in significant dose-dependent hypophosphatemia after 31 h and hyperphosphaturia occurred after 7 and 31 h. Similar results were obtained with PTH (positive control). Fractional excretion of phosphate was also significantly increased after 7 and 31 h with MEPE and PTH groups. The changes in phosphate handling observed in the MEPE groups were in agreement with the expected effects of a phosphatonin-like molecule.

Serum levels of 1,25-dihydroxy vitamin-D₃ were elevated in the MEPE-treated animals and as expected in PTH-treated animals after 31 h. This is consistent with the fact that 1,25-dihydroxy vitamin-D₃ suppresses MEPE and PHEX mRNA expression and markedly elevated levels of MEPE and PHEX also occur in vitamin D receptor (VDR) knock-out mice [2,19,60]. Also, we have reported an increase in 1,25-dihydroxy vitamin-D₃ synthesis in proximal tubule renal cells (Hu-CL8 cell line) exposed to OHO tumor conditioned media in vitro [73]. Thus, the increase in 1,25-dihydroxy vitamin-D₃ may be a physiological attempt to suppress the apparent acute increase in MEPE following bolus administration. Although

the elevation in 1,25-dihydroxy vitamin-D₃ is paradoxically the reverse to that found in HYP, it is not unexpected and can be explained. In this regard, HYP and OHO patients are chronically exposed to sustained levels of a phosphatonin-like factor(s) over long periods of time and this is in contrast to the intermittent experimental boluses administered in this study using normal mice. Of additional interest is the recent observation of increased mRNA levels for 25-(OH)-Vit-D₃-1- α -hydroxylase (1- α -hydroxylase) in Hyp mice [25]. Moreover, in Hyp-mice, a 1- α -hydroxylase post-transcriptional abnormality occurs that limits calcitriol production and this abnormality is likely independent of phosphatonin and recreated only in the in vivo Hyp-mouse milieu [25]. This is also consistent with our observed increase in serum 1,25-dihydroxy vitamin-D₃ levels in MEPE-treated normal mice and the fact that MEPE levels and 1- α -hydroxylase-mRNA levels are also elevated in murine-Hyp [25]. Finally, differences in physiological responses elicited by different modes of administration of PTH and 1,25-dihydroxy vitamin-D₃ (intermittent or continuous) are well known [31,36,44,65,70]. New bone formation occurs with intermittent bolus injections of PTH, whereas continuous administration of PTH results in bone resorption [31,36,44,70]. Further studies are required to see if this is also true for MEPE and its effects on serum 1,25-dihydroxy vitamin-D₃ levels, and also serum alkaline phosphatase.

The levels of serum calcium were significantly elevated in the PTH-treated animals after 7 h (1 h after third bolus administration) and the PTH animals were significantly hypocalcemic after 31 h compared to MEPE and control animals. There was a slight elevation of serum calcium in the high dose MEPE animals after 7 h but this was not significant. The lack of MEPE effects on calcium is consistent with the disease phenotype and putative phosphatonin effects. The slight but non-significant serum calcium increase in high dose MEPE animals may reflect the relatively reduced increase in 1,25-dihydroxy vitamin-D₃ compared to the PTH (1–34) mice. Consistent with the hypophosphatemia induced by MEPE in vivo, we observed an inhibition of phosphate uptake in vitro. The $V_{\max}$ percentage rate of inhibition was higher (54%) in primary human renal cells (RPTEC) compared to a human renal cell line (Hu-CL8) (9.1%). In contrast, K_m values were similar, suggesting that the number and/or accessibility of Na-dependent phosphate co-transporter molecules are reduced in the Hu-CL8 cell line relative to the primary RPTEC renal cells (Fig. 4). Replacement of choline chloride for sodium chloride consistently reduced phosphate uptake to less than 3%, suggesting that the bulk transport mechanism was sodium dependent. No significant differences were observed in uptake of ¹⁴C- α -methyl D-glucose between controls and experimentals, indicating that the Hu-MEPE and PTH (1–34) inhibition was specific for Na-dependent phosphate co-transport. These data suggest that Hu-MEPE-mediated hypophosphatemia in vivo is due to direct effects on renal Na-dependent phosphate co-transport.

The physiological substrate for PHEX remains elusive although FGF23 and MEPE are possible candidates [11,12]. However, although small fluorogenic peptides of MEPE and FGF23 are reported to be cleaved by PHEX [12], full-length MEPE appears resistant to proteolysis [29,40]. Also, the proteolysis of the full-length form of FGF23 by PHEX remains equivocal with one group reporting cleavage [11] and others no cleavage [29,40]. Recently, we reported that PHEX and a carboxy-terminal PHEX-fragment protect MEPE from cathepsin B degradation [29]. Cathepsin B is expressed in the osteoblast [1] and cathepsin B cleavage of MEPE results in the specific release of the MEPE ASARM peptide (Figs. 1 and 2). The cleavage sites are highly conserved in all cloned species (mouse, rat, monkey and human) (Fig. 1). We noted earlier that the ASARM peptide/motif is also a feature of osteopontin (OPN), dentin-matrix-protein-1 (DMP-1) and dentin-sialophosphoprotein (DSPP) (Fig. 8B) [71]. These proteins (including MEPE) all map to 4q21.1 and are now known as the SIBLING protein family (short-integrin-binding ligand-interacting glycoproteins) [24]. Recently, the osteopontin ASARM peptide was used by

Hoyer et al. [32] to investigate effects on calcium oxalate crystal formation and growth. This osteopontin-derived ASARM peptide was the most potent inhibitor compared to a series of overlapping and contiguous osteopontin short peptides. In our hands, the MEPE ASARM peptide is also a powerful inhibitor of von Kossa-positive BMP2-mediated mineralization of murine osteoblast (2T3) cells in vitro (Figs. 6A–C). A quite remarkable association, however, to an ancestral mineralization gene from this region is provided by our observation that statherin also contains an ASARM motif and maps to the same region of chromosome 4 (Figs. 8A and B). Statherin, a salivary protein, is a 62-residue molecule with asymmetric charge and structural properties. A key role is maintaining the mineral solution dynamics of enamel by virtue of its ability to inhibit spontaneous precipitation and crystal growth from supersaturated solutions of calcium phosphate minerals [5,43,64,78]. Statherin's role in preserving the calcium phosphate supersaturated state of saliva is crucial for re-calcification and stabilization of tooth enamel and for the inhibition of formation of mineral accretions on tooth surfaces. In addition, statherin has been proposed to function in the transport of calcium and phosphate during secretion in the salivary glands [5,64,78]. As with the MEPE ASARM peptide, a single cathepsin B site is present in statherin that would potentially release the highly charged and phosphorylated aspartate–serine-rich statherin ASARM peptide (see Fig. 2). Moreover, like the MEPE ASARM peptide, the statherin ASARM peptide is responsible for the inhibition of mineralization and binding to hydroxyapatite. Specifically, a short consensus peptide DSSEES/K is responsible for this in statherin [43,64] and this is repeated in the MEPE ASARM region (Fig. 8A) [71]. Thus, the potent inhibition of mineralization of osteoblasts mediated by full-length MEPE likely reflects cleavage of MEPE by osteoblastic cathepsin B or similar osteoblastic protease(s) in culture and concomitant release of protease-resistant MEPE ASARM peptide (Figs. 2 and 6C). In vitro (cell culture), addition of exogenous MEPE likely overloads the capacity of cell-surface expressed PHEX to protect MEPE from secreted cathepsin B and/or other osteoblast/matrix proteases. Further experiments are required to confirm this in vivo. Of additional interest is the finding that normal human-osteoblast cathepsin B production, secretion and activity are markedly stimulated by Interleukin- β -1, PTH and dexamethasone bone-resorbing agents [1,61].

PHEX is expressed on the osteoblast plasma membrane [50,76,87] and PHEX mutations interfere with processing and subsequent transmembrane docking of PHEX and extracellular extrusion [76]. Thus, sequestration and protection of MEPE and perhaps other matrix proteins by PHEX likely occurs on the extracellular osteoblast surface. Cathepsin D, neprilysin and ECEL-1/DINE proteases are markedly up-regulated in Hyp mice osteoblasts and bone marrow stromal cells (BMSC) [17,34]. The reported massive up-regulation of MEPE, the excess protease expression and the lack of functional PHEX would collectively increase the levels of MEPE ASARM peptide dramatically in Hyp (Fig. 2) particularly as this peptide is uniquely resistant to a vast array of proteases (trypsin, papain, proteinase K, carboxypeptidases, cathepsins, tryptase, etc). This in turn would cause the observed periosteocytic defects in mineralization leading to rickets, dental abnormalities, and/or osteomalacia. Physicochemically, the ASARM peptide is highly acidic with a *pI* of 2.3 and a net charge of –6.9 at pH 7. There are three casein kinase sites that would result in phosphorylation of three serine residues with a further increase in negative charge. Thus, this molecule (ASARM peptide) would predictably sequester free calcium with high avidity and like bisphosphonates inhibit mineralization at high doses [9,46,89]. Moreover, casein kinase II is present in osteoblasts as an ectokinase and phosphorylates extracellular matrix proteins [95,106].

Recently, Campos et al. [12] demonstrated that PHEX cleaves a synthetic fluorogenic MEPE peptide at the acidic residue distal to and adjacent to the MEPE ASARM peptide in a site, remarkably, that is in the same regional location as cathepsin B (Figs. 2 and 8A).

Moreover, PHEX has strict peptide-substrate S₁ specificity for acidic amino acids (Asp or Glu) with a strong preference for Asp [8,12]. This and the other requirements ideally suit cleavage release and/or protein–protein interaction of the ASARM peptide region of MEPE with PHEX. Thus, although we cannot exclude the possibility that PHEX cleaves MEPE in vivo, the notion of a PHEX-MEPE protein–protein interaction that does not necessarily lead to catalysis is supported strongly by recent published observations [8,12,29,40].

If the proposed ASARM peptide model is valid, then a MEPE knock-out should result in increased mineralization. Recently, an elegant study by Gowen et al. [26] described a MEPE (OF45) knock-out. The animal has increased bone mass, increased numbers and thickness of trabeculae and cortical bone mass. In vitro, osteoblasts exhibit an accelerated mineralization rate, and in vivo, the mineralization apposition rate (MAR) is dramatically increased compared to normals. However, although mineralization was accelerated in the knock-out, this also occurs whenever bone formation is increased. Further experimentation and more sophisticated analyses are therefore required to confirm whether mineral crystals are larger and/or abnormal in the MEPE knock-out. This would be consistent with MEPE's proposed role as a controller of the rate and size of mineral structures (ASARM peptide) as well as bone formation rate. For example, detailed and careful analysis of the osteopontin (Opn) knock-out (osteopontin 'ASARM peptide' inhibits calcification of oxalate) [32], using FTIRM analyses, revealed that the relative amount of mineral in the more mature areas of bone (central cortical bone) is significantly increased in these animals. Moreover, mineral maturity (mineral crystal size and perfection) throughout all anatomic regions of the Opn-deficient bone is significantly increased [10]. Further experiments are needed to determine whether this phenotype is also exhibited to a greater or lesser extent in the MEPE knock-out confirming the ASARM peptide model.

In summary, we would propose that PHEX plays a unique and acute role in modulating mineralization by sequestering MEPE and perhaps other matrix proteins and protecting them from proteolysis and activation/inactivation. In the case of MEPE, an interaction with PHEX would potentially prevent release of a mineralization inhibitor or '*minhibin*' ASARM peptide. The protein–protein non-protease role for PHEX is not unprecedented for the M13 Zn-metalloendopeptidase family. All members form homodimer and/or heterodimer complexes and some have no known physiological substrate [39,90]. KELL antigen (a member of the same M13 family) for example interacts with XK protein and the only protease activity (non-physiological) reported is very low efficiency conversion of big endothelin-3 to endothelin-3 [39]. Also, a chronic role for PHEX in mineralization may well be mediated by the reported PHEX suppression of MEPE expression [2,3,29]. Further studies, however, are needed to confirm this model.

Although the MEPE-PHEX protection model fits the observed mineralization defects in HYP and OHO, an explanation of the intrinsic molecular mechanisms responsible for the defective phosphate requires further thought. The OBPTN(s) (osteoblastic in provenance) have the capacity to inhibit renal phosphate handling and mineralization. We would propose that in the Hyp osteoblast, the documented overexpression of cathepsins, ECEL-1/DINE proteases [17,34] MEPE [2,3,29] and lack of functional PHEX [33] results in increased ASARM peptide (*minhibin*) and also an excess of MEPE-specific phosphate inhibitor peptides [OB-phosphatonin(s)] (Fig. 2). In vitro, inhibition of phosphate uptake requires prolonged incubation with MEPE 4–5 h to overnight. This likely allows partial proteolytic degradation of the excess recombinant MEPE added to the cells by nascent proteases (renal neprilysin for example) and release of small protease-resistant ASARM peptides. Moreover, MEPE null-mutant mice do not exhibit hyperphosphatemia, suggesting that these (MEPE-derived) OB-PTN peptides are specifically negative modulators of renal phosphate handling and other homeostatic mechanisms are able to retain phosphate balance in the absence of

MEPE. Many cytokines, growth factors and effector molecules impact positively or negatively on renal phosphate handling [85]. Recently, for example, the human stanniocalcins (large glycoprotein hormones) originally found in fish have been shown to either stimulate renal phosphate uptake (STC-1) or inhibit renal phosphate uptake (STC-2) [93,94]. Thus, the phosphate and mineralization activities of MEPE are proposed to be distinct bio-functional properties of specific MEPE peptides that are temporally and coordinately dependent on PHEX expression (directly or indirectly).

Our experimental findings confirm that MEPE (or MEPE peptides) inhibit renal phosphate handling in vivo and in vitro but they do not provide evidence for a role as a physiological phosphatonin. Indeed, MEPE (ASARM peptide) may be one of a number of pathophysiological factors inhibiting phosphate transport and/or mineralization in Hyp. The acidic MEPE ASARM peptide is resistant to a vast array of proteases, is phosphorylated, with a low pI, high charge, low molecular weight and distinct physicochemical similarities to bisphosphonates (etidronate) and phosphonoformic acid [9,38,42,46,47,53,63,83,84,89,91,92]. Moreover, etidronate, phosphonoformic acid (PFA) and phosphonoacetic acid affect renal phosphate handling (direct binding), vitamin D metabolism and mineralization in vivo and in vitro [9,38,42,46,47,53,63,83,84,89,91,92]. Thus, the ASARM peptide may also inhibit phosphate transport by directly binding to the phosphate transporter as demonstrated with PFA and etidronate [38,42,47,53,63,83,84,91,92] and in turn exacerbating the effects of other inappropriately processed phosphatonin(s) on NPT2 transcription (FGF-23?). Indeed, FGF23 and MEPE expression are closely correlated in Hyp [40]. Moreover, a number of investigators have reported small (<5 kDa), uncharacterized, phosphaturic molecules partially purified from OHO conditioned media [35,52,54–56,100]. Remarkably, the phosphaturic activity of these molecules is resistant to inactivation by a number of proteases. Thus, although the evidence is circumstantial, one may speculate whether these uncharacterized OHO, tumor-derived molecules are related to the protease-resistant ASARM peptide. It should also be noted that another unrelated factor, Frizzled related protein-4 (FRP4), is also phosphaturic in vivo with a potential role in renal–skeletal OHO tumor pathogenesis and possibly Hyp [6].

Evidence is now emerging that extracellular matrix proteins like MEPE may well have multifunctional roles (phosphatonins and minihibins, for example) and these roles may be subtly different between species as evidenced by the expression of MEPE in primate but not rodent brain. Moreover, our data support the notion that MEPE is at least one of the osteoblast-derived factors directly acting on renal Na-dependent phosphate transport and mineralization in HYP. The effects on renal phosphate handling and mineralization are likely mediated by distinct bioactive peptides and a novel PHEX–MEPE interaction may well be partly or wholly responsible for modulating the effects on the skeleton via the release of a distinct protease-resistant ASARM peptide or minihibin. Whereas these studies have documented unequivocal changes in renal phosphate handling commensurate to the addition of recombinant Hu-MEPE, we cannot be certain that the effects are physiological but may rather reflect pathological or pharmacological changes (Hyp, OHO). A detailed and more sophisticated analysis is required to confirm the relative physiologic importance of MEPE in calcium/phosphorus metabolism. Nevertheless, our data indicate that MEPE, like many other effector molecules, impacts on phosphate renal homeostasis and may be of importance for ongoing studies on bone renal phosphate metabolism. The future may well see the therapeutic exploitation of the distinct activities of these peptides for the control of renal phosphate handling and perhaps in the treatment of disorders of mineralization in teeth, bone, kidney and also in the prevention of ectopic mineralization.

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Fig. 1. Key features and localization of the acidic serine–aspartate-rich MEPE-associated motif (ASARM peptide and motif). (A) Scheme showing the last 50-amino acid COOH-terminal residues of MEPE in man, mouse and rat, respectively. The ASARM motif is highly conserved in man, macaque monkey, mouse and rat and is localized to the last COOH-terminal 18-amino acid residues of the large, approximately 500-residue MEPE proteins as depicted in the scheme [71]. Cathepsin B (an osteoblast protease) specifically cleaves MEPE at the COOH terminus releasing ASARM peptide. The cathepsin B cleavage site does not occur elsewhere in MEPE and is highly conserved between species. (B) Moreover, the ASARM peptide is uniquely resistant to many proteases (trypsin, papain, proteinase K, carboxypeptidases, tryptase, etc.). The ASARM motif is found in members of the SIBLING protein family (MEPE, DMP-1, osteopontin, DSPP) and in osteopontin occurs in the mid-region of the molecule [71] and also salivary statherin. The dark boxes represent the position of the ASARM motif in MEPE and osteopontin and the number of amino acid residues for each respective protein is indicated [71]. Recently, Hoyer et al. [32] demonstrated that the osteopontin ASARM motif [71] inhibits calcium oxalate crystallization and growth. Also, in vivo studies using hyperoxaluria induced osteopontin knock-out mice have confirmed that osteopontin/osteopontin peptides are critical inhibitors of renal stone formation [96] and mineral maturity (mineral crystal size and perfection) throughout all anatomic regions of the osteopontin knock-out mouse bone is significantly increased [10].

Fig. 2.

Scheme illustrating a proposed role and molecular mechanism for PHEX, MEPE and the ASARM peptide in mineralization. Cathepsin B is expressed in the osteoblast together with PHEX and MEPE [1,2,16,26,28,29,50,61,71,75,86]. NEP, ECEL-1/DINE and cathepsin D and MEPE are up-regulated in Hyp osteoblasts that have defective PHEX [2,3,17,29,34]. PHEX protects MEPE from cathepsin B specific cleavage [29] (possibly by sequestration on the cell surface) and prevents release of the ASARM peptide. Thus, in rickets, increased levels of ASARM peptide are proposed to inhibit mineralization (defective PHEX). Also, the ASARM peptide is resistant to a vast range of proteases due to its unusual sequence. The MEPE ASARM peptide (NH₂-FSSRRRDDSSSESSDSGSSSESDGD-COOH) [71] inhibits mineralization in vivo (this manuscript) and the osteopontin ASARM peptide (NH₂-DDSHQSDSHHSDESDDED-COOH) [32,71] potentially inhibits calcium oxalate crystallization and crystal growth [32]. Also, the salivary statherin ASARM peptide contains the specific peptide sequence shown to play a biological role in inhibiting spontaneous precipitation of supersaturated salivary calcium and phosphate and maintaining the mineralization dynamics of tooth enamel [5,43,64,78]. The MEPE knock-out (complete absence of MEPE ASARM peptide and MEPE) as expected has accelerated mineralization, increased bone density and bone formation [26]. Moreover, studies in vivo using hypoxaluria renal stone induction in osteopontin null-mutant mice confirm that osteopontin/osteopontin peptides are critical inhibitors of renal stone formation [96]. Mineral maturity (mineral crystal size and perfection) throughout all anatomic regions of the osteopontin knock-out mouse bone is also significantly increased [10]. Of additional interest is the recent finding that loss of function mutations in the cathepsin C gene is the primary cause of Papillon-Lefevre syndrome (PLS) [88]. PLS results in periodontopathia with loss of both deciduous and permanent dentitions and severe intracranial calcifications [88]. MEPE is abundantly expressed in both brain (man/primates) and odontoblasts/osteoblasts (rodents and man/primates) [26,45,71]. Confirmation of this model requires further studies.

Fig. 3. SDS-polyacrylamide electrophoretic separation and Western blotting of purified insect-expressed full-length human-MEPE (Hu-MEPE). A shows a Coomassie-stained gel with three lanes containing bovine serum albumin (BSA), a single lane of standard protein molecular weight markers (Ma) and two lanes containing MEPE. B shows the corresponding PVDF membrane blot containing immobilized proteins screened with mid-region (RGD peptide) MEPE polyclonal (see Materials and methods section). Confirmation of MEPE identity was achieved by N-terminal amino acid sequence analysis of the excised PVDF blot region corresponding to the positive Western band. Purified protein contained N-terminal amino acid residues APTFQ confirming cleavage of predicted nascent-MEPE signal peptide by the sf9 insect cells.

Fig. 4.

Dose-dependent in vitro inhibition of $^{33}\text{PO}_4$ uptake is induced by Hu-MEPE in primary human proximal tubule epithelial cells (RPTEC) and a human renal cell line (Hu-CL8). A and B graphically illustrate the inhibition using Lineweaver–Burk plots [inverse percentage rate of inhibition against the inverse MEPE concentration (ng/ml)]. The Hu-CL8 cell line (A) and human RPTEC primary renal cells (B) have similar K_m values (23.8 and 27.39 ng/ml, respectively) but differing $V_{\max}$ values (9.1% and 53.4%, respectively). Graph C (histogram) illustrates Hu-MEPE and PTH dose-dependent inhibition observed with RPTEC cells in vitro (see text for discussion). Normal; buffer solvent, MEPE-25 (25 ng/ml), MEPE-50 (50 ng/ml), MEPE-100 (100 ng/ml), PTH-10 (10 ng/ml), PTH-100 (100 ng/ml). Differences in C were assessed statistically by the use of Newman–Keuls multiple comparison equations after ANOVA (non-parametric). A P value of less than 0.05 was considered significant. The SEM was used as a representative measure of how far the sample mean differed from the true population mean (see bars). For A, B and C, each data point contained $N = 12$ and $N = 15$ replicates for MEPE and PTH, respectively.

Fig. 5.

MEPE and PTH induce hypophosphatemia and increase the fractional excretion of phosphate (FEP) when administered intraperitoneally (IP). (A) Histogram of serum phosphate measured after 31 h and four bolus IP injections of MEPE40 ($40 \mu\text{g kg}^{-1} 30 \text{ h}^{-1}$), MEPE400 ($400 \mu\text{g kg}^{-1} 30 \text{ h}^{-1}$) and PTH ($80 \mu\text{g kg}^{-1} 30 \text{ h}^{-1}$). (B and C) Histograms illustrating the changes in FEP after 7 h (three bolus injections IP) and 31 h (four bolus injections IP). See Materials and methods for calculations and detailed description of protocol. Differences were assessed statistically by the use of Newman–Keuls multiple comparison equations after ANOVA (non-parametric). A *P* value of less than 0.05 was considered significant. The SEM was used as a representative measure of how far the sample mean differed from the true population mean (see bars) and each group contained *N* = 7 animals.

Fig. 6.

Hu-MEPE and MEPE ASARM peptide (CFSSRRRDDSSSESSDSGSSSESDGD) inhibit BMP2-mediated mineralization of mouse osteoblast cell line 2T3. Wells were stained for mineralization nodule formation using von Kossa (see Materials and methods) and the results after 26 days culture are shown. Row A: upper two wells (BMP2), contain BMP2 (100 ng/ml); middle two wells (control), control cells with no BMP2 or peptide; lower two wells (BMP2 and control peptide), BMP2 (100 ng/ml) with control peptide (CGSGYTDLQERGDNDISPFSGDGQPF) at 300 ng/ml (108.6 pmol/ml). Row B: Upper two wells (BMP2 and MEPE 100), contain Hu-MEPE (100 ng/ml) plus BMP2 (100 ng/ml); middle two wells (BMP2 and MEPE 500), Hu-MEPE (500 ng/ml) plus human-BMP2 (100 ng/ml); lower two wells (BMP2 and MEPE 800), MEPE (800 ng/ml) plus BMP2 (100 ng/ml). Cells in which MEPE was added in the absence of BMP2 (data not shown) were indistinguishable to control cells (Row A, middle two wells). Row C: Upper two wells (BMP2 and ASARM 60), BMP2 (100 ng/ml) plus MEPE ASARM peptide (CFSSRRRDDSSSESSDSGSSSESDGD) at 60 ng/ml (22.7 pmol/ml); lower two wells (BMP2 and ASARM 300), BMP2 (100 ng/ml) plus MEPE ASARM peptide at 300 ng/ml (113.5 pmol/ml).

Fig. 7.

Quantification of mineralization inhibition of mouse osteoblast 2T3 cell line by Hu-MEPE as assessed by von Kossa staining. Concentrations above 100 ng/ml completely inhibited mineralization (see Fig. 6). Thus, effects at 10, 100 and 500 ng/ml MEPE are shown after day 13 (A) and day 20 (B), respectively. The mineralized bone matrix formation of 2T3 cells were quantitated by computer image analysis as previously described [14] and the data represent the mean for three samples (see Materials and methods). ANOVA non-parametric and Neuman–Keuls multiple comparison confirm highly significant inhibition of BMP2 mediated mineralization by Hu-MEPE (see graph). The SEM was used as a representative measure of how far the sample mean differed from the true population mean (see bars).

Fig. 8.

Salivary statherin and MEPE consensus ASARM motif: mineralization inhibition and ancestral genes on chromosome 4. The depicted scheme illustrates the remarkable association of MEPE, DMP-1 and the SIBLINGS to an ancestral mineralization gene that is also thought to play a key role in phosphate calcium transport in saliva (salivary statherin). Statherin maps to chromosome 4 in the SIBLING/MEPE region and also contains an ASARM motif. Statherin is a 62-residue peptide with asymmetric charge and structural properties [43,64]. The upper scheme (A) depicts a clustal alignment of the COOH-terminal region of human-DMP-1 human-MEPE, mouse-MEPE and rat-MEPE with human-Statherin. In MEPE, the ASARM peptide is the most distal region of the molecule encompassing the last 17 residues of the COOH terminus and the region is highlighted with a boxed cartouche labeled MEPE ASARM peptide (A). In DMP-1, the ASARM region is also at the carboxy terminus but ends at residue 480 slightly upstream of the distal COOH terminus (protein 513 residues long). The short 62-residue statherin molecule contains an ASARM motif region as depicted in the diagram and the key residues are highlighted in the consensus string shown at the bottom of A. The boxed cartouche labeled as statherin ASARM peptide contains the specific statherin ASARM peptide sequence shown to play a biological role in inhibiting spontaneous precipitation of supersaturated salivary calcium and phosphate and maintaining the mineralization dynamics of tooth enamel [5,43,64,78]. As with the MEPE ASARM peptide, a single cathepsin B site is present in statherin that would potentially release the highly charged and phosphorylated aspartate-serine-rich statherin ASARM peptide [indicated by line between statherin arginine (R) residues 29 and 30; (A)]. In Statherin, the cathepsin B cleavage site is adjacent and located COOH-terminal to the motif. In MEPE, the cathepsin B cleavage site is also adjacent to the ASARM motif but asymmetrically arranged NH₂-terminal to the motif between the arginine and aspartate. In

both cases (MEPE and statherin), cleavage would result in the release of a short phosphorylated aspartate/serine-rich acidic peptide of low *pI* and almost identical physicochemical properties. A feature of the MEPE ASARM region is the repeat (D) SSES/E sequence. This short sequence has been shown to be key inhibitor of hydroxapatite crystals formation and mineralization in salivary statherin [43,64]. The MEPE ASARM region is highly homologous to the DMP-1 but the single cathepsin B site in DMP-1 is located further upstream toward the NH₂ terminus (A). B schematically presents the remarkable clustering of MEPE, DMP-1, statherin and other SIBLING genes on chromosome 4. All contain an ASARM motif in differing structural contexts with many diverse structural and genomic features (exon–intron structure) and associations with bone dental functions.

Table 1
Selected serum and urine parameters following IP administration of Hu-MEPE, PTH and vehicle in mice

Values	7 h						31 h					
	Vehicle			PTH			MEPE40			MEPE400		
	Value	SEM		Value	SEM		Value	SEM		Value	SEM	
<i>Serum</i>												
Pi (mg/dl)	9.29	0.48		7.69	0.71		9.56	0.49		9.66	0.57	
Ca (mg/dl)	6.03	0.29		7.10 [*]	0.32		5.64	0.26		6.94 [*]	0.33	
Glucose	196.40	12.64		195.80	28.02		223.75	17.74		195.60	7.139	
Na	153.80	0.86		152.800	1.11		151.80	1.32		153.00	0.32	
K	9.06	0.34		8.96	0.44		8.48	0.46		8.30	0.19	
Cl	119.40	0.51		122.60	1.21		117.40	0.93		119.20	0.66	
Alk phos	67.89	7.39		64.01	5.17		62.07	7.82		58.84	3.56	
1,25 D3	–	–		–	–		–	–		–	–	
Creatinine	1.07	0.09		1.04	0.03		0.92	0.07		1.01	0.04	
FEP	7.79	0.94		27.42 ^{***}	3.38		6.27	0.30		14.43 ^{***}	0.89	
RPC	2.13	0.09		8.97 ^{***}	1.14		2.28	0.14		4.73 ^{***}	0.24	
PEI	3.47	0.11		7.47 ^{***}	0.95		3.96	0.24		5.34 [*]	0.27	
<i>Urine</i>												
Pi (mg/dl)	31.84	0.23		61.65	1.00		41.09	0.61		56.06	0.82	
Pi (mg)	0.66	0.005		2.19	0.03		0.72	0.01		1.50	0.02	
Ca (mg/dl)	16.59	0.35		17.57	0.48		15.28	0.38		14.50	0.46	
Creatinine	48.38	1.27		32.56	0.09		62.67	0.38		41.16	0.69	

Insect-expressed recombinant Hu-MEPE was injected IP into mice ($n = 7$) four times and at two different doses (see Materials and methods). Three boluses were given over 6 h and serum/urine collected 1 h after the third bolus (7 h time points) and a final fourth bolus was given 30 h after the first and serum/urine collected 1 h after the final injection (31 h time points). PTH was used as a positive control. Measurements were made on individual ($n = 7$) serum samples except for 1,25-dihydroxy vitamin-D₃ where group samples were pooled and the experiment repeated twice with the same results. Also, group urine samples from respective metabolic cages were pooled and the experiments repeated with the same experimental outcome (see Materials and methods). Urine total protein, K, Cl and glucose were not significantly different between groups and within the normal range (data not shown). Fractional excretion of phosphate (FEP), renal phosphorus clearance (RPC) and phosphate excretion index (PEI) calculations are provided in the Materials and methods section. Experimentals are coded as PTH-1-34 (PTH at 80 $\mu\text{gkg}^{-1} \text{ 30 h}^{-1}$), MEPE40 (40 $\mu\text{gkg}^{-1} \text{ 30 h}^{-1}$) and MEPE400 (400 $\mu\text{gkg}^{-1} \text{ 30 h}^{-1}$). The results shown represent standard error of the mean (SEM) and the following symbols represent P values calculated using analysis of variance (ANOVA) non-parametric and Neuman-Keuls multiple comparison test relative to normal/vehicle groups:

The following units are used to express the data: urinary and serum Pi, Ca, creatinine and glucose (mg/dl), urinary/serum Na, K, and Cl (MEQ/l), alkaline phosphatase (U/l) and serum 1,25-dihydroxy vitamin-D₃ (pg/ml). The measurements of renal phosphate handling were FEP (%), RPC (μl/min) and PEI (dl/mg).

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$.