Phosphorylated acidic serine–aspartate-rich MEPE-associated motif peptide from matrix extracellular phosphoglycoprotein inhibits phosphate regulating gene with homologies to endopeptidases on the X-chromosome enzyme activity

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Abstract

Inactivating PHEX (phosphate regulating gene with homologies to endopeptidases on the X chromosome) mutations cause X-linked hypophosphatemia in humans and mice (Hyp) through overproduction of fibroblast growth factor 23 (FGF23) a phosphaturic factor, by osteocytes. Matrix extracellular phosphoglycoprotein (MEPE) is also elevated in Hyp and other hypophosphatemic disorders. In addition, the administration of an ASARM (acidic serine–aspartate rich MEPE-associated motif) peptide derived from MEPE causes phosphaturia and inhibits bone mineralization in mice, suggesting that MEPE also plays a role in phosphate homeostasis. Since recent studies found that MEPE binds specifically to PHEX *in vitro*, we tested the effect of recombinant-MEPE and its ASARM peptide on PHEX enzyme activity *in vitro* and FGF23 expression in bone marrow stromal cell cultures *ex vivo*. We found that both recombinant MEPE and synthetic phosphorylated ASARM

peptide (ASARM-PO₄) inhibit PHEX enzyme activities in an *in vitro* fluorescent-quenched PHEX enzyme activity assay. The ASARM-PO₄ peptide inhibits PHEX enzyme activity in a dose-dependent manner with a K_i of 128 nM and $V_{\max-i}$ of 100%. Recombinant MEPE also inhibits PHEX activity ($K_i=2$ nM and $V_{\max-i}=26\%$). Long-term bone marrow stromal cell cultures supplemented with 10 μ M ASARM-PO₄ peptide resulted in significant elevation of FGF23 transcripts and inhibition of mineralization. These findings suggest that MEPE inhibits mineralization and PHEX activity and leads to increased FGF23 production. The resulting coordination of mineralization and release of a phosphaturic factor by MEPE may serve a physiological role in regulating systemic phosphate homeostasis to meet the needs for bone mineralization.

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Introduction

Fibroblast growth factor 23 (FGF23) is a novel phosphaturic hormone (Econs & Drezner 1994, ADHR Consortium 2000, Shimada *et al.* 2001) predominately produced by osteocytes in bone (Liu *et al.* 2006b). FGF23 regulates serum phosphate levels by inhibition of renal phosphate reabsorption and by suppression of 1,25(OH)₂D₃ production by the kidney. Overexpression of FGF23 causes hypophosphatemia and reduced 1,25(OH)₂D₃ levels and rickets/osteomalacia (Bai *et al.* 2004, Larsson *et al.* 2004, Shimada *et al.* 2004b). In contrast, FGF23 deficiency (Bai *et al.* 2003, Shimada *et al.* 2004a, Liu *et al.* 2006b) or mutations increasing FGF23 degradation (Larsson *et al.* 2005) results in hyperphosphatemia, increased serum 1,25(OH)₂D₃ levels, and soft tissue calcifications. FGF23 is a pathological phosphaturic factor in several hereditary hypophosphatemic disorders,

including X-linked hypophosphatemia (XLH) and autosomal dominant hypophosphatemic rickets (ADHR). XLH is caused by inactivating mutations of PHEX (phosphate regulating gene with homologies to endopeptidases on the X chromosome; The HYP Consortium 1995), a member of the M13 family of the type II cell-surface zinc-dependent proteases (Rowe *et al.* 1997). Serum levels of FGF23 are elevated in XLH (Yamazaki *et al.* 2002, Weber *et al.* 2003) and Hyp mice (Liu *et al.* 2003), the murine homologue of XLH. FGF23 is not the substrate for PHEX (Liu *et al.* 2003, Benet-Pages *et al.* 2004); instead loss of PHEX function results in overproduction of FGF23 by osteocytes in bone through mechanisms that remain to be defined (Liu *et al.* 2003, 2006c). ADHR is caused by mutations that prevent the degradation of FGF23 (White *et al.* 2001).

Matrix extracellular phosphoglycoprotein (MEPE) is a member of the SIBLING protein family (Fisher & Fedarko

2003) originally cloned from a patient with tumor-induced osteomalacia (TIO) (Rowe *et al.* 2000). MEPE is exclusively expressed in osteoblasts, osteocytes, and odontoblasts and markedly up-regulated in Hyp osteoblasts and TIO tumors (Petersen *et al.* 2000, Rowe *et al.* 2000, 2004, Argiro *et al.* 2001, Guo *et al.* 2002, Fisher & Fedarko 2003, Gowen *et al.* 2003). MEPE was originally proposed as candidate for the phosphaturic factor in TIO; however, MEPE deficiency results in accelerated osteoblast-mediated bone formation and mineralization without changes in serum phosphate levels (Gowen *et al.* 2003, Liu *et al.* 2005b). These findings suggest that its primary function is to regulate the mineralization process, although administration of recombinant MEPE is reported to induce phosphaturia in mice (Rowe *et al.* 2004). In addition, MEPE can be cleaved by cathepsin B to release a carboxy-terminal MEPE peptide (ASARM peptide) (Rowe *et al.* 2004) and the phosphorylated ASARM peptide inhibits mineralization *in vivo* and *in vitro* and binds to PHEX (Rowe *et al.* 2004, 2005), suggesting that MEPE also undergoes proteolytic processing.

MEPE is elevated in the bone of Hyp mice and positively correlated with FGF23 expression (Weber *et al.* 2003, Liu *et al.* 2005b), but the relationship between PHEX and MEPE is not clear. Similar to its proposed role in TIO, MEPE was also thought to be a candidate for the phosphaturic factor in XLH/Hyp. However, we have recently demonstrated that the Hyp phosphaturic phenotype is not corrected by transfer of MEPE-deficient mice on to the Hyp mouse background (Liu *et al.* 2005b), indicating that MEPE is not the phosphaturic factor in this disorder. While these studies indicate that MEPE is not the proximate cause of hypophosphatemia in Hyp mice, it does not preclude an upstream effect of MEPE that is potentially mediated through MEPE binding to PHEX via the MEPE ASARM-motif or a role of MEPE in the mineralization defect in Hyp mice (Rowe *et al.* 2005).

To further understand the relationship between PHEX, MEPE, and FGF23 in causing hypophosphatemia, we tested the ability of recombinant-MEPE and ASARM peptides to inhibit PHEX enzyme activity *in vitro*. We also determined whether MEPE and ASARM-PO₄ peptides were able to up-regulate FGF23 expression and inhibit mineralization in a bone marrow stromal cell culture system.

Materials and Methods

Assay of SecPHEX endopeptidase activity

A quenched fluorescence enzymatic assay used to measure PHEX endopeptidase activity was kindly offered by Dr Philippe Crine from Enobia Inc., Montreal, Canada (Campos *et al.* 2003). In this assay, a secreted form PHEX (SecPHEX) and a synthetic substrate (Abz)-GFSDYK-(Dnp) were used. The synthetic peptide substrate was labeled with a fluorogenic reagent aminobenzoic acid (Abz) in the amino terminus and a quenching moiety dinitrophenyl (Dnp) in the carbonic

terminus. The secPHEX dose-dependent activity was tested from concentrations of 10 to 100 ng with 10 μ M substrate. The reaction was followed by measuring the fluorescence from excitation at 320 nm and emission at 420 nm in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Full-length insect expressed human MEPE was purified and expressed as described previously (Rowe *et al.* 2004, 2005). Synthetic peptides derived from MEPE sequence, including MEPE-arginine glycine aspartate motif (RGD) peptide, phosphorylated ASARM (ASARM-PO₄), and non-phosphorylated ASARM peptide (Multiple Peptide Systems, Inc., San Diego, CA, USA) were used in the inhibition assay (Rowe *et al.* 2005). PHEX activities were expressed as an initial speed rate calculated by fitting the steepest linear slope over at least eight adjacent reading values. The slope was converted into nanomoles of substrate hydrolyzed per minute and kinetic parameters, K_i and $V_{\max-i}$, were calculated by the non-linear regression data analysis GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA, USA).

Animal care and genotyping

The FGF23-deficient/reporter mouse model was created by knocking in an enhanced green fluorescent protein (eGFP) construct following the adenine thymine guanine (ATG) in exon 1 of the FGF23 gene as previously reported (Liu *et al.* 2006b) and was crossed and maintained on C57BL/6J background. This eGFP reporter mouse was also transferred onto Hyp background. The heterozygous FGF23-deficient/reporter mouse will express eGFP under control of endogenous FGF23 promoter and mimic the expression pattern of FGF23. Genomic DNA tissue was extracted and purified from the tail of each mouse using a QIAGEN DNeasy Tissue Kit (QIAGEN Inc). Genotypes were determined by PCR as previously reported (Liu *et al.* 2006b).

All mice were fed with a standard rodent diet (8604 Harlan Teklad Rodent Diet) containing 1.01% phosphorus and 1.36% calcium (Harlan Teklad, Madison, WI, USA) and tap water. All mice were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86-23, 1985).

Bone marrow stromal cell (BMSC) culture

BMSCs from long bones isolated from 6- to 8- week-old male FGF23-deficient/eGFP reporter mice on both wild-type (WT) and Hyp background were cultured as previously described (Liu *et al.* 2006b). Briefly, bone marrow cells were isolated and plated in 35 mm dishes at a density of 7×10^6 per 35 mm plate and grown in 2-minimum essential medium (α -MEM; Invitrogen) containing 10% fetal bovine serum (FBS) for 5 days and then switched to the differentiation medium (α -MEM containing 10% FBS supplemented with

5 mmol/l, β -glycerophosphate, and 25 μ g/ml ascorbic acid) with and without phosphorylated ASARM peptide at 10 μ M concentration. At day 14, pictures of the cells were taken under bright field and fluorescent light microscopy. Cells were then either stained with Alizarin red-S or used for RNA extraction. Alizarin red-S stain was extracted with 10% cetylpyridinium chloride and quantified by absorbance measurement at 562 nm as previously described (Liu *et al.* 2005a).

RNA isolation and quantitative RT-PCR

Total RNAs were extracted from cultured cells with Trizol (Invitrogen) and then treated with RNase-free DNase using an RNeasy column (QIAGEN Inc.). First strand cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad). Total RNA (1 μ g) was used in each 20 μ l reverse transcription reaction. Input RNAs (400 and 20 ng) were used to amplify FGF23 and cyclophilin A respectively. The primers used for real-time quantitative RT-PCR are 5'-TGTCGCAGAAG-CATCA-3' and 5'-GTGGGCGAACAGTGTAGAA-3' for FGF23 and 5'-CTGCACTGCCAAGACTGAAT-3' and 5'-CCACAATGTTCATGCCTTCT-3' for cyclophilin A. The iCycler iQ Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad) were used for real-time PCR analysis as previously reported (Liu *et al.* 2006b).

Results

Recombinant MEPE and ASARM-PO₄ inhibit PHEX enzyme activities

To evaluate the functional significance of our previously reported MEPE and MEPE ASARM-peptide (phosphorylated and non-phosphorylated) interactions with PHEX (Rowe *et al.* 2005), we examined if addition of recombinant MEPE or its ASARM peptide (phosphorylated and non-phosphorylated) inhibited PHEX enzymatic activity *in vitro*. For these studies, we used the previously reported soluble secreted recombinant PHEX enzyme and a synthetic fluorescent-quenched peptide that is specifically cleaved by PHEX (Campos *et al.* 2003). We found that both recombinant MEPE (Fig. 1) and synthetic phosphorylated ASARM peptide (ASARM-PO₄; Fig. 2) inhibit PHEX enzyme activities. Recombinant full-length MEPE significantly inhibited PHEX activity at concentrations of 2 nM in a dose-dependent and saturable manner. The K_i and $V_{\max-i}$ were 2 nM and 26% respectively (Fig. 1). Synthetic ASARM-PO₄ peptide (derived from the C-terminal MEPE ASARM-motif) also resulted in a potent inhibition of PHEX activity *in vitro* with a K_i and $V_{\max-i}$ of 129 nM and 100% respectively (Fig. 2). Specifically, dose-, time-dependent, and saturable inhibition of PHEX activity was observed at nM concentrations for both recombinant MEPE and ASARM-PO₄. Control RGD peptide (Fig. 2) and non-phosphorylated

Figure 1 Kinetics of inhibition of PHEX enzymatic activity by recombinant MEPE. Michaelis-Menton graph (A) and corresponding Lineweaver-Burke plot (B) showing percentage inhibition of secPHEX activity as measured using the quenched fluorescence enzymatic assay (see Materials and Methods). Percentage inhibition represents the percentage change in enzyme velocity per unit time relative to control recombinant secPHEX in the absence of inhibitor peptides (0% inhibition of activity). Recombinant insect expressed MEPE-inhibited secPHEX activity in a dose-dependent and saturable manner with a maximum inhibition ($V_{\max-i}$) of 25.45% and a K_i of 2.1 nM. Each experimental dose point is statistically significant by one-way ANOVA analysis at P value <0.05.

ASARM peptide (data not shown) had no effect on PHEX activity at micromolar levels. The inhibitory effects of MEPE and ASARM-PO₄ on PHEX enzyme activity are consistent with previously reported surface plasmon resonance binding studies (Rowe *et al.* 2006).

ASARM-PO₄ stimulates FGF23 transcript and inhibits mineralization in BMSC cultures

Next, we explored whether ASARM-PO₄ inhibition of PHEX activity has biological significance. To evaluate the effects of ASARM-PO₄ on FGF23 production by osteoblasts, we incubated 10 μ M ASARM-PO₄ with BMSCs derived from FGF23-deficient/eGFP reporter mice, which express eGFP under the control of the endogenous FGF23 promoter. For controls, we assessed BMSCs not treated with peptide (normal controls) and BMSCs from *Hyp* mice (positive controls), which are PHEX deficiency and exhibit elevated FGF23 expression (Liu *et al.* 2006b). Incubation of these cells for 14 days in the presence of 10 μ M ASARM-PO₄ peptide resulted in a significant twofold increase in FGF23 mRNA levels compared with untreated control cultures (Fig. 3A).

Figure 2 Kinetics of inhibition of PHEX enzymatic activity by peptides derived from MEPE. (A) Time-course of phosphorylated ASARM peptide (ASARM-PO₄) inhibition of PHEX enzyme activity. Graph shows raw fluorescence data measurements of PHEX fluorogenic enzymatic assay in the absence and in the presence of different concentrations of ASARM-PO₄ peptide. The fluorescence strength in the reactions increased in linear relationship from 0 to 40 min. There was a dose-dependent inhibition of PHEX by ASARM-PO₄ (10–300 nM). (B) Dose-dependent effects of ASARM-PO₄ inhibition of PHEX. Percentage inhibition reflects the inhibition with peptide compared with the enzyme activity without peptide from $n=3$. (C and D) Kinetics of inhibition of PHEX enzymatic activity inhibition by ASARM-PO₄ peptide. Michaelis–Menton (C) and corresponding Lineweaver–Burke plot (D). RGD and non-phosphorylated ASARM control peptides had no effect on enzyme activity up to micromolar concentrations (data not shown). ASARM-PO₄ inhibited secPHEX activity in a dose-dependent and saturable manner with a maximum inhibition ($V_{\max-i}$) of 100% and a K_i of 128.7 nM. Each experimental dose point is statistically significant by one-way ANOVA analysis at P value <0.05 .

In addition, ASARM-PO₄ peptide increased eGFP expression that was limited to osteocytes embedded in mineralization nodule (Fig. 3B). However, the level of FGF23 expression was less than observed in Hyp controls.

The addition of the ASARM-PO₄ peptide also inhibited mineralization, as evidenced by alizarin red-stained nodules in ASARM-PO₄ peptide-treated BMSCs compared with untreated controls (Fig. 4A). In contrast to the attenuated reduction in FGF23, ASARM-PO₄ inhibited mineralization to a similar degree as Hyp-derived BMSCs (Fig. 4B).

Discussion

Our findings provide new insights into how PHEX, MEPE, and FGF23 potentially act in concert to regulate phosphate homeostasis and matrix mineralization. First, the ability of MEPE and the MEPE-derived ASARM-PO₄ peptide to inhibit PHEX activity *in vitro* (Fig. 2), and stimulate FGF23

production by BMSC (Fig. 3), suggests that MEPE is an important regulator of PHEX function. In previous studies, we have shown that PHEX inhibits MEPE degradation *in vitro* (Guo *et al.* 2002) and that MEPE and its ASARM-PO₄ peptide binds to the PHEX protein catalytic site (Rowe *et al.* 2005). The present findings support the possibility that the observed phosphaturic effects of MEPE *in vivo* (Rowe *et al.* 2004) are mediated, at least in part, through MEPE binding to and inactivation of PHEX. Both the ASARM-PO₄ peptide and MEPE maximally inhibit PHEX activity at nanomolar concentrations, in a dose-dependent and saturable manner (maximal inhibition 100% for ASARM-PO₄ and 25% for MEPE). Recent detection of micromolar levels of circulating MEPE (Bresler *et al.* 2004, Jain *et al.* 2004) suggests that the inhibition of PHEX represents a physiological concentration of MEPE. The observation that the phosphorylated ASARM peptide is more potent than full-length MEPE also raises the possibility that proteolytic processing and phosphorylation are important means to

Figure 3 Effects of ASARM-PO₄ on FGF23 expression in BMSC cultures. (A) FGF23 message levels measured by quantitative real-time RT-PCR in BMSC cultures from wild-type (controls), wild-type treated with ASARM-PO₄, and Hyp (positive controls). FGF23 mRNA levels are expressed relative to the levels of the cyclophilin A mRNA. Values represent the mean $\pm$ s.e.m. ($n=3$). ASARM-PO₄ significantly stimulated FGF23 mRNA expression. (B) Assessment of ASARM-PO₄ effects on eGFP expression driven by endogenous FGF23 promoter in BMSC cultures. The mineralization nodules in BMSC cultured in differentiation medium for 14 days were viewed under fluorescent microscope ($\times 200$). WT control cultures showed no eGFP (left panel), whereas WT BMSCs cultured with the ASARM-PO₄ peptide at 10 μ M concentration for 14 days showed demonstrable eGFP-positive cells embedded in the mineralization nodule (middle panel). eGFP expression was less in ASARM-PO₄-treated cultures compared with the abundant eGFP-positive cells in the mineralization nodules in Hyp-derived BMSCs (Hyp), which were used as positive controls (right panel).

regulate the biological functions of MEPE (Rowe *et al.* 2004, 2005). Recently, the importance of proteolytic processing in Hyp has been shown by the improved bone mineralization following the *in vivo* administration of cathepsin inhibitors (Rowe *et al.* 2006).

Secondly, we confirm the ability of the ASARM-PO₄ peptide to regulate mineralization. We found that the addition of ASARM-PO₄ peptide, in physiological concentrations, inhibited mineralization of BMSC cultures (Fig. 4). The MEPE-derived ASARM-PO₄ peptide has also been shown to inhibit mineralization *in vivo* in mice and *in vitro* using a murine osteoblast cell-line (2T3 cells; Rowe *et al.* 2004, 2005). Our prior observations also implicate MEPE in the mineralization

defect observed in Hyp mice. In this regard, mineralization of BMSC cultures is improved in MEPE-deficient Hyp mice *ex vivo*, in spite of persistent hypophosphatemia in the combined MEPE-deficient/Hyp mice (Liu *et al.* 2005a,b). The increased MEPE in Hyp mouse bone could account for the 'intrinsic' mineralization abnormality in this disorder. Thus, MEPE and/or its degradation products may directly interfere with the process of mineralization of the extracellular matrix independent of hypophosphatemia. The exact mechanism whereby the mineralization process is disrupted by the ASARM peptide, and the role of PHEX/MEPE interactions in the process, is not clear, but likely involves altering the ratio of inhibitors and/or inducer of mineralization (Murshed *et al.* 2005).

Figure 4 Effect of ASARM-PO₄ on BMSC mineralization. Alizarin Red-S staining of mineralized extracellular matrix in BMSC cultures (A) and quantification of mineralization (B). The addition of the ASARM-PO₄ (10 μM) to wild-type BMSCs for 14 days resulted in less mineralization nodules similar to BMSCs derived from Hyp mice. Numeric values represent the mean ± S.E.M. of Alizarin red-S from a 35 mm plate (n=3).

Although we demonstrate that MEPE and its ASARM peptide inhibit PHEX activity *in vitro* and is associated with increased FGF23 message expression in culture, our studies have not established that the reported phosphaturic actions of MEPE (Rowe *et al.* 2004) are mediated through increased FGF23 or inhibition of PHEX *in vivo*. Future studies that investigate whether FGF23-null mice are refractory to MEPE-induced phosphaturia would be necessary to establish the role of FGF23 in mediating MEPE actions. MEPE inhibition of PHEX, if physiologically relevant, places MEPE upstream of PHEX. This might explain not only the phosphaturic effect of recombinant MEPE, since excess MEPE would mimic the effects of inactivating PHEX mutations in Hyp, but potentially also explain why super-imposed MEPE deficiency failed to rescue the Hyp phenotype (Liu *et al.* 2005b). It is also possible that MEPE has effects that are independent of both FGF23 and PHEX both in bone and other sites.

Regardless, phosphate is an important determinant of mineralization of extracellular matrix in bone and soft tissues (Murshed *et al.* 2005) and a bone-kidney axis involving PHEX and FGF23 may regulate renal phosphate handling to meet the demand of phosphate for mineralization of bone (Quarles 2003, Liu *et al.* 2006a). Our findings of the dual regulation of PHEX and mineralization by MEPE degradation products suggest that MEPE may also be involved in this putative bone-kidney axis as an intermediate molecule in bone matrix that directly regulates

the mineralization process in bone and indirectly coordinates renal phosphate handling through interactions with PHEX and regulation of FGF23 production. Proof of this hypothesis will require additional studies that establish the respective roles of PHEX and FGF23 in mediating the effects of MEPE *in vivo*.

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